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Protecting the code: DNA double-strand break repair pathway choice

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Protecting the code: DNA double-strand break repair pathway choice

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Editorial: Protecting the code: DNA double-strand break repair pathway choice

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Editorial on the Research Topic
Protecting the code: DNA double-strand break repair pathway choice

The genetic information in our cells is constantly challenged by several sources that can cause DNA damage. Amongst the various lesions that can occur in the genome, DNA double-strand breaks (DSBs) are considered the most dangerous (Scully et al., 2019). Cells combat DSBs by activation of the DNA damage response (DDR), a complex evolutionary conserved cellular network that senses, signals and repairs DNA breaks, while coordinating DNA repair with chromatin regulation, gene expression, and cell cycle progression (Ciccia and Elledge, 2010). Defects in the DDR can lead to devastating diseases such as immune disorders and cancer (Jackson and Bartek, 2009). In addition, the targeted generation of DSBs can be exploited for CRISPR-mediated genome editing and gene therapy (Yeh et al., 2019; Nambiar et al., 2022). DSB repair is, therefore, one of the most critical tasks a cell must pursue to maintain genome integrity, and malfunction of this process has important clinical implications.

This Research Topic is focused on *Protecting the code: DNA double-strand break repair pathway choice* and features 18 articles that reflect the complexity of cellular processes that determine DNA repair pathway choice. It consists of topical reviews as well as original research and methods articles focusing on key DNA repair mechanisms, including the main DSB repair pathways non-homologous end-joining (NHEJ) and homologous recombination (HR), and on techniques to study these pathways and elucidate their relevance for human health and disease.

DSB repair pathway choice relies on multiple regulatory layers that can respond to environmental and cell-intrinsic cues (Chapman et al., 2012; Krenning et al., 2019). Amongst the latter are non-B DNA structures, which are formed at particular sequences

(e.g., at repetitive regions or at common fragile sites) and can exist in the form of G-quadruplexes (G4) and RNA-DNA hybrids (R-loops). In the article by Camarillo et al. an update and perspective is provided on the tight interconnection between G-quadruplexes and R-loops and their emerging role as roadblocks for DNA end-resection during DSB repair by HR. Regarding the temporal progression of the DDR, Kieffer and Lowndes propose that the response to DSBs can be divided into immediate-early, early, and late responses, in analogy to the events occurring upon viral infection. Their review provides an integrated view of these sequential DDR responses and how they are modulated by the complexity of the DSB end, chromatin context, cell cycle phase, and the availability of specific DSB repair factors to control DSB repair pathway choice.

The packaging of DNA into chromatin, the so-called 'chromatin barrier', complicates the efficient detection and repair of DSBs (Goodarzi and Jeggo, 2012). ATP-dependent chromatin remodelers and post-translational modifications (PTMs) of histones and other chromatin-associated proteins are therefore required to modulate chromatin structure around DSBs and facilitate repair. The review by Karl et al. covers the latest insights into the function of several chromatin remodelers and their impact on DNA end-resection, which is a critical determinant of DSB repair pathway choice. The authors describe recent advances in understanding the role of nucleosome sliding and positioning, editing, and eviction on resection and DSB repair.

Several mechanisms ensure that HR is restricted to the S and G2 phases of the cell cycle, including the antagonism between the DSB-responsive chromatin readers 53BP1 and BRCA1 (Hustedt and Durocher, 2016). The review by Sanchez et al. covers the latest insights on the diverse nature of protein interaction domains involved in the DDR, their crosstalk within chromatin, and how multiple, sometimes competing signals are integrated at the level of the chromatin scaffold for proper DSB repair. Further strengthening the role of chromatin structure and nuclear topology for repair, the review by Sebastian et al. describes the processes that shape the three-dimensional (3D) chromatin landscape and how they impact genome functions including DNA replication and DSB repair. Besides chromatin context and topology, DSB movement into repair-permissive environments and the potential role of phase separation are discussed. A particular challenge for DSB repair is posed by dense heterochromatin, and recent studies have revealed how heterochromatic features influence DSB repair. The review by Caron et al. covers the latest insights on this topic and discusses the interplay between heterochromatin marks and DSB repair, focusing on the role of both pre-existing heterochromatin domains and *de novo* establishment of heterochromatin features in euchromatic regions upon DNA damage.

Despite recent technical improvements, studying chromatin structure and dynamics at high spatial and temporal resolution remains challenging. The research article by Lou et al. describes a novel approach to look at nanoscale chromatin changes based on fluorescence lifetime imaging microscopy (FLIM) of Förster resonance energy transfer (FRET) between fluorescently labeled histones. Employing the DSB-inducible AsiSI cell system (DIvA), their approach has sufficient spatial resolution to map chromatin compaction nuclear-wide and the authors use this to elucidate how nanoscale chromatin architecture impacts the balance between competing DSB repair pathways such as NHEJ and HR.

According to current models, HR repair comprises DNA end-resection followed by homology search (Wright et al., 2018). Once homology is found, usually on the undamaged sister chromatin, a displacement loop (D-loop) is formed which allows DNA repair synthesis. However, after DNA repair synthesis is complete, HR can proceed via different HR sub-pathways. The review by Elbakry and Löbrich highlights these alternative sub-pathways, including the canonical sub-pathways of synthesis-dependent strand annealing (SDSA) and the Holliday junction (HJ) pathway, as well as the non-canonical break-induced replication (BIR) pathway, and discusses clinical implications of HR sub-pathway choice.

A central protein in the orchestration of HR is the tumor suppressor BRCA2. Mutations in the *BRCA2* gene are associated with breast and ovarian cancer, but how individual *BRCA2* mutations affect HR is incompletely understood. The research article by Jimenez-Sainz et al. sheds light on this issue by revealing that the pathogenic variant R3052W causes mis-localization of BRCA2 to the cytoplasm. The defect in nuclear localization can thus explain the HR deficiency, which results in genome instability and sensitization to PARP inhibitors and crosslinking drugs.

Besides gene mutations, changes in expression of DNA repair genes frequently contribute to tumor formation. In recent years it has become clear that tumors can reactivate genes whose expression is normally restricted to germ cells. The review by Lingg et al. discusses the function of meiotic genes and how their aberrant reactivation in somatic cancer cells affects DSB repair and genome stability. Considering that meiotic genes are transcriptionally repressed in somatic cells of healthy tissues, targeting reactivated meiotic genes could provide a therapeutic opportunity to specifically kill cancer cells.

The ability of cells to proliferate depends on the faithful duplication of their genome via DNA replication during S phase of each cell cycle. Upon replication stress, cells coordinate a variety of genome and cell cycle surveillance pathways to ensure the completion of replication and maintain genome stability (Panagopoulos and Altmeyer, 2021; Saxena and Zou, 2022). The review by Wootton and Soutoglou provides an overview on the many aspects of chromatin and nuclear environment such as topologically associated domains (TADs), non-canonical histone variants, and histone modifications, and how these affect replication fork stability, S-phase progression and repair

of replication-associated DNA damage. Extending this theme, the review by Nickoloff et al. focusses on the safe and unsafe pathways to repair broken replication forks, highlighting the danger of erroneous single-ended DSB repair by NHEJ, and describing mechanisms to ensure that broken forks are instead repaired faithfully by HR.

For most two-ended DSBs, however, NHEJ seems to be the predominant or fastest repair pathway in mammalian cells. This type of end-joining repair also plays an important role during V(D)J recombination, which occurs during lymphocyte differentiation to generate antibody diversity. During this process DSBs are introduced by the RAG nuclease, and the review by Libri et al. describes various parameters that constrain the repair of RAG-induced DSBs to NHEJ, including DSB-end structure, the presence of a post-synaptic cleavage complex, and protection against DSB end resection.

This Research Topic also features articles that discuss newly emerging methodologies to investigate how cells commit to a certain repair pathway. The review by Meyenberg et al. provides a comprehensive overview on recent developments in the context of tissue specific DNA repair upon CRISPR-induced DNA breaks. The authors also discuss the implications for genome editing and gene therapies to treat genetic diseases. Extending on the CRISPR methodology, the review by van de Kooij and van Attikum describes the advent of Cas9 nucleases in the construction of novel reporter systems to measure DSB-repair pathway usage. They compare single-pathway and multi-pathway DSB-repair reporters and highlight how the new Cas9-based reporter systems enhance the flexibility and design of reporter constructs in comparison to established I-SceI reporter systems. Finally, the methods article by Schep et al. provides a detailed protocol for DSB-TRIP, a technique that utilizes genomic scars left behind by DNA repair to study DSB repair pathway usage throughout the genome and correlate repair pathway choice with various chromatin features.

CRISPR-based screens have greatly facilitated the identification of synthetic lethal interactions relevant to DNA repair and replication in normal and cancer cells (Setton et al., 2021; Wilson and Loizou, 2022). Synthetic lethality, or sickness, describes a cellular condition in which a defect in either one of two genes has little or no effect on cellular fitness, whereas the combination of both gene defects results in cell death or severely compromised fitness, respectively (Setton et al., 2021). The review by Rossi et al. highlights recent studies on the importance of the repair protein RAD52 to keep HR-deficient cancer cells viable. The critical role of RAD52 in this context makes it an attractive target for the development of anti-cancer therapies to treat HR-deficient tumors. Apart from such targeted therapeutic approaches based on the concept of synthetic lethality, radiotherapy is widely used for the treatment of tumors, and particularly particle radiotherapy has received increasing attention due to dose distribution advantages. The review by van de Kamp et al. describes different types of ionizing radiation in the context of radiotherapy, and discusses the DNA lesions they induce and how these in turn impact DNA end processing and repair. Moreover, combination therapies and promising DDR targets that could improve particle radiotherapy are discussed.

Together, this article collection highlights the growing understanding of the fundamental principles of DNA repair pathways and their context-dependent regulation. At the same time, the collection also sheds light on the many unknowns that still exist about repair pathway and sub-pathway choice in different biological settings and disease conditions. Future research and emerging technologies, some of which are described in this collection, will aim at turning these insufficiently understood areas into new knowledge that can be used to harness DNA repair for targeted genome editing and precision cancer therapy to improve clinical outcomes in patients.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Homologous Recombination Subpathways: A Tangle to Resolve

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Homologous recombination (HR) is an essential pathway for DNA double-strand break (DSB) repair, which can proceed through various subpathways that have distinct elements and genetic outcomes. In this mini-review, we highlight the main features known about HR subpathways operating at DSBs in human cells and the factors regulating subpathway choice. We examine new developments that provide alternative models of subpathway usage in different cell types revise the nature of HR intermediates involved and reassess the frequency of repair outcomes. We discuss the impact of expanding our understanding of HR subpathways and how it can be clinically exploited.

Keywords: homologous recombination, pathway choice, synthesis-dependent strand annealing, ATRX, RECQ5, double-strand break, holliday junction, crossover

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REPAIR OF DSBS BY HR

Cells have evolved multiple mechanisms to preserve genome integrity and restore structural and functional properties of the genome following DNA damage. DNA double-strand breaks (DSBs) are critical lesions whose timely and accurate repair is important for cellular viability and genomic stability. Cells are equipped with multiple pathways to repair DSBs, the most prominent of which are non-homologous end-joining (NHEJ) and homologous recombination (HR). HR provides a high-fidelity mechanism for repair in cycling cells but is restricted to the S and G2 phases of the cell cycle. In contrast to NHEJ, which involves ligating the break ends together, HR involves copying sequences from an intact donor to restore any lost information. HR is also important for the faithful duplication of the genome by providing means of tolerating replication stress and overcoming lesions resulting from replication fork obstruction such as single-stranded DNA (ssDNA) gaps and one-ended DSBs.

Homologous recombination at DSBs can proceed in multiple subpathways, but the initial steps are functionally similar and involve common factors. Briefly, HR commences with the 5'–3' extensive resection of break ends by nucleases to generate 3' ssDNA overhangs, which are then coated by replication protein A (RPA). The breast and ovarian cancer susceptibility protein 2 (BRCA2) then loads the recombinase RAD51 to ssDNA, replacing RPA and forming a nucleoprotein filament to initiate the homology search for complementary sequences. Once homology is found, a displacement loop (D-loop) is formed, where a primer-template junction allows DNA repair synthesis to proceed. After repair synthesis completion, HR can proceed by the displacement of the extended break end from the D-loop and annealing to the complementary sequence at the non-invading end, a subpathway referred to as synthesis-dependent strand annealing (SDSA). An alternative mechanism involves the formation of a joint structure containing a four-way junction between the recombining strands, known as a

Holliday junction (HJ). This can occur through the annealing of the non-invading end to the displaced strand of the D-loop in a second-end capture step, or possibly by simultaneous invasion of the two resected ends into the donor and subsequent extension. To allow proper chromosome segregation, the two intertwining strands must be separated, which can occur through two mechanisms with distinct genetic outcomes. Double HJs (dHJs) are prominent HR intermediates and are predominantly processed by helicase- and topoisomerase-dependent dissolution that separates the recombining molecules without genetic exchanges. Alternatively, these joint molecules (JMs, used in this manuscript to refer to post DNA synthesis structures) can be resolved by the structure-selective nucleases to give rise to crossover (CO) or non-CO products at an expected equal frequency. HR can also proceed through a third, non-canonical subpathway termed break-induced replication (BIR), which is characterized by long-range conservative DNA synthesis from the invading DSB end without engagement of the second end and displaying a high propensity to form genomic rearrangements and point mutations. Over the past decade, our understanding of DSB repair pathway choice between NHEJ and HR was greatly enhanced (a topic also reviewed in this issue), which proved useful in many applications, including delineating mechanisms of cellular responses to cancer therapy and finding new drug targets. However, less attention has been paid to HR subpathway choice, our understanding of which falls short especially in human cells. This review aims to focus on the differences between the known HR subpathways, what is known about subpathway choice and the mechanistic and clinical implications of these distinct mechanisms.

HR SUBPATHWAY OUTCOMES

One feature that is often used to distinguish between the different HR subpathways is their propensity to cause genetic exchanges. Although canonical HR is known to be of high fidelity compared to NHEJ, COs are considered harmful as they can lead to loss of heterozygosity (LOH) if exchanges occur between homologous chromosomes (Moynahan and Jasin, 1997). Translocations, deletions, or inversions can result if COs occur between repeated DNA by non-allelic homologous recombination (Moynahan and Jasin, 1997; Wright et al., 2018). However, it is worth noting that these occur rarely as spontaneous events and while promoted by DSB induction, they are largely suppressed in somatic cells (Stark and Jasin, 2003). An important mechanism to attenuate possible detrimental outcomes is the predominant use of the sister chromatid as donor instead of the homologous chromosome, which renders COs genetically silent (Kadyk and Hartwell, 1992; Soutoglou et al., 2007). Additionally, cells can employ pathways that inherently avoid these products, such as SDSA, which is believed to be the predominant HR subpathway for DSB repair. However, as COs are mostly inconsequential, it is not sufficient to assume cells always favor a CO-avoiding pathway and so the propensity for causing genetic exchanges does not provide an adequate explanation for HR subpathway choice.

While SDSA altogether avoids the formation of HR intermediates that can lead to COs, the processing of such intermediates can also be regulated to favor non-CO products. Consistent with this, dHJs are predominantly dissolved by the BLM-TOPOIIIα-RMI1/2 (BTR) complex to non-COs, with CO-prone resolution acting as a last resort to handle these intermediates (Sarbajna and West, 2014). Yet whether dHJs are the only, or even the main, intermediates leading to COs is in question and it remains unclear under which conditions cells favor CO-forming vs. CO-avoiding pathways for DSB repair. Consequently, a more careful dissection of how HR subpathways are regulated and the factors involved are warranted for a better understanding of how distinct repair outcomes arise.

REVISITING HR SUBPATHWAY CHOICE

In recent years, work by us and others has aimed to define factors involved in promoting and regulating HR subpathway usage. Subpathway choice is often connected to helicases, which can function to either disrupt HR intermediates (such as RAD51 filaments and D-loops), or to promote DNA synthesis and D-loop extension. Therefore, it is important to understand what governs the stability of these intermediates and how they differ in the distinct subpathways. Multiple helicases have been implicated to regulate HR in human cells, including RTEL (Barber et al., 2008), BLM (van Brabant et al., 2000), FANCJ (Sommers et al., 2009), FBH1 (Chan et al., 2018), RECQ1 (Bugreev et al., 2008) and RECQ5 (Hu et al., 2007; reviewed in Huselid and Bunting, 2020). Helicases that disrupt the pre-synaptic RAD51 filaments by enhancing RAD51 removal are referred to as anti-recombinases due to their HR-limiting functions. Conversely, disruption of extended D-loops serves to limit the extent of DNA synthesis and displace the invading strand to channel repair toward SDSA. Often, helicases harbor both anti- and pro-recombinogenic biochemical functions, making it difficult to pinpoint the precise contribution of these helicases to DSB repair. Recently, we have found that at two-ended DSBs, RECQ5 promotes a repair pathway, likely to be SDSA, involving short-range repair synthesis and resulting in non-CO repair products. The role of RECQ5 in this context is unclear, although it has been implicated to involve RAD51 removal after strand displacement to prevent re-invasion cycles and allow strand annealing to promote SDSA (Paliwal et al., 2014). Other functions could relate to those of analogous yeast helicases, such as Srs2, that involve disrupting D-loops and limiting DNA synthesis (Burkovics et al., 2013; Liu et al., 2017). Additionally, some reports support a requirement for only the helicase domain of Srs2 for its SDSA function (Bronstein et al., 2018), and others find that also the RAD51-interacting domain has an effect on CO formation (Jenkins et al., 2019), rendering the precise role of the helicase uncertain. Furthermore, factors regulating strand annealing post displacement are not well-defined, although, differential processing of the non-invading break ends has been implicated. Successful engagement of the second end is important to terminate repair of two-ended breaks and its failure can activate one-ended DSB repair

mechanisms, such as BIR (Chandramouly et al., 2013). Consistently, the length of homology between the non-invading end and the displaced strand, influenced by the extent of resection, has been suggested to regulate subpathway choice between SDSA and BIR in human cells. Similarly, asynchronous resection and short homology tracts lead to failure of strand annealing and activation of BIR in yeast, a process regulated by the Mph1 and Sgs1 helicases, which dismantle D-loops (to favor SDSA) or short homology duplexes (to promote BIR), respectively (Mehta et al., 2017; Pham et al., 2021).

Alternatively, HR can proceed through a RAD54-dependent pathway with a propensity for CO formation (Spies et al., 2016). We have shown that the chromatin remodeler ATRX promotes this subpathway of HR that involves long stretches of DNA synthesis leading to the formation of a high frequency of COs visualized as sister-chromatid exchanges (SCEs; Juhász et al., 2018). Interestingly, the HR intermediates formed by this pathway, which can be detected as IR-induced ultra-fine bridges (UFBs), are completely dependent on the structure-selective nucleases MUS81 and GEN1 for resolution and are independent of BLM function (Elbakry et al., 2021). Since BLM has a well-documented role in suppressing endogenous SCEs, HR at two-ended DSBs can lead to distinct structures than those formed at replication-associated lesions that may not be classic dHJ and are therefore processed differently. This is consistent with studies reporting high CO levels and synthetic lethality of cells lacking GEN1 and SLX4 (essential for MUS81 function at HJs) even in the presence of BLM, indicating the presence of HJs that exclusively require resolution (Garner et al., 2013; Wyatt et al., 2013). Thus, it appears that one subpathway of HR DSB repair strictly forms a type of JM that requires resolution, the mechanism of which remains unclear (discussed below).

Strikingly, cells lacking ATRX expression, such as U2OS cells, rely completely on RECQ5 for HR-mediated repair of DSBs but are able to switch to the ATRX subpathway upon the induction of ATRX expression (Elbakry et al., 2021). The regulation of pathway choice seems to be dependent on proliferating cell nuclear antigen (PCNA) interaction, as both ATRX and RECQ5 possess PCNA-interaction peptide (PIP) domains that are essential for their HR function. Repair studies using mutants suggest that ATRX and RECQ5 compete for PCNA binding, possibly involving post-translational modifications (PTMs) that could regulate the downstream processes (Elbakry et al., 2021). The possibility of PTM-mediated regulation would be consistent with a role of RECQ5-dependent PCNA ubiquitination as well as PCNA-SUMO2 conjugation during transcription-replication conflict resolution, which serve to remove PCNA and RNA polymerase II from chromatin, respectively (Urban et al., 2016; Li et al., 2018). Additionally, it has been shown that yeast Srs2 interacts with SUMO-PCNA to promote SDSA by regulating the DNA polymerase, or by dissociating heteroduplex DNA (hDNA) at the D-loop and allowing second-end annealing and repair completion (Burkovics et al., 2013; Liu et al., 2017). Whether these PTMs or others influence HR outcome remains to be determined and would provide valuable insights about the regulation of subpathway choice during HR. This is particularly relevant considering that different cell types utilize the subpathways to various extents. For example, while ATRX-deficient cancer cells seem to rely on RECQ5 for HR, normal untransformed cells do not use RECQ5 and rely completely on ATRX for HR (Elbakry et al., 2021). Conversely, ATRX-proficient cancer cells, like HeLa cells, exhibit an uneven contribution from the two subpathways (**Figure 1**). This discrepancy in HR subpathway usage warrants a re-examination of a general one-size-fits-all model for the repair of breaks *via* HR and demands a more careful attention to the model systems and cell lines used. Differential subpathway usage also provides a novel way to assess HR proficiency in cancer cells that have a particular reliance on one subpathway or the other. Therefore, instead of solely focusing on upstream factors like BRCA1/2 and RAD51, we should also consider the downstream processes that define which subpathways are operating in the cell.

HR SUBPATHWAYS: MAKING ENDS MEET

Understanding how HR subpathways function and how the choice between them is regulated requires the consideration of the repair outcomes these pathways produce. The preference of a pathway favoring the formation of SCEs to one that avoids them challenges the common dogma that cells inherently avoid COs at all costs. To explain this discrepancy, it is worth examining how these conclusions were established. Many of the studies reporting rare CO occurrence were carried out using HR reporters in mammalian cells, and while they highly contribute to understanding pathway choice, the limitations of these systems could mask or skew these frequencies. One such limitation is reliance on ectopic or integrated artificial constructs that detect unequal recombination events or recombination between homologous chromosomes, all of which do not reflect the natural HR substrate of the identical sister (Johnson and Jasin, 2000; LaRocque et al., 2011; Zapotoczny and Sekelsky, 2017). Indeed, CO frequencies close to 50% can be observed in yeast, where the homologous chromosome represents a more natural recombination substrate (Ho et al., 2010; Yim et al., 2014). Furthermore, genetic analysis of the products in reporter systems in mammalian cells often differentiates only between long-tract gene conversion (LTGC) events and COs arising from short-tract gene conversion (STGC) and do not take into consideration that CO events arising from longer DNA synthesis tracts would be indistinguishable from non-CO LTGC events (Johnson and Jasin, 2000). This likely underestimates the frequency of CO in these systems, since COs have been associated with pathways involving longer tracts of DNA synthesis (Elliott et al., 1998; Mitchel et al., 2010; Yim et al., 2014). Additionally, the genetic background of cells used, such as U2OS cells that lack an HR factor, should be considered as this also affects the results from these reporters (Juhász et al., 2018; Elbakry et al., 2021). Therefore, while the notion that CO-avoiding pathways are preferred may or may not be valid, it is imperative to consider other factors influencing pathway choice and repair outcome. Cells deal with the various DNA-damaging lesions in distinct mechanisms, and those arising during S phase, which give rise to the majority

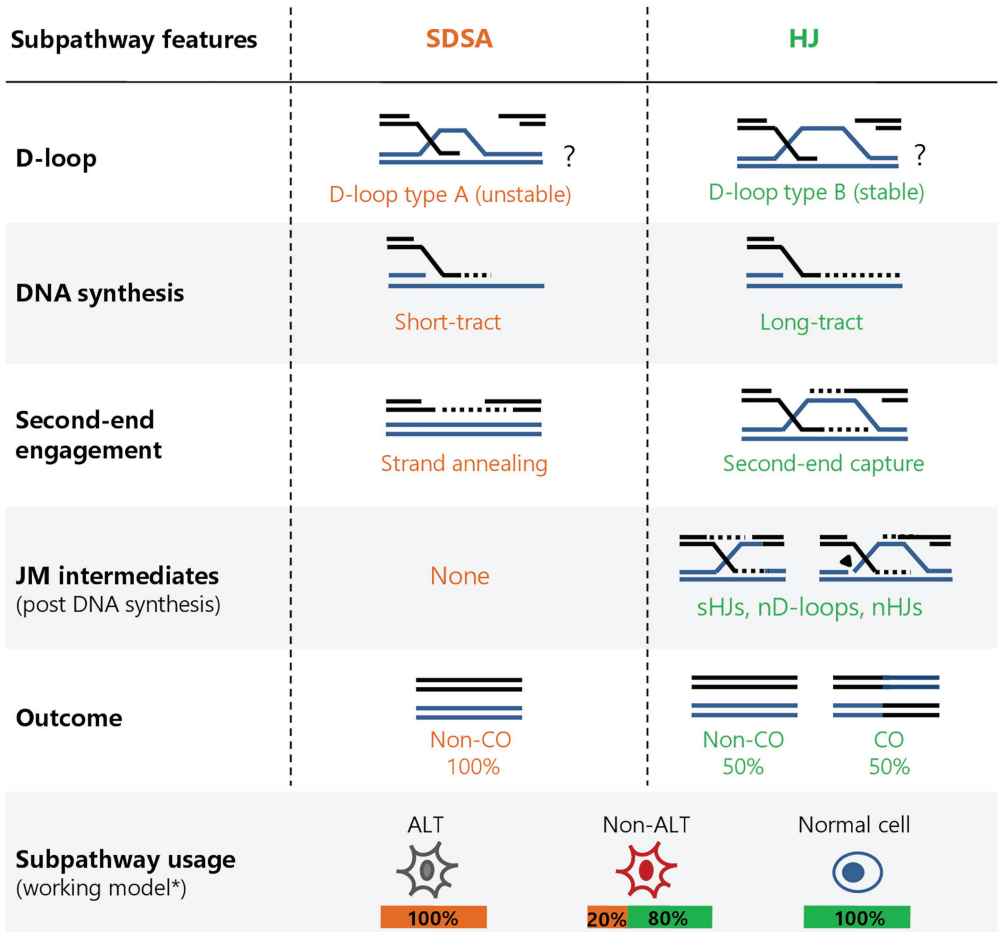

FIGURE 1 | Homologous recombination (HR) subpathway features at two-ended DNA double-strand breaks (DSBs). HR at two-ended breaks can proceed in distinct subpathways after RAD51-mediated strand invasion: synthesis-dependent strand annealing (SDSA; orange) and the Holliday junction (HJ; green) subpathway. Distinguishing features include the type and stability of displacement loop (D-loop; based on yeast models), extent of DNA repair synthesis, mode of second-end involvement, formation of joint molecules (JMs) post DNA synthesis (including single or nicked HJs and nicked D-loops) and repair outcome [crossover (CO) or non-CO]. Different types of cells use the subpathways to varying extents, largely influenced by their alternative lengthening of telomeres (ALT) status (see this paper). *These frequencies are non-comprehensive and are subject to change as more cell lines are analyzed.

of the spontaneous SCEs, could be handled differently from those at two-ended DSBs. As has been observed in yeast, recombination at ssDNA gaps results in dHJs requiring dissolution by Sgs1, while DSB-generated structures rely on nuclease-mediated resolution (Ho et al., 2010; Giannattasio et al., 2014). Therefore, the structure and nature of the lesion could influence the HR intermediates formed and how they are processed and consequently, whether or not they lead to genetic exchanges. Similarly, the genomic location of the lesion can dictate outcome, as has been shown for DSB repair pathway choice between HR and NHEJ (Beucher et al., 2009; Goodarzi et al., 2010; Aymard et al., 2014). Consistent with this, locus-specific SCE analysis showed that early and late replicating fragile sites exhibit differential SCE frequencies, suggesting that genomic locus and chromatin architecture could also influence HR subpathway choice (Waisertreiger et al., 2020). Furthermore, recent studies have demonstrated distinct mechanisms of HR-mediated repair of DSBs occurring in transcribed regions vs. transcriptionally silent loci, implicating the formation of DNA:RNA hybrids as novel regulators of HR (Yasuhara et al., 2018; Ouyang et al., 2021).

Notwithstanding the underlying mechanism, the preference of CO-forming pathways in certain contexts suggests that this is probably a less toxic outcome than products from alternative pathways. It is not yet clear how this could be the case for SDSA vs. a pathway involving a more complicated HR intermediate joining the two chromatids, as the fidelity of either subpathway has not been closely examined in the specific context of two-ended breaks. It is possible that factors such as D-loop stability, polymerase choice, and the fidelity of second-end engagement may play a role in ensuring accurate repair, even at the cost of an increased risk of CO formation. Not much is known about the regulation of the annealing step during SDSA and how the cell ensures the involvement of the correct ends. As HR normally deals with endogenous breaks that arise at replication forks that have one end, employing pathways that require two ends dictates the need to "wait"

until a second end is generated by an approaching replication fork. In this context, the premature displacement of the invading strand could cause its annealing to a non-matching break end, leading to translocations, a more detrimental outcome than a genetically silent CO (Ensminger and Löbrich, 2020). Therefore, a subpathway that has a more stringent second-end annealing condition, like second-end capture by the displaced D-loop strand, could be preferred. Second-end capture ensures enough homology is met, and also involves a structure refractory to termination by other pathways, like end-joining and/or single-strand annealing.

Furthermore, one pathway may involve a more stable intermediate structure that is resistant to dissociation and thereby could be favored to ensure repair completion. For example, studies utilizing novel D-loop analysis assays in yeast have shown the formation of two distinct D-loop species, whose lengths and abundance are regulated by Rad54 and its paralog Rdh54/Tid1 (Piazza et al., 2019; Shah et al., 2020). The features of the different D-loop species make them resistant to specific helicases and alterations in these properties influence HR outcome and survival. It is tempting to speculate that a similar mechanism may occur in human cells and can dictate subpathway choice (**Figure 1**). In this context, ATRX may cooperate with human RAD54 to form more stable intermediates (Spies et al., 2016; Juhász et al., 2018), possibly through a mechanism involving histone deposition within the D-loop (Elbakry et al., 2018). To investigate these possibilities, the required tools (e.g., D-loop capture and extension assays) need to be adapted and optimized for human cells. Furthermore, D-loop stability and extension can also be promoted by RNA:DNA hybrids arising during HR at transcribed regions (forming DR-loops), a feature that could influence subpathway choice (Ouyang et al., 2021).

While multiple factors can skew HR outcome by influencing subpathway usage, frequent COs during DSB repair can arise during the processing of HR intermediates that are preferentially channeled toward resolution instead of the CO-avoiding dissolution (Elbakry et al., 2021). This is a scenario where the structure-selective nucleases represent the main, rather than the back-up, pathway to handle JMs. Although, the activation of the MUS81-SLX4 and GEN1 complexes during late G2/M phase of the cell cycle (Pfander and Matos, 2017) could explain the preferential use of the nucleases at this stage, it does not exclude a role for the BTR dissolvasome, and raises the question if these JMs are intact dHJs, or in fact, dHJs at all (**Figure 1**). The preferential formation of COs from HR junctions has been indicated by the analysis of hDNA tracts in yeast and suggested a bias toward resolution explained by the presence of nicked or single HJs, which are more suitable substrates to the nucleases (Mitchel et al., 2010). Additionally, the formation of anaphase bridges arising from non-canonical HJs has been observed in yeast and was found to be specific to resolvase-deficient cells (García-Luis and Machín, 2014). Therefore, alternative JMs that are distinct from the canonical dHJ have been proposed in various contexts of HR by us and others and potentially occur more frequently than previously thought (Wright et al., 2018; Machín, 2020; Elbakry et al., 2021). The presence and frequency of these structures is yet to be determined and would both reflect the usage of distinct subpathways and dictate the requirement of specific downstream processing factors. Therefore, we find the use of the more general term "HJ pathway" more accurate when dealing with pathways involving JMs in DSB repair.

CLINICAL IMPLICATIONS OF HR SUBPATHWAY CHOICE

Homologous recombination deficiency has been used to target cancer cells for therapy ever since the concept of synthetic lethality has been elegantly shown in BRCA1/2 deficient cells treated with PARP inhibitors (Bryant et al., 2005; Farmer et al., 2005). This success has fueled further studies to identify other synthetically lethal targets in BRCA-defective cells, as well as cells deficient in other HR factors. Therefore, with an even deeper understanding of HR and the different subpathways involved, new strategies can be employed to effectively kill cancer cells. For example, cells that are defective in canonical HR subpathways and over-rely on other subpathways, such as BIR, can be selected by targeting BIR-specific factors. Alternatively, tumors lacking factors involved in particular subpathways can be targeted by identifying new synthetic lethal interactions specific to these tumors (**Figure 2**). Further, as

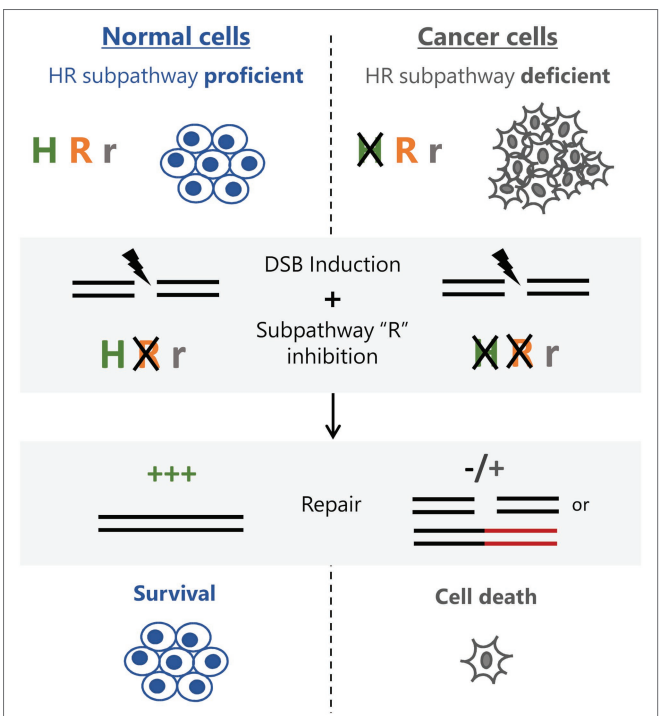

FIGURE 2 | Exploiting HR subpathway usage for cancer therapy. Normal cells have intact homologous recombination repair (HRR) and use predominantly subpathway "H" for DSB repair. Cancer cells lacking subpathway "H" rely predominantly on subpathway R for repair. Inhibiting subpathway "R" does not affect normal cells, which can repair normally and survive treatment. Cancer cells lose their main HRR functionality and either fail to repair or depend on more erroneous pathways (subpathway "r") leading to accumulation of breaks and/or toxic lesions and subsequent cell death.

demonstrated recently, the loss of the BIR factor PIF-1 can be exploited for selective killing of cells made to rely on this HR subpathway by the concurrent deletion of FANCM, revealing a new synthetic lethality relationship and an approach to target PIF-1 mutant cancer cells (Li et al., 2021). Also, it is known that the HR factor ATRX is defective in a variety of tumors that are commonly using the alternative lengthening of telomeres (ALT) mechanism of telomere maintenance (representing around 10–15% of all cancers; Dilley and Greenberg, 2015). While it is still not completely clear how loss of ATRX contributes to the ALT phenotype, exploiting a possible HR pathway imbalance (i.e., higher dependence on SDSA in tumors lacking ATRX), regardless of ALT status, could prove an effective approach to target these cells (**Figure 2**). This is particularly attractive if, as demonstrated, normal cells rely on the ATRX pathway for repair. Therefore, as the interplay between the HR subpathways becomes clearer and more defined, the therapeutic window of exploiting HR subpathways will expand, justifying a need for a better understanding of the mechanisms governing pathway choice.

AUTHOR CONTRIBUTIONS

AE and ML designed, revised, and edited the manuscript. AE wrote the first draft. All authors contributed to the article and approved the submitted version.

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DNA Double-Strand Break Repair: All Roads Lead to HeterochROMAtin Marks

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In response to DNA double-strand breaks (DSBs), chromatin modifications orchestrate DNA repair pathways thus safeguarding genome integrity. Recent studies have uncovered a key role for heterochromatin marks and associated factors in shaping DSB repair within the nucleus. In this review, we present our current knowledge of the interplay between heterochromatin marks and DSB repair. We discuss the impact of heterochromatin features, either pre-existing in heterochromatin domains or *de novo* established in euchromatin, on DSB repair pathway choice. We emphasize how heterochromatin decompaction and mobility further support DSB repair, focusing on recent mechanistic insights into these processes. Finally, we speculate about potential molecular players involved in the maintenance or the erasure of heterochromatin marks following DSB repair, and their implications for restoring epigenome function and integrity.

Keywords: chromatin mobility, chromatin remodeling factors, DNA double-strand break repair pathway choice, heterochromatin, histone variants, histone modifications

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INTRODUCTION: DIVERSITY AND FUNCTIONAL IMPORTANCE OF HETEROCHROMATIN MARKS

The organization of the genome into chromatin in the nuclear space serves to precisely orchestrate cellular functions by controlling gene expression. While the euchromatin compartment is generally accessible and associated with active gene transcription, heterochromatin is more condensed and mostly transcriptionally silent (Allshire and Madhani, 2018). Beyond this general definition, heterochromatin domains are actually quite diverse, in localization, regulation, and function. Constitutive heterochromatin is highly conserved between different cell types, rich in repeated sequences, and plays critical roles in the maintenance of chromosomal architecture and stability (Janssen et al., 2018; Penagos-Puig and Furlan-Magaril, 2020). The bulk of constitutive heterochromatin forms at pericentromeric regions, which are involved in the control of chromosomal segregation (Saksouk et al., 2015). Likewise, telomeres adopt a specific constitutive heterochromatin structure that serves to shield chromosomal ends from aberrant DNA repair, thus protecting chromosome integrity (de Lange, 2002; Allshire and Madhani, 2018; Lim and Cech, 2021). Facultative heterochromatin, in contrast, is developmentally regulated and varies across cell types. Its main function is to silence gene regions that should not be expressed in a specific developmental or somatic context (Trojer and Reinberg, 2007). The inactive X chromosome (Xi) is a typical example of facultative heterochromatin, which is established early during female mammalian development for the dosage compensation of X-linked genes

(Galupa and Heard, 2018). Heterochromatin also forms at the nuclear periphery through interactions with the nuclear lamina leading to lamina-associated domains (LADs), which play an important role in chromosome organization and gene repression (van Steensel and Belmont, 2017).

The establishment and maintenance of the silent state in heterochromatin domains involve DNA methylation and repressive histone post-translational modifications (PTMs). While constitutive and facultative heterochromatin are both enriched in DNA methylation, they show specific histone PTMs. Constitutive heterochromatin is enriched in H3K9me2/3, which is bound by heterochromatin protein 1 (HP1), a factor that plays a crucial role in heterochromatin assembly (Saksouk et al., 2015). Facultative heterochromatin instead shows an enrichment of H3K27me3 and H2AK119ub (Galupa and Heard, 2018), and the facultative heterochromatin mark H3K27me3 is also enriched in LADs (van Steensel and Belmont, 2017). In addition to DNA and histone modifications, heterochromatin domains incorporate specific histone variants (Martire and Banaszynski, 2020), such as centromere protein A (CENP-A) at centromeres and macroH2A1 in the Xi, and associate with architectural factors that determine the three-dimensional chromatin structure. In this review, DNA and histone modifications, histone readers, histone variants, and architectural factors enriched in heterochromatin domains are collectively referred to as heterochromatin marks or features. Remodeling factors (Clapier et al., 2017) provide another layer of regulation of heterochromatin accessibility by affecting nucleosome positioning. All these factors come into play to shape heterochromatin domains and mediate their function (Allshire and Madhani, 2018).

One of the major functions of heterochromatin is to ensure a tight control of transcriptional states, which is key for maintaining genome integrity and cell fate (Janssen et al., 2018). Nevertheless, heterochromatin domains also represent challenging environments for DNA metabolic activities, including DNA replication and DNA damage repair (Fortuny and Polo, 2018). Indeed, these domains are late replicating, highly compact, and often encompass repetitive sequences, which contributes to replication stress and fuels genome instability. Heterochromatin repeats are also prone to instability through ectopic recombination leading to deletions or translocations. Moreover, the highly compacted state and the low transcriptional activity in heterochromatin domains impede several repair pathways (Fortuny and Polo, 2018). These obstacles can be circumvented by alterations of the heterochromatin structure during the repair process.

Among the many types of DNA lesions, highly cytotoxic DNA double-strand breaks (DSBs) are repaired by multiple pathways with different levels of fidelity. Non-homologous end joining (NHEJ) is predominant and proceeds by direct ligation of DNA ends, while homologous recombination (HR) requires an initial resection of the DNA ends followed by recombination with a homologous template, usually the sister chromatid, which restricts HR to the S and G2 phases of the cell cycle (Chen et al., 2018; Zhao et al., 2020). Single-strand annealing (SSA) is based on homology on the same DNA strand and repairs DSBs between repeated sequences, leading to large deletions (Bhargava et al., 2016). DNA double-strand break repair by microhomology-mediated end joining (MMEJ) is also highly mutagenic, as it relies on short microhomology sequences that are exposed after end resection, and always generates small indels (Sallmyr and Tomkinson, 2018). The choice between several DSB repair pathways with different degrees of mutagenicity is thus decisive for the maintenance of genomic stability and is subject to complex regulatory mechanisms (Scully et al., 2019), including at the chromatin level. Recent studies have uncovered the key role of heterochromatin marks in dictating DSB repair pathway choice. DNA double-strand break repair, in turn, involves alterations in heterochromatin organization and heterochromatin marks, which need to be reverted to preserve epigenome integrity.

Here, by focusing on recent discoveries in the field, we provide an overview of our current knowledge of the interplay between heterochromatin marks and DSB repair and discuss potential mechanisms that preserve the integrity of heterochromatin domains.

HETEROCHROMATIN FEATURES DIRECT DSB REPAIR PATHWAY CHOICE

Heterochromatin marks and associated factors not only play critical roles in transcriptional silencing but also contribute to regulate DSB repair pathway choice, in part by controlling the recruitment of DSB repair factors. This regulation has been observed both in heterochromatin domains where heterochromatin marks are present before damage infliction and in euchromatin domains where DSBs trigger the deposition of specific heterochromatin marks. In this section, we discuss recent studies that provided new mechanistic insights into the regulation exerted by heterochromatin marks on DSB repair.

Role of Heterochromatin-Specific Histone Modifications in DSB Repair Pathway Choice

Histone PTMs constitute an important layer of epigenomic information with a broad impact on chromatin organization and function; some of these marks define heterochromatin domains and have been shown to regulate DSB repair responses.

For instance, a well-known PTM enriched in constitutive heterochromatin is H3K9me3. As previously described, an increase of this mark was observed at break sites in mammalian cells, both in heterochromatin and in euchromatin regions (Ayrapetov et al., 2014; Tsouroula et al., 2016; Natale et al., 2017), and several players in the H3K9me3 pathway – writers (SUV39H1/2, SETDB1) and readers (HP1, TIP60) – were shown to promote DSB repair by HR (Sun et al., 2009; Baldeyron et al., 2011; Soria and Almouzni, 2013; Tang et al., 2013; Alagoz et al., 2015; Jacquet et al., 2016). In line with these studies, the H3K9 methyltransferase SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) was also shown to regulate alternative lengthening of telomeres (ALT) in mouse

cells by creating an H3K9me3-rich heterochromatin environment that facilitates recombination (Gauchier et al., 2019).

Recent studies have elucidated the mechanism underlying the deposition of H3K9me3 around DSBs and described new interactions between this histone modification and the regulation of DSB repair. It was shown that H3K9me3 actually depends on another damage-induced PTM on histone H4. The DSB sensor complex MRE11-RAD50-NBS1 indeed recruits UFM1-specific ligase 1, leading to the conjugation of a ubiquitin-like protein to histone H4 lysine 31, a process known as ufmylation. This histone PTM is bound by the serine/threonine-protein kinase 38 (STK38), which in turn recruits the H3K9 methyltransferase suppressor of variegation 3–9 homolog 1 (SUV39H1) leading to the trimethylation of H3K9 around DSBs (Qin et al., 2019, 2020). The local increase of H3K9me3 at DSB sites seems to be crucial for HR as shown in human cancers with elevated levels of oncometabolites that inhibit the lysine demethylase KDM4B. This causes an aberrant constitutive hypermethylation of H3K9 instead of a local increase at break sites, which diverts TIP60 away from the DSBs, thereby impairing HR activation (Sulkowski et al., 2020).

The local increase of H3K9me3 points toward a heterochromatinization phenomenon that may be necessary for HR repair. However, one also has to consider how the DSB repair machinery handles breaks in heterochromatin regions that are already decorated with this mark. Indeed, HR could lead to mutagenic recombination in heterochromatin compartments due to their highly repetitive nature. Several strategies actually serve to prevent HR in H3K9me3-containing heterochromatin domains. In mouse cells for instance, resection of the DNA ends leads to their relocalization to the periphery of pericentromeric heterochromatin, where recombination takes place (Tsouroula et al., 2016). In *Drosophila melanogaster* in contrast, DSBs in pericentromeric heterochromatin trigger the recruitment of *Drosophila* lysine demethylase 4a, which demethylates H3K9me3 and H3K56me3, another conserved pericentromeric heterochromatin mark (Jack et al., 2013). This demethylase channels repair to NHEJ by inhibiting the recruitment of early HR factors to heterochromatic DSBs (Janssen et al., 2019).

In addition to H3K9me3, H3K27me3 also decorates heterochromatin regions, such as those associated with the lamina, and DSBs in these H3K27me3-enriched regions show increased repair by MMEJ (Lemaître et al., 2014; Schep et al., 2021). Interestingly, chemical inhibition of H3K27 and not of H3K9 methyltransferases shifted the MMEJ/NHEJ balance toward NHEJ (Schep et al., 2021), arguing that the H3K27me3 heterochromatin mark either stimulates MMEJ or inhibits NHEJ. However, the underlying molecular mechanisms still need to be elucidated and the impact of H3K27me3 on HR is unknown. Similar to H3K9me3, H3K27me3 was also found increased at DSBs in some studies but with conflicting results in other studies (reviewed in Ferrand et al., 2020), so further work is needed to clarify the status of this mark at DSBs in and outside heterochromatin.

Besides H3K9me3 and H3K27me3, other histone modifications play a role in DSB repair regulation in heterochromatin domains. For instance, several H3K36me2-specific histone methyltransferases, including multiple myeloma SET domain-containing protein (MMSET), promote NHEJ at deprotected telomeres in mouse cells. Interestingly, the involvement of H3K36me2 seems to occur downstream of DSB recognition and repair pathway choice (de Krijger et al., 2020).

While the mechanisms through which H3K9me3 impacts DSB repair are now quite well characterized, how other histone marks, like H3K27me3 and H3K36me2, influence this process is still unknown. Further studies are necessary to determine whether those marks modulate the recruitment of specific DSB repair factors to chromatin. In addition, it will be interesting to investigate whether histone PTMs also underlie the differential regulation of HR between centromeric and pericentromeric heterochromatin observed in mouse cells (Tsouroula et al., 2016). Homologous recombination of centromeric DSBs is indeed licensed in G1, in addition to S/G2, which could rely on histone marks decorating centromeres, such as H3K4me2, H3K36me2, and H3 acetylation (Chan and Wong, 2012).

Role of Heterochromatin-Specific Histone Variants in DSB Repair Pathway Choice

Besides histone modifications, another layer of chromatin regulation builds upon the incorporation of histone variants (Martire and Banaszynski, 2020), some of which are enriched in heterochromatin and regulate DSB repair. Such regulation occurs in already histone variant-enriched heterochromatin domains and also upon the accumulation of these variants at euchromatic DSBs.

The histone variant MacroH2A1, for instance, is enriched in facultative heterochromatin domains on autosomes and on the inactive X chromosome (Costanzi and Pehrson, 1998; Changolkar and Pehrson, 2006; Gamble et al., 2010). Remarkably, the macroH2A1 gene expresses two splicing isoforms: macroH2A1.1 and macroH2A1.2, which exhibit antagonistic properties in the regulation of DSB repair pathway choice in mammalian cells. MacroH2A1.2 accumulates at DSBs in an ataxia telangiectasia mutated (ATM)-dependent manner and stimulates DSB repair by HR by promoting the recruitment of breast cancer type 1 susceptibility protein (BRCA1; Khurana et al., 2014). Similarly, macroH2A1.2 deposition at sites of replication stress by the histone chaperone facilitates chromatin transcription (FACT) forms a chromatin environment amenable for BRCA1 recruitment (Kim et al., 2017). In human cells lacking the chromatin remodeler Alpha thalassemia/mental retardation syndrome X-linked (ATRX), macroH2A1.2 is also highly enriched at telomeres and contributes to ALT, a HR-mediated process (Kim et al., 2019). Mechanistically, macroH2A1.2 collaborates with the histone demethylase KDM5A to promote both DSB repair by HR and transcriptional silencing at breaks (Kumbhar et al., 2021). MacroH2A1.1 in contrast supports MMEJ, a mutagenic DSB repair pathway (Sebastian et al., 2020). The preferential interaction of macroH2A1.1 with MMEJ repair factors, including poly(ADP-ribose) polymerase 1 (PARP1), is likely linked to the ability of this isoform to bind ADP-ribose, a property

that is not shared with macroH2A1.2 (Sebastian et al., 2020). MacroH2A1.2-deficient cells display X-chromosome instability due to defective HR and enhanced MMEJ. Interestingly, loss of macroH2A1.1 rescues the X-chromosome instability observed in macroH2A1.2-deficient cells (Sebastian et al., 2020). This nicely illustrates how histone variants exert antagonistic control on DSB repair pathway choice and genome integrity in facultative heterochromatin.

Another histone variant that may regulate DSB repair is CENP-A, which defines centromeric heterochromatin. There is conflicting evidence regarding CENP-A accumulation post DSBs (Zeitlin et al., 2009; Ambartsumyan et al., 2010; Helfricht et al., 2013), and the link between CENP-A and DSB repair has not yet been explored. It will be interesting to investigate whether CENP-A can contribute to licensing HR in G1 at centromeric DSBs (Tsouroula et al., 2016). Furthermore, the CENP-A chaperone HJURP may have functional connections to DSB repair (Kato et al., 2007).

The Role of Heterochromatin-Associated Factors in DSB Repair Pathway Choice

In addition to histone variants and modifications, several heterochromatin-associated factors play a central role in DSB repair, including histone readers, architectural factors, and chromatin remodelers.

Chromatin remodelers were shown to regulate chromatin relaxation at heterochromatic DSBs in mammalian cells. In this respect, imitation switch (ISWI)-class and chromodomain-helicase-DNA binding (CHD)-class chromatin remodelers play antagonistic roles. CHD3 promotes heterochromatin compaction, but is released from chromatin following ATM activation, while ACF1 and SNF2H (ISWI class) are recruited to the damage site and lead to heterochromatin decompaction, which allows Artemis-dependent NHEJ (Klement et al., 2014). A recent study put forward the involvement of another chromatin remodeler in stimulating DSB repair by HR in heterochromatin. The human chromatin remodeler lymphoid-specific helicase (HELLS), through its ATPase activity, indeed promotes HR of heterochromatic DSBs in G2 cells exposed to ionizing radiation by facilitating end resection through CTBP-interacting protein (CtIP) recruitment (Kollárovič et al., 2020). Whether the function of HELLS in HR repair of heterochromatic breaks is linked to its ability to promote macroH2A1.2 deposition (Ni et al., 2020; Xu et al., 2021) is an intriguing possibility that deserves further investigation. The involvement of multiple remodelers, some of which having opposing activities, likely allows a fine-tuning of heterochromatin compaction during DSB repair, with dynamic changes over time after DSB induction.

Among readers of heterochromatin-specific modifications, HP1 is recruited to DSBs arising in euchromatin and heterochromatin domains, and a major regulator of DSB repair, with HP1 isoforms having different effects on DSB repair pathways: HP1α and β stimulate HR at the resection step, while HP1γ inhibits this pathway (Baldeyron et al., 2011; Soria and Almouzni, 2013). In line with these findings, HP1γ depletion, but not that of HP1α and β, negatively impacts Ku80 recruitment to heterochromatic DSBs in mouse cells (Tsouroula et al., 2016), suggesting that the HP1γ isoform may play a role in NHEJ. A possible mechanism through which some HP1 isoforms channel DSB repair toward HR might rely on the direct binding of HP1 to BRCA1-associated RING domain 1 (BARD1) in response to DSBs, which promotes retention of the BARD1-BRCA1 complex stimulating CtIP-dependent resection (Wu et al., 2015).

Finally, the heterochromatin-enriched architectural factor structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) contributes both to transcriptional silencing and to DSB repair. It is still unknown if both functions of SMCHD1 are mechanistically connected. This protein indeed plays a crucial role in mammalian X chromosome inactivation (Blewitt et al., 2008) and is involved in the silencing of specific autosomal genes (Gendrel et al., 2013; Mould et al., 2013). In addition, SMCHD1 is recruited to DNA damage foci (Coker and Brockdorff, 2014; Tang et al., 2014) and is highly enriched on deprotected telomeres in human cells (Vancevska et al., 2020), pointing to a role in the DSB response. SMCHD1 actually contributes to DSB repair pathway choice by promoting NHEJ while inhibiting HR, as shown using cell reporter systems (Tang et al., 2014). Consistent with this, SMCHD1 stimulates 53BP1 foci formation and impairs BRCA1 foci formation following cell treatment with the radiomimetic drug zeocin (Tang et al., 2014). SMCHD1 also promotes the fusion of unprotected telomeres, which relies on NHEJ; however, the function of SMCHD1 seems to be upstream of DSB repair at telomeres through the stimulation of ATM-dependent damage signaling (Vancevska et al., 2020).

Together, these studies illustrate that several heterochromatin marks, including histone trimethylation, histone variants, and non-histone proteins, regulate DSB repair pathway choice (**Figure 1**). Interestingly, some marks with opposing activities on DSB repair are enriched in the same heterochromatin domain, as observed for macroH2A1.1, 1.2, SMCHD1, and H3K27me3 on the Xi. This might suggest an interplay between heterochromatin marks, which could allow a fine regulation of DSB repair pathways. Notably, in addition to HR, NHEJ, and MMEJ, DSB repair by SSA also operates in heterochromatin, in particular when HR is compromised (Janssen et al., 2016; Tsouroula et al., 2016), but whether heterochromatin marks stimulate SSA is still unknown. Further studies are necessary to clarify the mechanisms through which heterochromatin marks modulate DSB repair and to assess the combinatorial effects of these marks.

ALTERATION AND MAINTENANCE OF HETEROCHROMATIN FEATURES IN RESPONSE TO DSBs

While the choice of repair pathway is influenced by the chromatin context, DSB repair itself leads to changes in heterochromatin

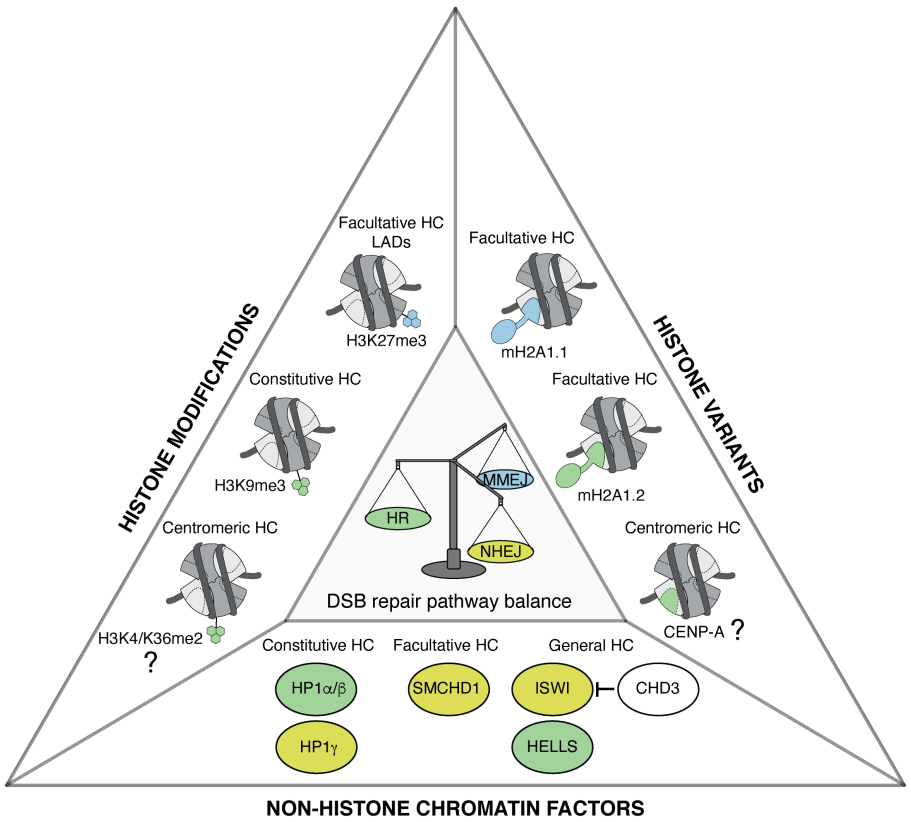

FIGURE 1 | Heterochromatin features govern DNA double-strand break (DSB) repair pathway choice. Representation of heterochromatic (HC) histone modifications, histone variants, and non-histone chromatin factors that modulate DSB repair pathway choice in mammalian cells. Features that favor homologous recombination (HR), non-homologous end joining (NHEJ), and microhomology-mediated end joining (MMEJ) are shown in green, yellow, and blue, respectively. The types of heterochromatin enriched in these features are indicated when known. The contribution of centromeric histone variant and modifications to promoting HR in G1 is still to be determined, as indicated by the question marks.

organization, which can have a profound impact on its function and thus on the maintenance of genomic integrity.

Heterochromatin Decompaction and Mobility of Heterochromatic Breaks

Heterochromatin is a highly compacted nucleoprotein structure that can be seen as an obstacle for the detection of DNA lesions and their repair. However, in mammals and *D. melanogaster*, it was observed that DSB repair kinetics were comparable between heterochromatin and euchromatin (Goodarzi et al., 2008; Janssen et al., 2016). This is achieved, at least in part, through the decompaction of heterochromatin, which facilitates DSB signaling and repair. Mechanistically, heterochromatin decompaction is regulated by chromatin remodelers, as discussed above, and by the ATM kinase, which phosphorylates KRAB-associated protein 1 (KAP-1) thus triggering its eviction from chromatin (Ziv et al., 2006; Goodarzi et al., 2008). Heterochromatin decompaction has been observed in several heterochromatin compartments in response to DSBs induced by ionizing radiation or by site-specific nucleases, including the Xi compartment in female mammalian cells (Müller et al., 2013) and pericentromeric heterochromatin domains in *Drosophila* and mouse cells (Chiolo et al., 2011; Tsouroula et al., 2016). Of note, the expansion of pericentromeric regions is not specific to the DSB response as it is also observed upon UV damage detection by DNA damage-binding protein 2 (DDB2), which triggers the eviction of linker histone H1 from chromatin (Fortuny et al., 2021). H1 eviction is also reported in response to DSBs (Strickfaden et al., 2016; Clouaire et al., 2018; Li et al., 2018), but whether linker histone eviction participates to heterochromatin decompaction post DSBs is not yet known.

Mechanistically, chromatin decompaction is accompanied by DSB repositioning outside of pericentromeric heterochromatin domains, favoring repair completion through HR, as shown in both *Drosophila* and mouse cells (Chiolo et al., 2011; Tsouroula et al., 2016; Amaral et al., 2017). In both systems, the resection of the broken ends occurs within pericentromeric heterochromatin and triggers their migration to the periphery of these domains in mouse cells, and to the nuclear periphery in *Drosophila* cells, where RAD51-mediated recombination takes place (Chiolo et al., 2011; Tsouroula et al., 2016). A similar process may occur in response to DSBs in the Xi. Indeed, while 53BP1 is

found both within and outside of the Xi, phosphorylated RPA localizes at the Xi periphery, indicating a possible relocalization of the breaks undergoing resection, to be repaired by HR (Müller et al., 2013). Functionally, such relocalization of breaks outside heterochromatin domains is thought to prevent aberrant ectopic recombination between repeated sequences.

A role for the nucleoskeleton and molecular motors in the relocalization of heterochromatic breaks was put forward in several species (**Figure 2**). In *Drosophila* for instance, nuclear actin filaments and myosins promote the relocalization of pericentromeric DSBs to the nuclear periphery (Caridi et al., 2018; Dialynas et al., 2019), with a contribution of sumoylation and of structural maintenance of chromosomes 5/6 proteins (SMC5/6) in anchoring the breaks to the nuclear periphery (Ryu et al., 2015). Mechanisms appear to be distinct in mammalian cells where SMC5/6 proteins are dispensable, and anchoring of breaks to the nuclear periphery does not occur (Tsouroula et al., 2016). Nuclear actin drives the migration of a subset of breaks undergoing HR also in mammalian cells (Schrank et al., 2018), but whether those correspond to heterochromatic breaks is not known. Similar to DSBs in pericentromeric heterochromatic repeats, DSBs in nucleolar repeats trigger chromatin mobility. Indeed, in human cells, nucleolar DSBs relocalize to the periphery of nucleoli where they are repaired by HR (van Sluis and McStay, 2015). A recent study provided the first clue to molecular players controlling the relocalization of nucleolar breaks, with a role for myosin chaperones and actin-related proteins (Marnef et al., 2019). The linker of the nucleoskeleton

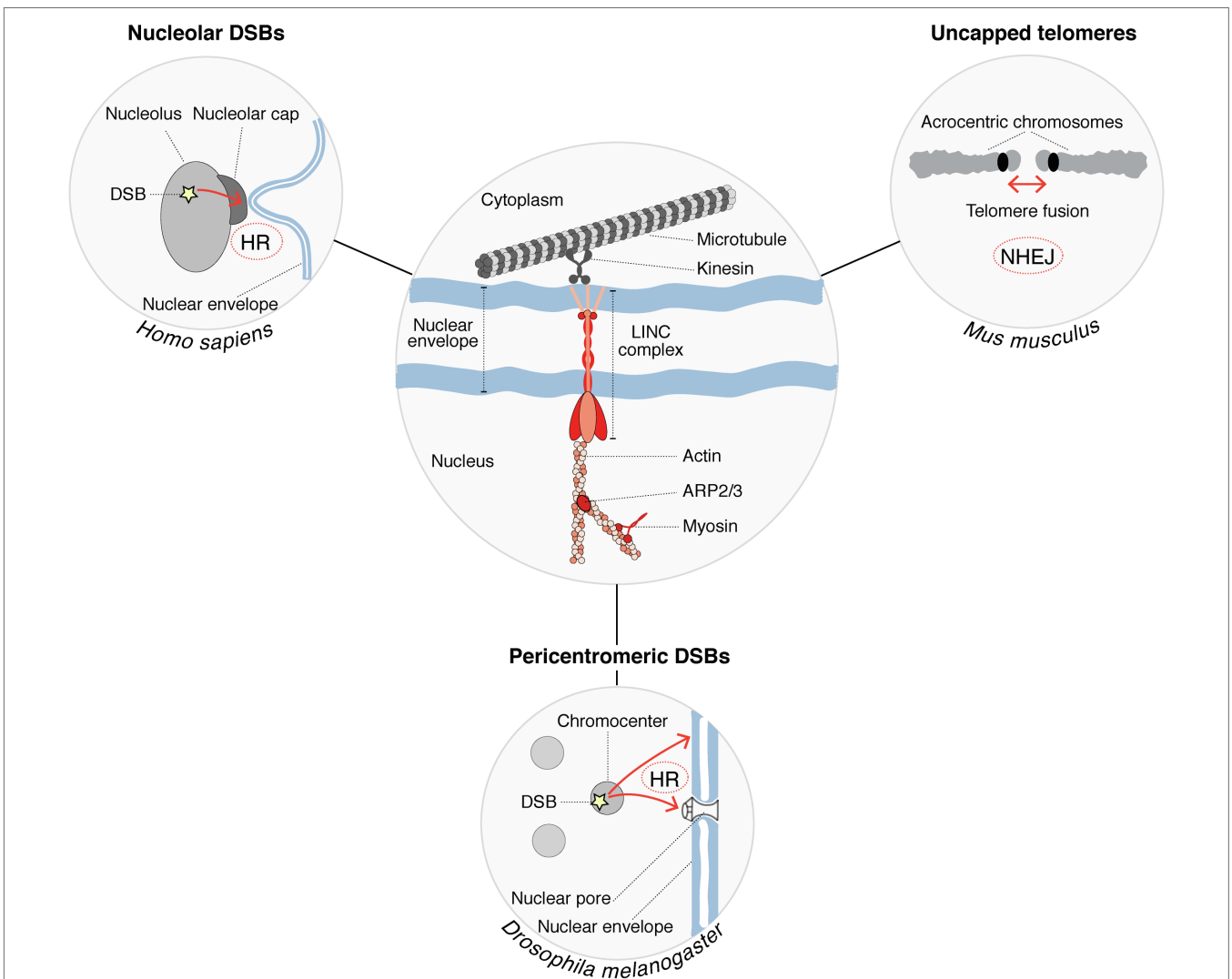

FIGURE 2 | Cytoskeleton factors regulate the mobility of damaged heterochromatin to support DSB repair. Nucleoskeleton factors and molecular motors (shown in red in the center circle) are involved in the mobility of heterochromatic DSBs (DSB mobility is represented by red arrows in the peripheral circles). Chromatin mobility is further supported by microtubules, which provide mechanical forces. This process is conserved in several species and promotes the repair of DSBs occurring within different heterochromatin compartments. DSB repair pathways operating in each case are indicated.

and cytoskeleton (LINC) complex, embedded in the nuclear envelope, also contributes to nucleolar DSB mobility, which involves nuclear envelope invaginations that connect nucleoli (Marnef et al., 2019). Similarly, DSB repair by NHEJ in heterochromatin domains invokes microtubule-mediated chromatin mobility, as reported for the fusion of uncapped telomeres in mouse cells, which is promoted by 53BP1-dependent chromatin mobility through the LINC complex (Dimitrova et al., 2008; Lottersberger et al., 2015). This microtubule-mediated heterochromatin mobility stimulates NHEJ of dysfunctional telomeres (Lottersberger et al., 2015). Together, these studies put forward the role of the nucleoskeleton in regulating damaged heterochromatin mobility to support DSB repair in several contexts (**Figure 2**). Considering the emerging role of phase separation in regulating DNA damage responses (Spegg and Altmeyer, 2021), we can envision molecular condensates as part of an alternative or cooperative mechanism to control DSB dynamics.

Maintenance of Heterochromatin Organization in Response to DSBs

Heterochromatin regions play a crucial role in silencing transposable elements and in regulating the segregation and stability of chromosomes (Allshire and Madhani, 2018). Thus, the maintenance of heterochromatin features is essential to preserve genome integrity and cell identity. However, very little is known regarding whether and how heterochromatin organization is faithfully re-established after DSB repair. One would assume that the heterochromatin compaction state would be restored and that repaired loci would retrieve their original positions inside heterochromatin domains. To address these questions, long-term kinetic analyses should be carried out post DSB induction in order to follow changes in heterochromatin organization during the course of the repair process and even beyond DSB repair completion. A refined analysis of heterochromatin folding during DSB repair would also be needed. Several recent studies have shed light on the impact of DSBs on chromatin folding in the nuclear space and on the importance of 3D chromatin organization in shaping DSB responses by exploiting chromatin conformation capture and super-resolution microscopy (Natale et al., 2017; Ochs et al., 2019; Sanders et al., 2020; Arnould et al., 2021). Similar approaches would help to determine whether heterochromatin compartments retrieve their original topology after DSB repair and to dissect the underlying molecular mechanisms.

Despite dramatic changes in heterochromatin organization following DNA breaks, some heterochromatin marks are maintained during the DSB repair process, as shown for H3K9me3 in mouse pericentromeric heterochromatin (Tsouroula et al., 2016; Natale et al., 2017) but not in *Drosophila*, where H3K9me3 levels decrease post DSB (Janssen et al., 2019). The mechanisms supporting the maintenance or the restoration of H3K9me3 within these heterochromatic domains are not yet elucidated; however, it is tempting to envision a similar response to what is observed in UV-damaged pericentromeric heterochromatin domains in mouse cells, where the histone methyltransferase SETDB1 is recruited and coordinates the maintenance of H3K9me3 with new H3 deposition during UV damage repair (Fortuny et al., 2021).

Removal of Heterochromatin Features From Euchromatin Domains Following DSB Repair

DNA double-strand breaks within transcribed genes trigger the incorporation of heterochromatin-specific histone variants and histone post-translational modifications, leading to a transient heterochromatinization of the damaged locus, which contributes to transcriptional silencing (**Figure 3**). Among the many regulators of transcriptional silencing at DSBs (Caron et al., 2019), heterochromatin marks play a pivotal role. For instance, mono-ubiquitylation of H2A on Lys 119 is induced in the vicinity of DSBs and governs transcriptional silencing (Shanbhag et al., 2010). Interestingly, the removal of H2AK119ub involves the deubiquitinase ubiquitin-specific peptidase 16 (USP16) and is crucial for transcription restart after DSB repair (Shanbhag et al., 2010). Silencing at DSBs is also contributed to by the deposition of H3K27me3 through the PARP1-EZH2 axis (Abu-Zhayia et al., 2018). However, how this histone mark is removed once the break is repaired and whether it is required to license transcription restart is still unknown. The histone demethylase ubiquitously-transcribed tetratricopeptide repeat X (UTX) was shown to diminish H3K27me3 levels in response to ionizing radiation (Rath et al., 2018). It is thus tempting to speculate that the removal of H3K27me3 upon DSB repair may be mediated by UTX, contributing to transcription restart. The transient heterochromatinization at euchromatic breaks also involves H3K9me2/3 deposition (Ayrapetov et al., 2014; Khurana et al., 2014). H3K9 dimethylation is deposited by PR/SET domain 2 (PRDM2), recruited to DSBs in a manner dependent on the histone variant macroH2A1.2 (Khurana et al., 2014). This histone variant also inhibits transcription at DSBs by stimulating H3K4me3 demethylation by KDM5A (Kumbhar et al., 2021). Further studies will be needed to investigate reversal mechanisms of these heterochromatin marks after DSB repair, including the removal of macroH2A1.2, H3K9me2/3, and their importance for transcription recovery.

CONCLUSION AND FUTURE DIRECTIONS

During the last decade, a growing number of studies have highlighted the key contribution of histone post-translational modifications and factors implicated in heterochromatin formation in the response to DSBs. Heterochromatin marks, either pre-existing in heterochromatin domains or *de novo* established in euchromatin, indeed play a central role in regulating DSB repair pathway choice. Thus, heterochromatin features should not be considered as barriers to DSB repair

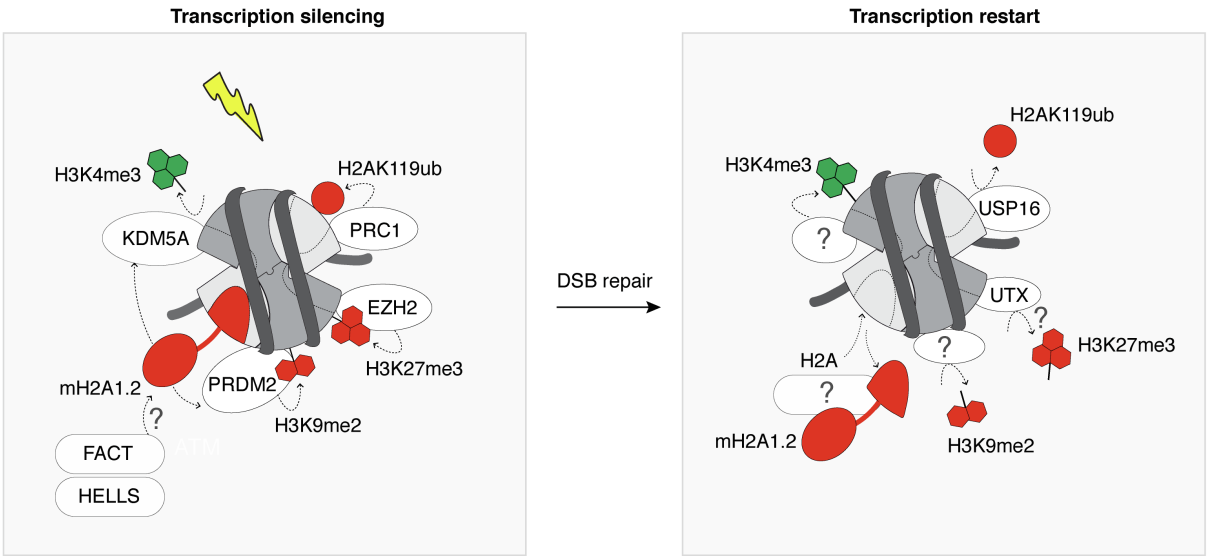

FIGURE 3 | Heterochromatin features are transiently established following DSBs. Following DSB induction, the chromatin surrounding the lesion is modified by the deposition of heterochromatin-specific histone variants and post-translational modifications (red) at the expense of active marks (green). This contributes to silence transcription in the vicinity of the DSB. For simplicity, all chromatin marks are represented on a single nucleosome flanking a DSB. Most of the players that promote the recovery of the pre-existing marks after completion of DSB repair are still unknown, as indicated by the question marks. White ovals and rectangles represent histone modifying enzymes and histone chaperone/remodeler, respectively.

but as fine-tuners of the DSB response. While our knowledge of the DSB repair pathways that operate in different heterochromatin domains is increasing, the players involved in restoring heterochromatin organization and in erasing heterochromatin marks from euchromatin regions after DSB repair are still unknown. Beyond histone variants and modifications, another crucial epigenetic mark enriched in heterochromatin domains is DNA methylation. Interestingly, DSB repair alters DNA methylation patterns (Sriraman et al., 2020), but little is known about the mechanisms allowing DNA methylation restoration. Furthermore, the DNA methyltransferase DNMT1 can read heterochromatin histone marks thus protecting cells against ionizing radiation (Ren et al., 2020, 2021). These findings suggest a potential role for DNA methylation in controlling DSB repair responses, which is still to be elucidated. Future work will shed light on these mechanisms and on the interplay between different heterochromatin marks in regulating DSB responses. This will help move toward a better characterization of genome and epigenome maintenance processes whose defects underlie pathological disorders.

AUTHOR CONTRIBUTIONS

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Tissue Specific DNA Repair Outcomes Shape the Landscape of Genome Editing

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The use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 has moved from bench to bedside in less than 10 years, realising the vision of correcting disease through genome editing. The accuracy and safety of this approach relies on the precise control of DNA damage and repair processes to achieve the desired editing outcomes. Strategies for modulating pathway choice for repairing CRISPR-mediated DNA double-strand breaks (DSBs) have advanced the genome editing field. However, the promise of correcting genetic diseases with CRISPR-Cas9 based therapies is restrained by a lack of insight into controlling desired editing outcomes in cells of different tissue origin. Here, we review recent developments and urge for a greater understanding of tissue specific DNA repair processes of CRISPR-induced DNA breaks. We propose that integrated mapping of tissue specific DNA repair processes will fundamentally empower the implementation of precise and safe genome editing therapies for a larger variety of diseases.

Keywords: CRISPR-Cas9, genome editing, DNA double-strand break, homology directed repair, non-homologous end-joining, microhomology mediated end-joining, tissue specific DNA repair, tissue stem cells

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DNA DOUBLE-STRAND BREAK REPAIR: THE FOUNDATION FOR GENOME EDITING

Genome stability is constantly challenged by endogenous and exogenous factors that threaten the integrity of DNA. If DNA damage is incorrectly repaired, this leads to mutations or widespread genome aberrations that impair cell function and survival. Intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS), reactive metabolites, and replication stress synergise with exogenous genotoxic sources of damage, such as radiation, chemical exposure, viral, or bacterial infections to challenge genomic stability. In order to protect genome integrity, cells have evolved sophisticated mechanisms to detect, signal, and repair diverse DNA lesions, known as the DNA damage response.

Biological Significance of DNA Double-Strand Breaks

DNA double-strand breaks (DSBs) are amongst the most toxic lesions cells can encounter, as both DNA ends become topologically separated. For this reason, DSBs are induced in cancer therapy, either through ionising radiation or by preventing their repair *via* topoisomerase inhibition. In contrast, formation of endogenous DSBs is an integral part of fundamental

cellular processes, such as the generation of immune receptor diversity, meiosis, and ageing (Jackson and Bartek, 2009). Therefore, DSB repair is an essential and vital cellular process. Overall, DSBs are repaired in two ways: re-ligation of the DNA ends through pathways such as non-homologous end-joining (NHEJ) and microhomology-mediated end-joining (MMEJ), or templated repair from a separate donor DNA molecule, through a process called homology directed repair (HDR; Yeh et al., 2019). A key aspect in the repair of DSBs in human cells is the competition between these two types of repair, with end-joining pathways being favoured over templated repair, in a cell-cycle dependent manner.

Cas9-Induced DNA Double-Strand Breaks: The Genome Editing Revolution

During the early 2000s, site-specific DSB generation, induced by engineered endonucleases, became an increasingly useful approach to edit the genome. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been successfully used as genome editing tools in mammalian cells (Miller et al., 2011; Hossain et al., 2015). However, inherent difficulties with protein design, synthesis, and validation remained a challenge to the widespread implementation of these nuclease-based editing technologies. This limitation was solved upon the discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), a breakthrough that revolutionised the field of genome editing (Jinek et al., 2012). CRISPR and the associated Cas9 endonuclease (CRISPR-Cas9) were initially identified as an antiviral defence mechanism in prokaryotes, but rapidly became a powerful genome editing tool in eukaryotic cells (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). The CRISPR-Cas9 system, guided by a single-guide RNA (sgRNA), targets a particular region of the genome, generating a DNA DSB that subsequently activates the cellular DNA repair machinery. The considerable ease of manipulating the sgRNA, compared to ZFNs and TALENs, has served an important role in the CRISPR revolution, creating the possibility to edit a wide variety of cell types and organisms, with unprecedent precision and efficiency. Importantly, besides being a powerful approach for functional genetic studies, CRISPR-Cas9 approaches hold great promise for the correction of genetic disorders caused by specific alterations in the genome, with recent clinical trials reporting promising results (Wang et al., 2020; Frangoul et al., 2021). However, most current clinical applications are still based on the disruption of a genetic sequence, rather than a precise edit. Moreover, the safety and efficiency of CRISPR-based therapies still need to be closely addressed and an important step is the fundamental understanding of the tissue specific DNA repair pathway choice, following a Cas9-induced DSB. The focus of this review will be on the DSB-dependent genome editing technologies which make use of *Streptococcus pyogenes* Cas9 (SpCas9), generating a blunt end at a targeted genomic site. We direct readers to the following additional technical advances that have expanded the CRISPR-toolbox and fall outside the focus of this review: engineered Cas9 nucleases with higher fidelity (Kleinstiver et al., 2016) and broader specificity (Kleinstiver et al., 2015; Walton et al., 2020), DSB-independent applications that increase the range of possible editing outcomes, such as DNA base editors (Komor et al., 2016; Gaudelli et al., 2017) and prime editing (Anzalone et al., 2019), CRISPR-mediated regulation of gene expression (Gilbert et al., 2013; Qi et al., 2013; Nuñez et al., 2021), and new CRISPR nucleases repurposed for genome editing (Zetsche et al., 2015).

REPAIR OF Cas9-INDUCED DNA DOUBLE-STRAND BREAKS

Cell Cycle Regulates DNA Double-Strand Break Repair Pathway Choice

After a Cas9-induced DSB, repair pathway choice is a crucial factor in determining the editing outcome. The blunt ends of the DNA break can be protected by the Ku70/80 heterodimer, fating the lesion for repair by NHEJ. Conversely, 5'–3' resection of DNA ends reveals sequence homologies that direct repair toward HDR or MMEJ (Yeh et al., 2019). Therefore, the processing of DSB ends from blunt ends to overhangs, *via* end-resection, is the major factor dictating repair pathway choice. Although HDR faithfully repairs lesions, the end-joining pathways are preferentially upregulated through several mechanisms following DSB formation. This is because NHEJ is active throughout all phases of the cell cycle, predominating in G0 and G1 (Shrivastav et al., 2008), whereas factors that promote extensive end-resection are more active during S and G2 phases, favouring HDR when a sister chromatid is present (Chang et al., 2017). The balance between HDR and NHEJ is further regulated by reciprocal inhibition between these two pathways. While 53BP1 and RIF1 mostly promote NHEJ by blocking end-resection, BRCA1 and CtIP direct break processing toward HDR or MMEJ (Escribano-Díaz et al., 2013).

End-Joining Repair

In the absence of a repair template, a Cas9-induced DSB is predominantly repaired in an error-prone manner, resulting in insertions and deletions (indels) within the targeted genomic sequence. If these indels give rise to frameshift mutations, they result in loss-of-function alleles. This type of repair outcome has been largely attributed to the use of NHEJ, which directly ligates the two DNA ends following cleavage, leading to the generation of small indels (<10 bp; Bothmer et al., 2017). More recently, MMEJ has been shown to contribute to a large fraction of the edited alleles observed after genome editing (Shen et al., 2018). The MMEJ-mediated repair of Cas9-induced DSBs is characterised by a distinct indel profile where larger deletions are the predominant outcome (>10 bp; Ferreira da Silva et al., 2019; **Figure 1A**). Similar to NHEJ, MMEJ ligates the DNA ends in the absence of an exogenous repair template but, unlike NHEJ, MMEJ requires initial and short-distance DSB end-resection to reveal regions of microhomology (Seol et al., 2018). The initial resection (5–25

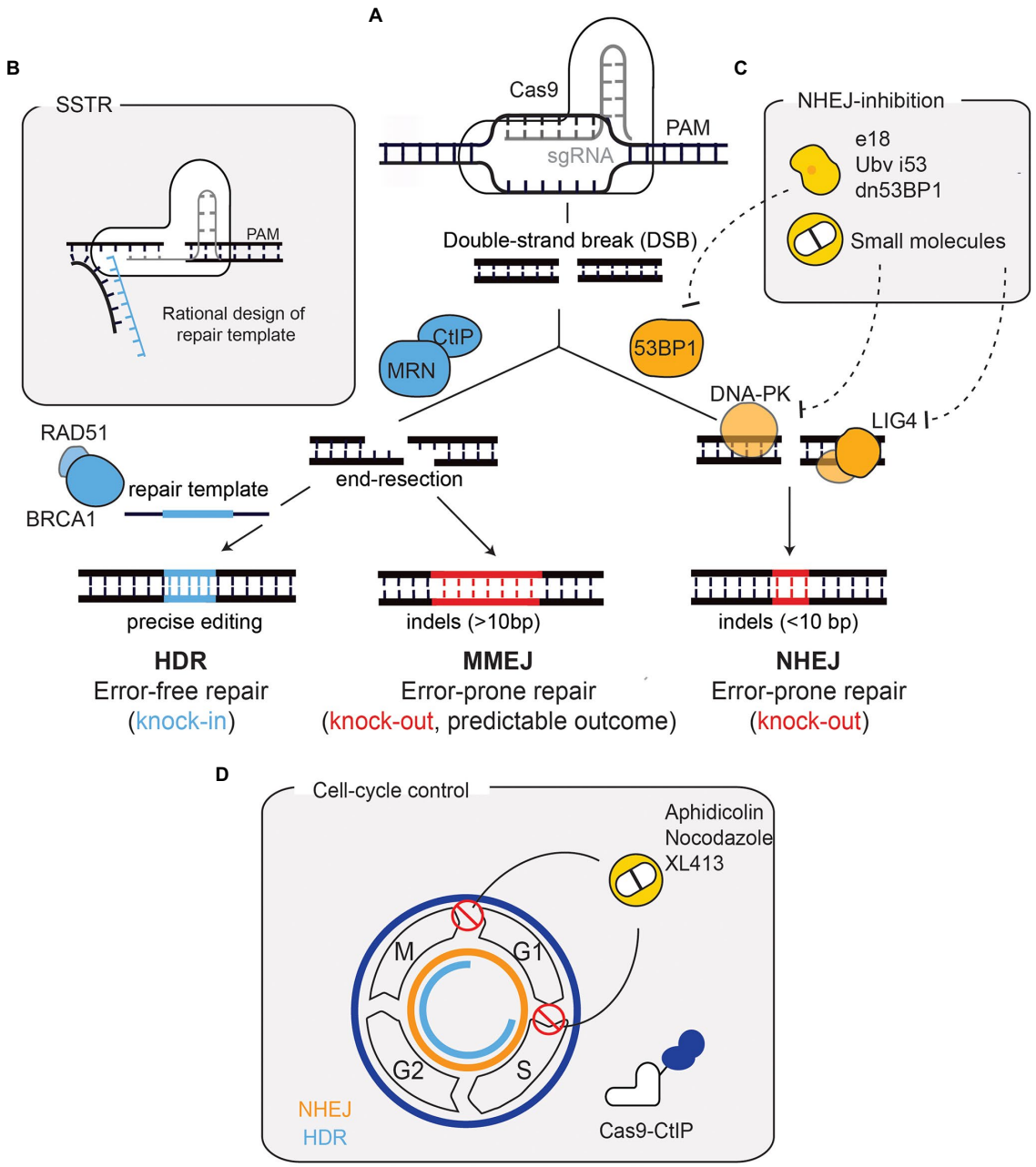

FIGURE 1 | Repair outcomes after a Cas9-induced DNA double-strand break (DSB) and strategies for enhancing precise repair. **(A)** Cas9, targeted by a sgRNA, induces a DSB in a precise region of the genome. Non-homologous end-joining (NHEJ), promoted by 53BP1, is the default repair pathway. Through the coordinated action of factors such as DNA-PK and LIG4, NHEJ repairs the DSB by re-joining the DNA-ends in an error-prone manner. This results in small insertions and deletions (indels) that can generate a loss-of-function allele if a frameshift is generated. If end-resection occurs [mediated by CtIP and MRE11-Rad50-NBS1 (MRN)], microhomology-mediated end-joining (MMEJ), or homology-directed repair (HDR) function. The repair outcome following MMEJ-mediated repair can vary, although this can be predicted since it depends on regions of microhomology and leads to larger indels. HDR, mediated by factors such as BRCA1 and RAD51, relies on a repair template and hence is error-free, leading to precise genomic alterations. **(B)** The use of ssDNA oligonucleotides (ssODN) as donor templates has also been developed to harness HDR. This process is called single-stranded templated repair (SSTR). SSTR is generally more efficient due to the asymmetry of the Cas9-DNA complex, which leads to the release of the PAM-distal non-target strand. Therefore, a rational design of the ssODN donor template complementary to the strand that is first released improves precise editing. **(C)** The inhibition of NHEJ has been used to improve precise repair following Cas9-breaks. 53BP1 inhibition through ubiquitin variants, dominant negative forms, or expression of factors that displace 53BP1, has proven useful. Small molecule inhibitors against DNA-PKcs and LIG4 have also been used. **(D)** Cell cycle manipulation has also proved useful for enhancing HDR. HDR (depicted in blue) is only active in S/G2/M phases, contrary to NHEJ (depicted in orange), which is active throughout the cell cycle. Strategies to improve HDR have included the use of compounds (such as XL413, aphidicolin, and nocodazole) to block cells in HDR-permissive phases. A Cas9-CtIP fusion allows end-resection (and subsequently HDR) to occur throughout the entire cell-cycle. PAM, protospacer adjacent-motif.

base pairs) is performed by the MRE11-Rad50-NBS1 (MRN) complex, which is activated in a cell-cycle dependent manner by CtIP (Truong et al., 2013). This exposes microhomologies on opposite strands that anneal to one another. DNA polymerase θ (POLQ) stabilises the annealed single-stranded DNA and fills the gaps, *via* templated synthesis. The early resection steps that occur in MMEJ are shared with HDR. However, annealing and extension of overhanging ends during MMEJ function to prevent HDR. Moreover, HDR requires extended end-resection, which depends on additional factors, such as the helicase Bloom syndrome protein (BLM) and Exonuclease 1 (EXO1; Truong et al., 2013).

Albeit being generally considered as an alternative pathway, studies based on the pharmacological and genetic ablation of NHEJ have shown that MMEJ can fully compensate for the absence of NHEJ in the repair of Cas9-induced DSBs (Brinkman et al., 2018; Ferreira da Silva et al., 2019). Despite the error-prone nature of end-joining pathways, there is mounting evidence indicating that the pattern of DNA repair following a Cas9-induced DSB is not stochastic (van Overbeek et al., 2016; Shou et al., 2018). Based on this observation, several studies have systematically analysed how sequences flanking the DSB impact repair outcome, leading to the important conclusion that template-free Cas9 editing can be predicted and applied to achieve a specific outcome (Allen et al., 2018; Shen et al., 2018).

Homology-Directed Repair

In contrast to the end-joining pathways, and within the context of genome editing, HDR depends on an exogenous repair template, allowing cells to integrate specific and precise alterations in their genome (**Figure 1A**), thus making it more relevant for therapeutic applications. HDR efficiency, however, remains a challenge and several approaches have been developed to overcome this limitation. Biochemical modelling of the Cas9-DNA interaction has been fundamental to prove that the efficiency of HDR can be improved through rational design of the repair template, concluding that the use of single-stranded DNA (i.e., synthetic oligonucleotides) as a repair template improves HDR (Richardson et al., 2016; Aird et al., 2018). This sub-type of HDR is commonly called single-stranded templated repair (SSTR; **Figure 1B**).

Importantly, transcriptional and genetic differences impact the efficiency of CRISPR-Cas9 editing and therefore the effectiveness of genome editing approaches. Screens performed in human cancer cell lines have shown that the Fanconi anaemia (FA) pathway diverts repair toward SSTR, playing an important role in HDR efficiency (Richardson et al., 2018). The Fanconi anaemia group D2 protein (FANCD2) has been shown to have a direct role on genome editing, by physically localising to Cas9-induced DSBs. This finding has important therapeutic implications for future genome editing applications in FA patients. Moreover, the involvement of FA, a pathway that repairs interstrand cross-links, on the repair of Cas9-mediated DSBs highlights how little is known about the interplay between DNA repair pathways in the context of different CRISPR-mediated technologies.

Rewiring DNA Double-Strand Break Repair Towards Homology-Directed Recombination

The importance of DNA repair for genome editing applications is further illustrated by the different approaches that modulate DNA repair pathways to improve HDR efficiency. For example, since NHEJ is the default pathway in human cells, its inhibition has been exploited to favour HDR. This has been achieved through the use of small-molecules targeting LIG4 or DNA-PKcs (Robert et al., 2015; Riesenberg and Maricic, 2018), ubiquitin-variants targeting 53BP1 (Canny et al., 2017), expression of factors that displace 53BP1 from DSBs (Nambiar et al., 2019), or 53BP1 dominant negative forms (Paulsen et al., 2017; **Figure 1C**). Another strategy to promote HDR is through cell cycle modulation, thereby increasing precise editing and minimising undesirable indels (**Figure 1D**). One of such strategies makes use of a Cas9 fused with the protein CtIP (Charpentier et al., 2018). This construct bypasses the requirement for cell cycle dependent activation of CtIP (by CDK1/2), necessary for end-resection and subsequent HDR. Pharmacological cell cycle arrests in HDR-permissive phases (S/G2) with aphidicolin, nocodazole, or the small molecule XL413, can also improve the efficiency of precise editing (Lin et al., 2014; Wienert et al., 2020). Overall, the modulation of DNA repair pathway choice, either through direct inhibition of NHEJ or cell-cycle regulation, comprises a potent strategy to boost precise editing.

CRISPR-Cas9 Editing Outcomes Are Shaped by DNA Repair Processes

The DNA damage response is a highly interconnected signalling network, which is modulated by cell cycle stage, gene expression changes, chromatin states, differentiation status, and cell type (Blanpain et al., 2011; Fortini et al., 2013; Klement and Goodarzi, 2014; Polak et al., 2015; Hustedt and Durocher, 2017; Weeden and Asselin-Labat, 2018; Yimit et al., 2019).

In the pursuit of safe and precise genome editing, next generation sequencing (NGS) technologies have empowered researchers to look for off-target effects beyond commonly predicted sites, enabling high standards for quality control of *ex vivo* edited cell populations (Li et al., 2019). Even in the near absence of off-target editing, the challenge of achieving precise editing outcomes at the desired target site remains. Investigating CRISPR-Cas9 outcomes in mouse embryonic stem cells, mouse hematopoietic progenitors, and differentiated human cells lines with intact DNA repair, Kosicki et al. (2018) found frequent large-scale deletions around the cut site, as well as crossover events with distant sites. Notwithstanding the advanced technologies to limit off-target effects, these surprising results revealed that more research is required to understand possible editing outcomes and how to avoid unwanted on-target effects.

A recently developed approach termed Repair-Seq was used to systematically map DNA repair outcomes, and hence editing outcomes, after Cas9 and Cas12a mediated genomic editing across several loci (Hussmann et al., 2021). This revealed that

genetic dependencies driving repair outcomes are determined by the exact type of DNA lesion present. Predicting editing outcome is thus dependent on the understanding of lesion conformation and its interplay with DNA repair factors.

In summary, recent insights into the complex interplay between DNA break configuration and DNA repair factors, highlighted how the landscape of genome editing outcomes remains underexplored. The studies discussed above made their observations in a few cellular models but found a surprising variety of lesions and repair outcomes generated. The level of complexity further increases when one takes cell type and tissue specific effects of DNA repair into consideration. It becomes apparent that the full control of CRISPR-mediated genome editing is only possible with full understanding of the intricacy of endonuclease generated lesion conformation in combination with DNA repair regulation in a tissue dependent context.

SUCCESS OF CRISPR-BASED THERAPIES DEPENDS ON UNDERSTANDING TISSUE SPECIFIC DNA REPAIR

DNA Repair Outcomes Are Tissue Specific

Outside the CRISPR field, it has long been noted that the balance between the type of DNA lesion and DNA repair activity determines tissue specific repair outcome. Germline mutations in DNA repair genes cause disease phenotypes, which often manifest in a tissue specific manner. A classic example are *BRCA1/2* mutations, which cause a defect in HDR, yet predispose primarily to breast and endometrial cancers. Similarly, defects in DNA single strand break repair (SSBR), predominantly affect neuronal cell types, while, for instance defects in crosslink repair (Fanconi anaemia pathway) precipitate bone marrow failure and neurological degeneration (Tiwari and Wilson, 2019). The differential effect certain DNA repair defects have on specific cell types cannot be fully explained. Part of the explanation may be tissue specific differences in terms of which type of DNA damage is encountered, for instance, due to differential cellular metabolism or hormone levels (Langevin et al., 2011; Garaycoechea et al., 2012; Singh and Yu, 2020). However, DNA damage is only one side of the coin, while DNA repair is the other. Indeed, different cell types, even within tissues, have been found to show divergent propensity for DNA repair. Differential sensitivity to DSBs, for instance, has been observed among human hematopoietic stem cells (HSCs) and progenitor cell populations (Milyavsky et al., 2010). Compared to progenitor populations, HSCs showed delayed repair kinetics and higher levels of p53 activation, leading to increased apoptosis after DSB induction.

How the cell type affects the specificity of DNA repair outcomes across tissues is thus another level of consideration for designing CIRSPR applications. Although the intricate tissue specific response to DNA DSBs complicates design of gene editing therapies, in-depth characterization of tissue specific DNA repair mechanisms is key for developing safe and efficient therapies. We discuss recent insights which advanced the understanding of underlying mechanisms effectuating tissue specificity of DNA repair, and how this might influence CRISPR applicability.

Tissue Specific Cell Cycle Effects

Since cell cycle stage impacts repair pathway choice, only actively cycling cells have full accessibility to NHEJ, MMEJ, and HDR. Other cells, quiescent or post-mitotic, must re-enter the cell cycle to access DSB repair and other repair pathways (Nouspikel and Hanawalt, 2000; Shin et al., 2020). Upon exit of G0, NHEJ is the predominant repair pathway for DSBs, increasing the possibility of mutagenic repair (Mohrin et al., 2010; Shin et al., 2020). The inaccessibility of HDR coupled with the preference for NHEJ in some cell types poses a problem for the utility of CRISPR therapeutics. To achieve a long-lasting therapeutic effect, targeting long-lived stem cell populations offers the best strategy. However, many somatic stem cells across tissues are quiescent and therefore HDR-based therapies aimed at introducing specific edits are challenging and might limit the applicability of CRISPR technology in the clinics. A recent study, however, has demonstrated that detailed knowledge of DNA repair and cell cycle regulation can significantly increase the HDR-editability of the target cell population. Shin et al. demonstrated that quiescent HSCs can be edited with HDR up to an overall efficiency of 30% if they are stimulated to enter the cell cycle before commencing editing.

Tissue Specific Effects of Differentiation and Chromatin Status

It has been established that many different cell lineages across tissues exhibit slower rates of DNA repair and generally have reduced capacity to maintain their genome. This can be seen as an adaptive advantage, as highly differentiated cells do not spend energy on whole genome maintenance and instead focus on the conservation of actively transcribed genes (Nouspikel and Hanawalt, 2002). Most terminally differentiated cells are not of interest for CRISPR therapeutics, apart from long-lived differentiated cells such as neurons and intermittently mitotic hepatocytes. For the most part, tissue specific stem cells will be the target for clinical CRISPR applications by virtue of their ability to populate the tissue with gene-edited cells. Because DNA repair, from signalling to pathway choice, is tightly interconnected with epigenetic regulation, it must be appreciated that the distinct chromatin profiles of differentiated and non-differentiated cells might influence how a DNA lesion is repaired. HDR, in contrast to NHEJ, requires end-resection, which happens more effectively in open chromatin regions. Consequently, HDR is favoured in genomic regions with open chromatin conformation, marked by H4 acetylation and HeK36me3. NHEJ, on the other hand, is preferred in heterochromatic regions and at sites where H4 is demethylated at lysine 20 (H4K20me2; Karakaidos et al., 2020). Recently, the pathway balance between NHEJ and MMEJ as influenced by chromatin configuration has also been mapped

(Schep et al., 2021). This study showed that MMEJ is more active than NHEJ in specific heterochromatin contexts, namely late replicating regions, lamina associated regions, and at H3K9me2 sites. Moreover, MMEJ was shown to compete with SSTR (Schep et al., 2021). Therefore, systematically mapping chromatin environments across cell types can inform avenues for regulation to successfully install CRISPR edits which rely on the incorporation of repair templates.

The advances in mapping and understanding intrinsic differences in DNA repair regulation across cell types will undoubtedly promote design of more efficient CRISPR therapies, which can be applied *ex vivo* using induced pluripotent stem cells (iPSCs) and organoid-based approaches (Schwank et al., 2013; Xie et al., 2014; Li et al., 2015), while keeping unwanted on-target effects to a minimum. Especially when targeting long lived and actively dividing stem cells, *ex vivo* editing offers a safer route over *in vivo* editing, because edited cells can be thoroughly investigated and selected for the desired editing outcome, prior to transplantation into the patient. However, some diseases may require *in vivo* editing due to the plurality of tissues and cell types affected, adding another layer of complexity, since tissue context must be considered as well.

Editing Outcomes Are Influenced by Tissue Architecture

One disease in which *in vivo* editing would likely be necessary is cystic fibrosis, which is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The function of this chloride/bicarbonate channel is to regulate the exchange of electrolytes and thus the hydration levels of secretory epithelia. Loss or reduction of function in this protein leads to cycles of mucus accumulation, inflammation, and infection in the lung, progressively destroying the airway epithelium (Ensinck et al., 2021).

With 360 reported pathogenic mutations, editing strategies for cystic fibrosis need to be tailored to each patient and draw on an integrated understanding of DNA repair. In order to achieve a long-term cure, the resident tissue stem cells, i.e., basal cells, must not only be studied in terms of their response to CRISPR-induced DNA breaks and subsequent repair, but also where they are situated within their host tissue. This is especially relevant because, within the lung, an intra-tissue variance in response to DNA damage exists. Along the airway epithelium of the trachea and larger bronchi, basal stem cells are responsible for renewing the epithelium, giving rise to ciliated and club cells (Rock et al., 2009; Asselin-Labat and Filby, 2012; Hogan et al., 2014). It should be noted that basal cells are the most active stem cell pool along the trachea, whereas in the bronchi, club cells have also been shown to self-renew and give rise to ciliated cells (Rawlins et al., 2009). Within the lung tissue, there is also the highly specialised alveolar epithelium, which consists of elongated type 1 cells and secretory type 2 cells (alveolar type 2 = AT2), the latter being the resident stem cell (Barkauskas et al., 2013; Yamamoto et al., 2020). Surprisingly, it has been observed that basal stem cells exhibit a greater capacity for repair of DSBs compared to AT2 cells. Basal cells utilise NHEJ more efficiently than AT2 cells, allowing them to resist apoptosis and to begin proliferation. In the disease context, the pathologic changes and inflammatory environment of the tissue also play a role in how efficient CRISPR editing might function. Hence, to avoid a mixture of editing outcomes across different cell types within one tissue, the utilisation of DNA repair pathways and their relative efficiency in the target cells must be taken into consideration for CRISPR-Cas9 editing.

As the CRISPR field advances, it has become ever increasingly interwoven with the DNA repair field, because it is recognised that genome editing is dependent on the activity of the cellular DNA repair machinery. We focused on CRISPR-Cas9 technologies, which depend on DSB repair pathways and reviewed the emerging research on the complexity of tissue specificity of DNA repair. The outcome of a genomic edit builds upon the complex interplay of the DNA repair machinery, which is specific to the type of lesion generated, and differs across cell types and within tissue environments, owing to cell cycle effects, differentiation status, and chromatin configurations. The power to translate genome editing to the clinic increases with a progressive understanding of all aspects of DNA repair.

CRISPR IN THE CLINICS: CHALLENGES AND LIMITATIONS DUE TO DNA REPAIR TISSUE SPECIFICITY

With ever improving CRISPR-based technologies, gene-editing treatment has become a reality in the clinics. The dream to cure diseases by correcting the causative mutations is far simpler than its implementation. For a few applications, including engineering T-cells for cancer therapy, inborn blood disorders, transthyretin (TTR) amyloidosis, and heritable blindness, CRISPR-therapies have become available to patients. We review recent achievements in clinical trials and consider the applicability of tissue specific DNA repair.

CRISPR in Cancer Therapy

Recently concluded clinical trials have successfully shown delivery of CRISPR-Cas9-based *ex vivo* therapies to patients and demonstrated safety and feasibility of these treatments. Yet, these trials have also demonstrated that the mere reduction of off-target editing is not sufficient to achieve the desired outcome. One trial (NCT02793856) studying the therapeutic effect of knocking out the programmed cell death protein 1 (PD-1) in patient derived T-cells *via* NHEJ in refractory non-small-cell lung cancer, found a good ratio of 48.7 of on-target over off-target editing. Even so, 28.8% of all on-target edits did not match the predicted outcome (Lu et al., 2020). Another trial (NCT03399448), also focused on enhancing anti-tumor immunity of T-cells, set out to simultaneously edit four loci encoding for the endogenous T-cell receptor (TCR), and PD-1, while introducing a transgene (NY-ESO-1), which is more efficient at recognising tumor cells than the TCR. While

off-target editing events were rare, simultaneous editing of multiple loci led to translocations and large deletions. Of 12 possible translocation events, the most abundant rearrangement caused a 9.3 kb deletion, which was evident in all edited samples and remained detectable in patients up to 170 days post-transfusion (Stadtmauer et al., 2020). While all observed translocations persisted in peripheral blood, the frequency of detected rearrangements declined with time, indicating no specific growth advantage introduced by the unintended edits.

In summary, both trials demonstrated the utility of CRISPR-Cas9 based treatment approaches in patients, in addition to moderate clinical benefit. The editing strategy in both trials minimised off-target effects, while still introducing unwanted on-target effects. For transient cell populations such as engineered T-cells, this might be acceptable. However, for clinical applications which require precise editing of resident stem cell populations, better control over editing outcome is needed.

CRISPR for Hereditary Disease Therapy

Targeting Tissue Stem Cells

An important milestone in the development of therapeutic genome editing was reached in two CRISPR-based trials for β-thalassemia and sickle cell anemia (NCT03655678 and NCT03745287, respectively). Targeting CD45-positive hematopoietic stem and progenitor cells, the *ex vivo* editing strategy relied on error prone NHEJ to achieve gene knockout of *BCL11A*, a transcriptional repressor of foetal hemoglobin (Frangoul et al., 2021). Precise correction of the causative point mutations for these diseases seems like a more obvious choice compared to disrupting a transcription factor (**Figure 2A**). However, considering the relative ineffectiveness of HDR in the target cells and their propensity to utilize NHEJ, deliberate indel generation offers a more effective editing strategy. Both trials proved that minimising off-target effects, while carefully predicting and evaluating indels generated at the on-target

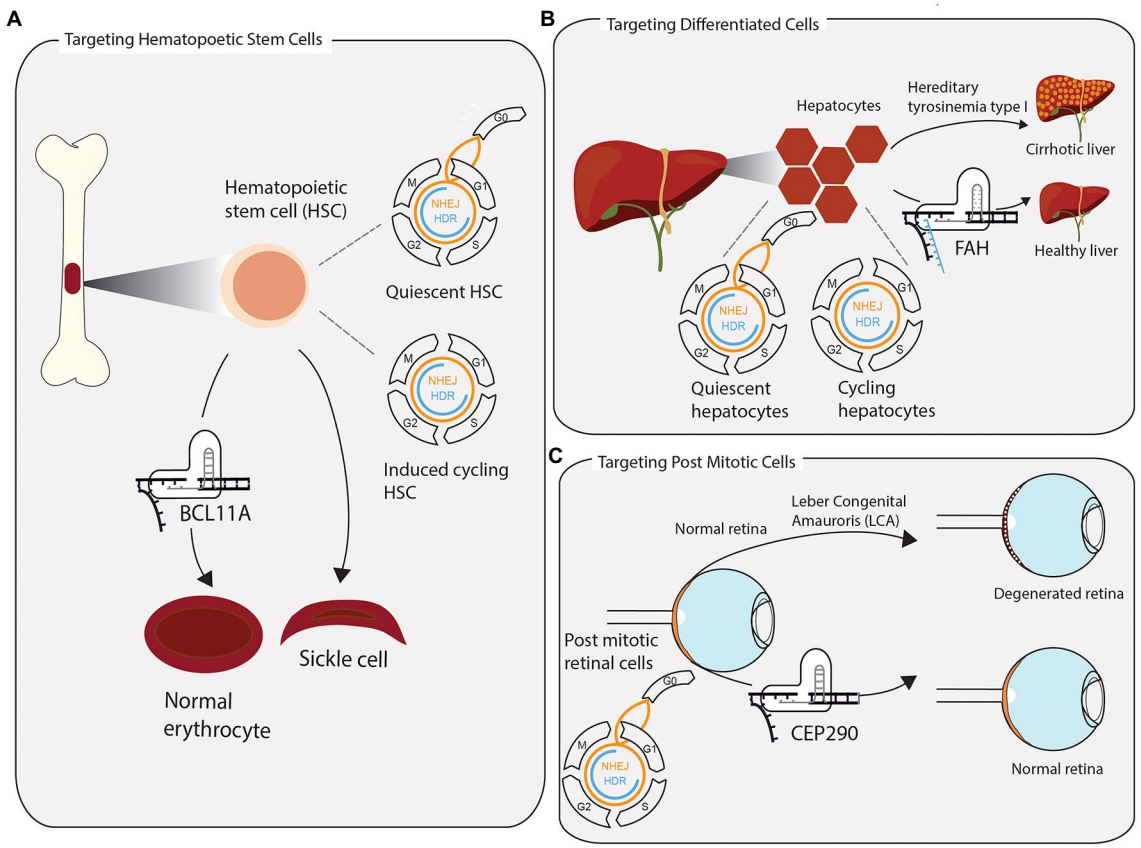

FIGURE 2 | Successful clustered regularly interspaced short palindromic repeats (CRISPR) applications require consideration of tissue-specific DNA repair and repair pathway accessibility. **(A)** Hematopoietic stem cells (HSCs) are extracted from the bone marrow and edited *ex vivo* for the treatment of sickle cell anaemia and β-thalassemia. Without stimulating cells to enter the cell cycle once before CRISPR-Cas9 editing, quiescent HSCs rely on error prone NHEJ to repair induced DSB. In a clinical application, the preference for NHEJ is leveraged to disrupt the transcription factor BCL11A, which represses the expression of foetal hemoglobin. The re-expression of foetal hemoglobin allows for the formation of normally shaped erythrocytes. **(B)** The toxic accumulation of fumarylacetoacetate in fatal hereditary tyrosinemia type I (HTI) leads to liver cirrhosis and liver failure due to a mutation in the fumarylacetoacetate hydrolase gene (FAH). Highly differentiated quiescent cells can be stimulated to re-enter the cell cycle upon DNA, or tissue, damage. Provided with a single-stranded repair template, few cycling hepatocytes have access to repair DSB *via* homologous directed repair. Precisely edited hepatocytes have a growth advantage over non-edited cells and reconstitute tissue homeostasis. **(C)** Leber congenital amauroris (LCA) is the first disease treated with an *in vivo* CRISPR approach. The post mitotic light sensitive cells in the retina degenerate with age, leading to impaired vision early on in life. Appropriating the propensity of post mitotic cells to repair DSBs *via* NHEJ, the therapy aims to disrupt an aberrant splicing site in exon 26 of CEP290, maintaining a functional retina.

site, are valid strategies to utilise NHEJ for safe editing of stem cells. Edited cells engrafted in patients' bone marrow, demonstrating the feasibility of editing long lived stem cells and replenishing stem cell compartments of interest with corrected cells (Frangoul et al., 2021). In future applications, which require precise editing, controlling quiescent and cycling states of HSCs might prove useful to increase HDR (Shin et al., 2020).

Targeting Differentiated Cells

Integrated knowledge of tissue architecture and DNA repair outcomes can help designing better CRISPR therapies. A prime example of this is the fatal genetic disease hereditary tyrosinemia type I (HTI). HTI is caused by a G>A point mutation in the fumarylacetoacetate hydrolase (FAH) gene, which causes skipping of exon 8, leading to a dysfunctional protein and accumulation of the toxic metabolite fumarylacetoacetate in hepatocytes, ultimately leading to cirrhosis, acute liver failure, and increased risk of hepatocellular carcinoma (Yin et al., 2014; King et al., 2017). The liver consists largely of highly differentiated hepatocytes, while the population of hepatic progenitor cells (HPCs) is considerably smaller. Although fully differentiated, in response to disturbances to homeostasis, quiescent hepatocytes can enter the cell cycle and begin proliferating to repair tissue injury (**Figure 2B**; Kiseleva et al., 2021). In their study on HTI, Yin and colleagues demonstrated that precise correction of the mutation can be achieved in mice *via* delivering CRISPR-Cas9 along with a single-stranded DNA repair template into hepatocytes, using hydrodynamic tail vein injection. Once stimulated to proliferate, actively cycling hepatocytes can utilize HDR to make the edit of interest. Although only one in 250 liver cells were successfully edited, corrected cells have a selective advantage and begin to outgrow unedited cells and repopulate the liver, effectively ameliorating the disease. Therefore, considering tissue architecture along with DNA repair pathway choice, results in a therapy which is more effective than the initial editing efficiency.

Gene editing of hepatocytes has recently found application in a clinical trial using *in vivo* editing (Gillmore et al., 2021). TTR amyloidosis (ATTR) is a progressive fatal disease, which may be inherited in an autosomal dominant manner through inheritance of one of more than 100 recognised pathogenic mutations in the TTR protein. Misfolding of mutant TTR promotes the accumulation of insoluble protein fibers, which are deposited predominantly in heart and nervous tissue, leading to cardiomyopathies and polyneuropathies. TTR has normal, but dispensable, functions in vitamin A transport and is almost exclusively produced in the liver. Thus, targeted knockout of the TTR gene in hepatocytes, coupled with vitamin A supplementation, is a viable treatment strategy to reduce systemic levels of TTR and curb the deposition of pathogenic TTR fibers (Gertz et al., 2015).

Gillmore et al. (2021) describe the intermediate results of an ongoing clinical study seeking to reduce TTR protein level in patients with hereditary ATTR (Gillmore et al., 2021). Extensive pre-clinical screening for off-target effects was conducted to allow for the optimal selection of an efficient sgRNA and the formulation of the editing drug "NTLA-2001." The CRISPR editing machinery, encoded in mRNA, and the TTR sgRNA was delivered encapsulated in lipid nanoparticles with liver tropism. Patients showed a dose dependent effect of TTR serum level reduction after 28 days, between 47–56 and 80–96% for the lower and higher dose of NTLA-2001, respectively. Thus far, patients have not exhibited serious adverse effects. Long-term monitoring of protein level reduction, side effects, and outcomes on disease progression and mortality will show the safety and applicability of this therapy. The liver is an optimal target organ for the first *in vivo* therapy targeting differentiated cells. It consists mostly of intermittently mitotic hepatocytes, which at once reduces the risk of pathogenic outgrowth, compared to consistently cycling cells, and simplifies the complexity of having to consider many cell types in the design of the editing strategy. Aside from the rarity of hereditary ATTR, pathogenic accumulation of wild type TTR fibers in the heart is also observed in patients and has been recognised as a cause for cardiomyopathy and eventual heart failure (Gertz et al., 2015). Hence, a successful CRISPR therapy for transthyretin amyloidosis may be the first to find broad application beyond rare diseases.

Targeting Post Mitotic Cells

Since specificity of editing outcomes and safety are still major technological hurdles, there are currently few ongoing clinical trials utilising *in vivo* CRISPR Cas9 editing. One trial is seeking to treat Leber congenital amaurosis (LCA; ClinicalTrials.gov, 2019). LCA manifests in degeneration of the retina and is caused by mutations in more than 25 genes (Daich Varela et al., 2021). The CRISPR-based drug, EDIT-101, targets a heterozygous mutation in intron 26 of the LCA gene CEP290 to remove an aberrant splicing site *via* generating an indel through NHEJ (**Figure 2C**; Maeder et al., 2019). While it is exciting that *in vivo* CRISPR editing begins to move into the clinic, it is pertinent to keep in mind that LCA constitutes an ideal model disease for this approach. The post-mitotic nature of the targeted cells ensures a greater propensity for utilising NHEJ to repair the induced break and reduces the risk of selective pathogenic outgrowth of edited cells, when compared to actively cycling somatic stem cells. Furthermore, there is reduced risk of inflammation or adverse reactions to introduction of Cas9, due to the immunoprivileged status of the eye.

The examples above illustrate the potential and versatility of CRISPR-based therapies. The success of such approaches, however, relies on careful consideration about the biology of targeted cells and a deep understanding about the tissue specific mechanisms of DNA damage signalling and repair.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The successful implementation of CRISPR-Cas9 technologies in a clinical setting relies on a deeper understanding of the DNA repair mechanisms and pathways responsible for genetic

replacement outcomes, as well as the activity and accessibility of these pathways in specific cell types and tissues. Following the generation of a DSB, cell cycle regulation, and DNA repair pathway choice play major roles in determining the editing outcome. Therefore, genome editing approaches have begun to harness DNA repair control and modulation for more efficient and predictable outcomes.

Overall, the genome and transcriptome of target cells impact the effectiveness of genome editing approaches. Moreover, cell identity and tissue context are important considerations in designing effective editing strategies. While *ex vivo* editing strategies allow for extensive quality control, *in vivo* editing strategies could target multiple cell types at once, but must be safe and accurate, especially when targeting long-lived somatic stem cells. Recent successes in therapeutic editing achieved in β-thalassemia and sickle cell anemia demonstrated the feasibility of utilising CRISPR-Cas9 editing in stem cells to alleviate disease. While these reports are encouraging, there is a large margin for improving treatment strategies for diseases which require editing of multiple loci or precise editing of one locus across multiple tissues. CRISPR technologies that do not rely on the generation of DSBs, such as DNA base editors and prime editing, are promising avenues for future precision medicine. These technologies are independent of cell cycle stage and hence have the potential to correct multiple cell types. However, both base editors and prime editing introduce unique types of DNA damage products, such as DNA single-strand breaks and base mismatches, to facilitate genome editing. Hence these approaches rely on other DNA repair pathways that must be understood, in tissue-specific contexts, for further expansion and improvement of these technologies (Gu et al., 2021).

The expansion of the tools available to understand and control the CRISPR-Cas9 system has continuously fuelled the development of new therapeutic strategies and has brought a fundamental discovery into the clinics in less than a decade. The implications for personalised medicine are immense. However, for this steep trajectory to continue and to broaden the applicability and impact of these technologies, the focus of future developments must shift to include the investigation of tissue specific DNA repair. Knowledge of the underlying mechanisms of how the DNA repair machinery reacts to a CRISPR break within a distinct cellular context is a key to mapping the landscape of genome editing.

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MM and JF wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Safe Path at the Fork: Ensuring Replication-Associated DNA Double-Strand Breaks are Repaired by Homologous Recombination

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Cells must replicate and segregate their DNA to daughter cells accurately to maintain genome stability and prevent cancer. DNA replication is usually fast and accurate, with intrinsic (proofreading) and extrinsic (mismatch repair) error-correction systems. However, replication forks slow or stop when they encounter DNA lesions, natural pause sites, and difficult-to-replicate sequences, or when cells are treated with DNA polymerase inhibitors or hydroxyurea, which depletes nucleotide pools. These challenges are termed replication stress, to which cells respond by activating DNA damage response signaling pathways that delay cell cycle progression, stimulate repair and replication fork restart, or induce apoptosis. Stressed forks are managed by rescue from adjacent forks, repriming, translesion synthesis, template switching, and fork reversal which produces a single-ended double-strand break (seDSB). Stressed forks also collapse to seDSBs when they encounter single-strand nicks or are cleaved by structure-specific nucleases. Reversed and cleaved forks can be restarted by homologous recombination (HR), but seDSBs pose risks of mis-rejoining by non-homologous end-joining (NHEJ) to other DSBs, causing genome rearrangements. HR requires resection of broken ends to create 3' single-stranded DNA for RAD51 recombinase loading, and resected ends are refractory to repair by NHEJ. This Mini Review highlights mechanisms that help maintain genome stability by promoting resection of seDSBs and accurate fork restart by HR.

Keywords: genome instability, DNA damage, DNA double-strand breaks, structure-specific nucleases, replication stress

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INTRODUCTION

Cells maintain relatively stable genomes during cell division to prevent accumulation of potentially oncogenic mutations. Cells proliferate despite copious DNA damage caused by endogenous and exogenous agents. Endogenous agents include reactive oxygen species (ROS) from oxidative metabolism, nucleases and other enzymes such as members of the AID/APOBEC DNA deaminase family, mis-incorporated ribonucleotides, and DNA chemical lability (Gates, 2009; Ciccia and Elledge, 2010; Nick Mcelhinny et al., 2010; Williams et al., 2013; Petljak and Maciejowski, 2020; Juan et al., 2021). DNA damage is induced directly or indirectly by exogenous chemical agents including alkylating agents and other DNA-reactive chemicals including cancer chemotherapeutics, and pollutants in food, water and air. Physical agents that

damage DNA include ultraviolet light and ionizing radiation (Friedberg et al., 2014; Nickoloff et al., 2020a). DNA damage comprises chemical changes to bases and the sugar-phosphate backbone, base loss, single-strand breaks, double-strand breaks (DSBs), and intra- and interstrand crosslinks. Protein-DNA crosslinks arise when topoisomerases are trapped in covalent linkages to DNA by topoisomerase poisons, commonly used in cancer therapy (Pommier et al., 2006; Deweese and Osheroff, 2009; Friedberg et al., 2014; Thomas and Pommier, 2019; Riccio et al., 2020). DNA damage detection, signaling and repair systems evolved to manage these threats, termed the DNA damage response (DDR). Nearly all DNA lesions block replicative polymerases (Pol ε, Pol δ), causing fork stalling and fork collapse, and cells manage this replication stress by activating S phase-specific DDR pathways. Replication stress is also caused by depletion of nucleotide pools by hydroxyurea, and DNA polymerase inhibitors (Vesela et al., 2017).

Unstressed cells suffer > 100,000 DNA lesions per day, with a steady state of ~10,000 lesions per cell (Tubbs and Nussenzweig, 2017). Thus, human cells manage an average of ~2000 DNA lesions per day in each chromosome, or roughly one lesion per 30 kbp per day. Given that typical human replicons are 75–175 kbp (ranging from 30–450 kbp) (Ligasova et al., 2009) and S phase comprises ~30% of a 24 h cell cycle, each active replicon will harbor > 2 DNA lesions (assuming lesions arise at similar rates throughout the cell cycle). The DNA replication machinery faces many other challenges in addition to DNA damage. The replisome helicase complex (CDC45, MCM2-7 and GINS), or more often replicative polymerases, slow or stall at unusual structures such as G-rich sequences that form G-quadraplexes, common fragile sites, and hairpins at inverted repeats and CAG/CTG triplet repeats (Bochman et al., 2012; Barlow et al., 2013; Zeman and Cimprich, 2014; Gadaleta and Noguchi, 2017; Kaushal and Freudenreich, 2019; Spiegel et al., 2020; Poggi and Richard, 2021). Replication stress is also caused by conflicts with R-loops formed during transcription, particularly at fragile sites, telomeres, and ribosomal DNA, and by proteins that bind tightly to DNA (Ivessa et al., 2003; Bermejo et al., 2012; Kotsantis et al., 2016; Billard and Poncet, 2019; Gomez-Gonzalez and Aguilera, 2019). These so-called 'difficult-to-replicate' sequences are encountered by replisomes in every S phase. Activated oncogenes in cancer cells also increase replication stress through de-regulated replication origin firing (Hills and Diffley, 2014). Persistent fork stalling can cause replisome dissociation, or forks may be cleaved by nucleases to yield seDSBs, both of which have been termed 'fork collapse' (Cortez, 2015). We discuss the distinct challenges associated with repair of frank, two-ended DSBs vs replication-associated seDSBs, and recent studies that illuminate mechanisms that ensure accurate, timely repair and restart of stressed replication forks.

Double-Strand Break Repair: A Double-Edged Sword

DSBs are dangerous DNA lesions that can cause genome instability and cell death. These threats are mitigated by several DSB repair pathways with different levels of accuracy. In mammalian cells, classical non-homologous end-joining (cNHEJ) is the dominant DSB repair pathway (**Figure 1**). An early cNHEJ step involves Ku70/Ku80 binding to broken ends. Ku protects ends from degradation and recruits DNA-PKcs, activating the DNA-PK holoenzyme, which promotes end-alignment and rejoining by LIG4-XRCC4 and other factors (Chang et al., 2017). cNHEJ operates on blunt or short overhanging ends and is error-prone, typically producing small insertion/deletion (indel) mutations that may be deleterious, but in certain contexts are quite beneficial, as in the generation of diverse antibody receptor genes (Arya and Bassing, 2017). Alternative NHEJ (aNHEJ) is a backup NHEJ pathway mediated by DNA ligase III when ends anneal at microhomologies. aNHEJ is more error-prone than cNHEJ, producing larger deletion mutations and translocations (Simsek et al., 2011; Iliakis et al., 2015; Sallmyr and Tomkinson, 2018). HR repair of DSBs is generally error-free as it employs an undamaged homologous sequence as repair template. HR initiates when broken ends are resected by > 50 nt, exposing long 3' ssDNA extensions initially bound by RPA that is exchanged with RAD51 which catalyzes strand invasion into homologous duplex DNA (**Figure 1**). End resection is regulated and mediated by many factors. CtIP, phosphorylated by CDK, ATM and ATR (limiting resection to S/G2 phases), activates MRE11 nuclease (in complex with RAD50-NBS1) to effect limited end resection (Anand et al., 2016). BRCA1-BARD1 promotes end resection in part by ubiquitination of H2A and by blocking the anti-resection factors 53BP1-RIF1/Shieldin (Mirman et al., 2018; Densham and Morris, 2019). Extensive resection is effected by EXO1 and by DNA2-BLM (Zhao et al., 2020).

Limiting HR largely to S/G2 phases promotes sister chromatid use as HR templates. RAD51 loading and strand invasion are mediated by many factors including BRCA1, BRCA2, five RAD51 paralogs (RAD51B/C/D, XRCC2/3), members of the Fanconi's anemia (FANC) protein family, RAD54/B, and RAD51AP1 and its paralog NUCKS1 (Pires et al., 2017; Wright et al., 2018; Niraj et al., 2019; Maranon et al., 2020). After repair synthesis, the extended strand reanneals with the resected end on the other side of the DSB, and gaps are filled and ligated to complete repair. Alternative HR pathways yield double Holliday junctions that can be resolved with or without crossovers (Piazza and Heyer, 2019). Single-strand annealing (SSA) is a RAD51-independent HR pathway that requires RAD52 and is observed, for example, in BRCA2-mutated breast cancer cells (Tutt et al., 2001). SSA is error-prone as resected ends anneal at complementary sequences, either between repeats flanking a DSB deleting one repeat and intervening sequences, or between repeats on different chromosomes causing translocations (Weinstock et al., 2006; Nickoloff et al., 2008; Bhargava et al., 2016). Failure to repair DSBs can cause chromosome loss and cell death, but it's clear that DSB repair also poses significant risks to genome integrity, including genome rearrangements, lethal dicentric chromosomes, and bridge-breakage-fusion cycles (Fenech et al., 2011; Murnane, 2012; Feijoo et al., 2014).

DNA Damage and Replication Stress Responses

The DDR elicits checkpoint responses that arrest or slow cell cycle progression and stimulate DNA repair. Checkpoints arrest or slow cell cycle progression at the G1/S transition, within S phase (intra-S checkpoint) and the G2/M transition. The DDR also promotes programmed cell death if damage is excessive (Roos and Kaina, 2013; Tian et al., 2015; Mladenov et al., 2016;

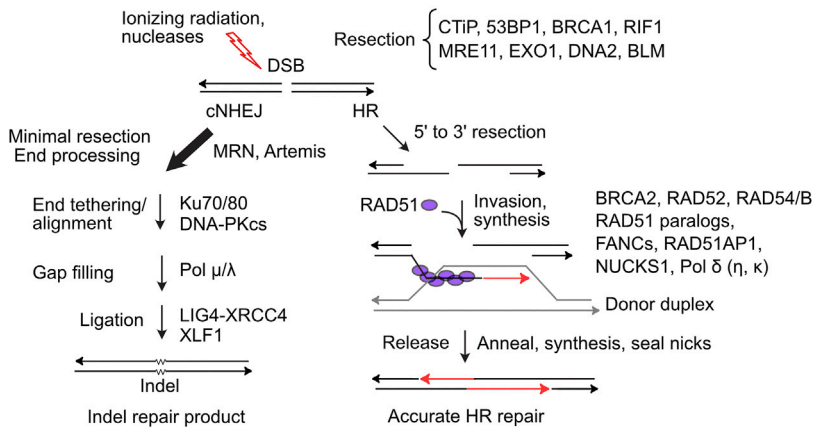

FIGURE 1 | Dominant two-ended DSB repair pathways. **(Left)** cNHEJ is the dominant pathway for repairing two-ended DSBs. cNHEJ acts on blunt or minimally processed ends bound by Ku70/Ku80 and DNA-PKcs. Short gaps are filled and ends are ligated to complete repair, typically with small insertions or deletions at the repair junction **(Right)** HR initiates with 5′-3′ resection and binding of ssDNA by RPA, which is replaced by RAD51 in a reaction mediated by BRCA2 and RAD51 paralogs. The RAD51 nucleoprotein filament invades homologous duplex DNA, assisted by RAD54, RAD54B and other factors. RAD51 dissociates and the invading end is extended and then released allowing pairing to ssDNA on the opposite side of the DSB. Gaps are filled and ends ligated to complete accurate DSB repair.

Blackford and Jackson, 2017). Cells initially respond to replication stress by protecting stalled forks and maintaining replisomes until the stress is resolved, otherwise various mechanisms are employed to restart or rescue the fork to ensure timely completion of DNA replication before mitosis. The DDR is important because defects in DDR proteins typically cause genome instability that can drive carcinogenesis (Tubbs and Nussenzweig, 2017), and because DDR proteins are important targets to augment cancer therapy (Nickoloff et al., 2017; Desai et al., 2018; Pilie et al., 2019; Nickoloff et al., 2020b; Baillie and Stirling, 2021). Central to the DDR are three members of the phosphatidyl inositol 3′ kinase-related kinase (PIKK) family, DNA-PKcs, ATM, and ATR, all of which play important roles in DSB repair. These PIKKs are structurally related and phosphorylate target proteins at canonical serine/threonine-glutamine (S/T-Q) sites, as well as non-canonical sites. PIKKs auto- and cross-phosphorylate each other and other DDR proteins to regulate checkpoints and repair (Liu et al., 2012; Marechal and Zou, 2013; Ashley et al., 2014; Boohaker and Xu, 2014; Blackford and Jackson, 2017). Although PIKKs phosphorylate overlapping targets, they have distinct roles in specific DSB repair contexts.

DNA-PKcs plays a critical role in cNHEJ repair of two-ended DSBs induced, for example, by ionizing radiation or nucleases (Chang et al., 2017). The catalytic subunit of DNA-dependent protein kinase, DNA-PKcs, is activated when complexed with Ku70/Ku80-bound DSB ends. DNA-PKcs mediates cNHEJ and checkpoint responses by phosphorylating itself, Ku, MRE11, RAD50, XRCC4-6, XLF, artemis, and histone H2AX, as well as proteins involved in transcription, cell growth, heat shock responses, and viral DNA integration (Anisenko et al., 2020). ATM (ataxia telangiectasia mutated) is also activated by DSBs, mediated by the MRE11-RAD50-NBS1 complex. ATM promotes frank DSB repair by HR, phosphorylating hundreds of targets including itself, BRCA1, NBS1, H2AX, p53, MDC1, and Chk2 kinase (Blackford and Jackson, 2017). Phosphorylated/activated Chk2 phosphorylates effector proteins that mediate HR and cell cycle arrest (among other processes), including BRCA1, BRCA2, p53, CDC25A, and RB (Marechal and Zou, 2013; Zannini et al., 2014). ATM autophosphorylation promotes ATM binding to MDC1 which binds to phospho-S139 S H2AX (γ-H2AX), and promotes spreading of the γ-H2AX signal to Mbp chromatin domains flanking DSBs (Savic et al., 2009). ATR plays a central role in replication stress responses (Yazinski and Zou, 2016), and is activated by ssDNA formed when blocked DNA polymerase decouples from the helicase and DNA unwinding continues ahead of the fork (Cortez, 2005). ATR is activated by a multi-step process that involves ATR-ATRIP recruitment to RPA-bound ssDNA, RAD17-RFC, Claspin, TopBP1, and 9-1-1 (Yazinski and Zou, 2016). ATR is also activated by an NBS1-dependent mechanism (Shiotani et al., 2013). Recently, ATR activation was shown to be mediated by the RPA-binding factor ETAA1 in an TopBP1-independent manner; dual inactivation of ETAA1 and TopBP1 abrogates ATR signaling and is synthetically lethal (Haahr et al., 2016). Activated ATR phosphorylates Chk1 which slows cell cycle progression in S/G2 phases and delays late origin firing (Yazinski and Zou, 2016).

All three PIKKs respond to DSBs and phosphorylate residues in RPA (Anantha et al., 2007; Oakley and Patrick, 2010; Ashley et al., 2014), yet they also have kinase-independent DDR roles. For example, distinct phenotypes result from DNA-PKcs null mutations vs kinase genetic inactivation or drug inhibition (Allen et al., 2003; Shrivastav et al., 2009; Menolfi and Zha, 2020), and cells lacking DNA-PKcs compensate by downregulating ATM expression (Peng et al., 2005; Shrivastav et al., 2009). Despite this crosstalk, current evidence indicates that DNA-PKcs promotes frank DSB repair by cNHEJ, ATM promotes frank DSB repair by HR,

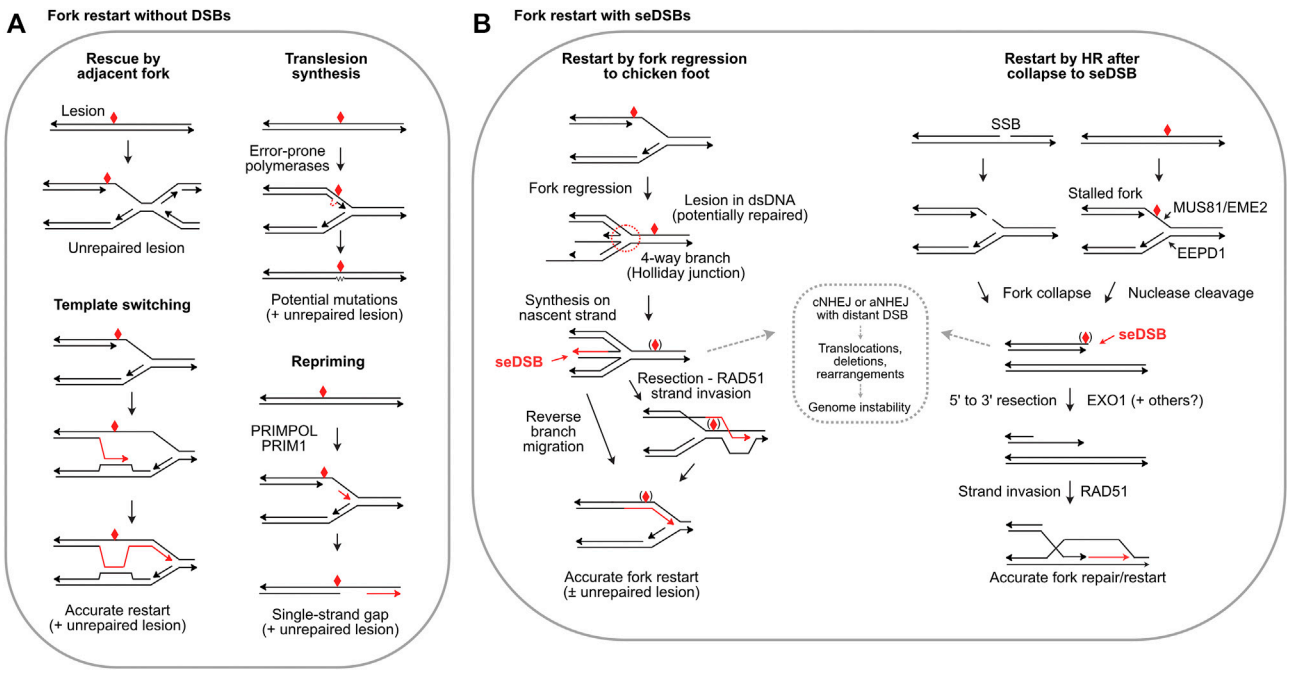

FIGURE 2 | Fork restart mechanisms. **(A)** Fork restart mechanisms that do not create seDSBs. Illustrated are rescue by an adjacent fork, TLS, template switching, and repriming. Blocking lesions are shown by red symbols and repair or bypass synthesis is shown by red arrows. **(B)** Fork restart by fork regression, fork encounters with a single-strand break (SSB), or fork cleavage, which create seDSBs. Regressed forks allow synthesis past the blocking lesion using the nascent strand as template. Reverse branch migration restarts the fork, or RAD51 may load onto a resected end allowing strand invasion downstream of the blocking lesion. Blocking lesions may be bypassed or repaired, indicated by symbols in parentheses. Collapsed forks due to encounter with single-strand breaks or fork cleavage can restart by RAD51-mediated strand invasion, i.e., break-induced replication. The strand invasion restart pathways are mediated by HR; fork regression/reversal is not an HR pathway, but RAD51 is still required to protect the nascent strands in the chicken foot. HR defects and HR inhibitors may shunt seDSB intermediates to cNHEJ or aNHEJ (dashed box) causing genome instability.

and ATR promotes repair of replication-associated DSBs by HR (Blackford and Jackson, 2017; Pilie et al., 2019).

Restarting Stalled and Collapsed Replication Forks

Given the central importance of DNA replication, it is not surprising multiple replication stress response mechanisms evolved. The initial response to replication stress is to stabilize replisomes at stalled forks to prevent fork collapse (Tye et al., 2021). Stalled forks often reverse to a so-called 'chicken foot' structure wherein nascent strands anneal, producing a four-way junction resembling a Holliday junction, but with a seDSB (**Figure 2B**). Fork protection involves the end resection inhibitor RIF1 (Mukherjee et al., 2019); MRNIP (Bennett et al., 2020); the de-ubiquitinating enzyme USP1 which suppresses translesion synthesis (TLS) by regulating PCNA (Lim et al., 2018); HR proteins RAD51, BRCA1, BRCA2 and FANCD2 (Schlacher et al., 2012; Rickman and Smogorzewska, 2019; Rickman et al., 2020); and the RAD51 regulator RADX (Bhat et al., 2018). Fork protection defects generally increase cytotoxicity of replication stress agents and are thus targets to augment cancer therapy. Fork protection can provide sufficient time to repair blocking lesions, but if not resolved in timely manner, adjacent forks may rescue stressed forks (**Figure 2A**), passively or through checkpoint activation of an adjacent dormant origin (Yekezare et al., 2013; Brambati et al., 2018).

Several fork restart mechanisms do not repair the blocking lesion, and thus are damage tolerance pathways. TLS involves transient replacement of replicative DNA polymerases with error-prone, TLS polymerases including Pol β, κ, η, τ, and ζ, and Rev1 (Goodman and Woodgate, 2013; Ma et al., 2020) (**Figure 2A**). Repriming by PRIMPOL and PRIM1 restarts replication downstream of blocking lesions, bypassing lesions and leaving single-strand gaps in nascent DNA (Quinet et al., 2021) (**Figure 2A**). Template switching uses sister chromatids to bypass blocking lesions and is generally error-free (**Figure 2A**), but poses risks of genome rearrangement from replisome switching to non-sister templates (Lehmann et al., 2020). Two additional restart pathways are fork reversal to a Holliday junction-like structure followed by fork restoration, and fork cleavage by structure-specific nucleases including the 3′ nuclease MUS81 (with EME2) (Pepe and West, 2014) and the 5' nuclease EEPD1 (Wu et al., 2015; Sharma et al., 2020) (**Figure 2B**). Metnase is a structure-specific nuclease that promotes fork restart, but Metnase does not cleave stalled forks and instead may process flaps that arise later (Sharma et al., 2020). SLX1-SLX4 is another structure-specific nuclease that resolves branched replication intermediates, and although it cleaves many types of branched structures including replication fork

structures *in vitro*, direct evidence that it cleaves stalled forks *in vivo* is lacking (Falquet and Rass, 2019; Xu et al., 2021). seDSBs at cleaved forks can be repaired and the fork re-established/restarted accurately if the end is resected and invades the sister chromatid (**Figure 2B**), often termed break-induced replication (BIR). Repair of collapsed forks by BIR may function primarily during S or G2 phases to ensure complete DNA replication prior to mitosis, but recent studies show that BIR also operates during mitotic DNA synthesis (MiDAS), an important mechanism for completing replication in common fragile sites, telomeres, and other under-replicated DNA during mitosis (Epum and Haber, 2021).

Minimizing Risks Associated With Replication Stress

Each fork rescue pathway poses risks: TLS is error-prone, producing point mutations, repriming yields vulnerable single-strand gaps, template switching poses risks of genome rearrangements (**Figure 2A**), and fork reversal/cleavage creates seDSBs which pose risks of aberrant cNHEJ causing genome rearrangements (**Figure 2B**). Interestingly, cNHEJ factors are present at seDSBs; similar to their presence at telomeres, cNHEJ factors at seDSBs may protect ends but further cNHEJ steps are suppressed (Sui et al., 2020; Audoynaud et al., 2021). Rescue by an adjacent fork might seem the least risky pathway: simply waiting for rescue by an adjacent fork (or repair of blocking lesion) would eliminate risks posed by other pathways. However, cells tightly regulate replication timing (and cell cycle progression), especially during embryonic development. The importance of timely fork restart is illustrated by increased genome instability, developmental defects, and cell death when fork restart is delayed by as little as 10 min (Kim et al., 2014; Kim et al., 2015; Wu et al., 2015; Chun et al., 2016). Timely fork restart probably limits the formation of toxic (cell lethal) recombination intermediates, thought to be unresolvable branched structures. In yeast, HR proteins and helicases drive formation of toxic recombination intermediates, and they are prevented or resolved by several factors including Srs2, Sgs1-Top3, Smc5/6, *Mus*81-Mms4, and Dna2 (Menolfi et al., 2015; Keyamura et al., 2016; Falquet et al., 2020). For example, toxic recombination intermediates cause synthetic lethality in *sgs1*Δ *mus81*Δ double mutants, but viability is restored by defects in HR genes including *RAD51*, *RAD52*, *RAD54*, *RAD55* and *RAD57* (Gangloff et al., 2000; Fabre et al., 2002; Bastin-Shanower et al., 2003).

Although it remains unclear how cells choose among various lesion bypass and fork restart pathways, it is likely that the types of blocking lesions, and the extent (local vs genome-wide) and duration of replication stress are determining factors. For example, certain blocking lesions may be promptly bypassed by TLS with sufficient accuracy, such as UV-induced T-T dimers (Washington et al., 2000), whereas specific types of lesions, high lesion loads, or persistent replication stress may require potentially riskier choices.

Despite the risks associated with seDSBs generated during fork regression and fork cleavage, these mechanisms are very common, particularly in human cells (Pepe and West, 2014; Zellweger et al., 2015; Meng and Zhao, 2017). The accuracy of HR-mediated fork restart may outweigh risks of error-prone bypass mechanisms like TLS. Given this, how might cells mitigate risks of cNHEJ acting on resulting seDSBs? A recently described mechanism operates at forks stalled by collision with opposing transcription R-loops in which MUS81 cleaves the fork, and the resulting ends are rejoined by LIG4-XRCC4 with assistance by RAD52-mediated strand annealing and PolD3, a non-catalytic subunit of Pol *d* (Chappidi et al., 2020). This pathway does not involve the core cNHEJ factor Ku, and thus prevents joining of the seDSB to other DSBs and genome instability. As noted above, resected DNA ends are poor cNHEJ substrates. As shown in Figure 2B, regressed forks initially have overhanging ends, unless or until nascent strand synthesis creates blunt ends. Thus, the overhanging ends in early fork regression intermediates are intrinsically protected from cNHEJ, and end-protection by RAD51 and other factors appears to reinforce cNHEJ suppression. In addition, ATM promotes dissociation of DNA-PK from seDSBs, suppressing cNHEJ (Britton et al., 2020). In the case of fork cleavage by either MUS81-EME2 or EEPD1 (or fork collapse at nicks), the initial state is a blunt, or nearly blunt seDSB, i.e., an excellent cNHEJ substate. Recent studies have shown that 5' fork cleavage by EEPD1 is strongly biased toward end-resection because EEPD1 recruits the key resection nuclease EXO1 to cleaved forks (Wu et al., 2015; Kim et al., 2017). EEPD1 first appeared in late chordates/early vertebrates ~450–670 Mya (Zerbino et al., 2018), and it may have been selected to augment MUS81-mediated fork restart to manage increased replication stress associated with expanding genomes. Metnase evolved even more recently, ~50 Mya, (Cordaux et al., 2006), and Metnase also recruits EXO1 to cleaved forks (Kim et al., 2016). Thus, EEPD1 and Metnase both promote HR-mediated fork restart by recruiting EXO1 to promote resection, minimizing cNHEJ of seDSBs at cleaved forks. It is unknown whether resection is also promoted during MUS81-mediated fork restart. MUS81 is not known to interact with EXO1, although EXO1 and MRE11 degrade (unprotected) reversed forks that force fork rescue by MUS81 (Lemacon et al., 2017). The expansion of genomes in higher eukaryotes may have created selection pressure for EEPD1/Metnase fork processing nucleases coupled to EXO1, driving a shift toward accurate, HR-mediated fork restart.

CONCLUDING REMARKS

Replication stress is intimately tied to cancer etiology and treatment. Replication stress causes genome instability that drives cancer progression, and it is caused by oncogenic stress and damage induced by genotoxic chemo- and radiotherapeutics. The DDR plays critical roles in managing replication stress, and inhibitors of DDR factors are promising targets to effect tumor-specific cell killing in mono-therapy or as adjuncts to genotoxic therapy (O'Connor, 2015; Pearl et al., 2015; Kirsch, 2018; Pilie et al., 2019; Trenner and Sartori, 2019; Nickoloff et al.,

2020b). Several specific and broader questions remain. For example, are forks cleaved by MUS81-EME2 also preferentially resected (by EXO1? by DNA2-BLM?) as with EEPD1-cleaved forks? How do different types of lesions, lesion loads, or the DDR determine choices among stressed fork restart mechanisms? And can we manipulate DDR signaling or structure-specific nucleases to more effectively and selectively kill tumor cells, in monotherapy or by augmenting conventional chemo- or radiotherapy? Clarifying these questions will promote the development more effective targeted cancer therapeutics.

AUTHOR CONTRIBUTIONS

JN, NS, LT, SA, and RH wrote the manuscript and JN prepared the figures.

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Making Connections: Integrative Signaling Mechanisms Coordinate DNA Break Repair in Chromatin

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DNA double-strand breaks (DSBs) are hazardous to genome integrity and can promote mutations and disease if not handled correctly. Cells respond to these dangers by engaging DNA damage response (DDR) pathways that are able to identify DNA breaks within chromatin leading ultimately to their repair. The recognition and repair of DSBs by the DDR is largely dependent on the ability of DNA damage sensing factors to bind to and interact with nucleic acids, nucleosomes and their modified forms to target these activities to the break site. These contacts orientate and localize factors to lesions within chromatin, allowing signaling and faithful repair of the break to occur. Coordinating these events requires the integration of several signaling and binding events. Studies are revealing an enormously complex array of interactions that contribute to DNA lesion recognition and repair including binding events on DNA, as well as RNA, RNA:DNA hybrids, nucleosomes, histone and non-histone protein post-translational modifications and protein-protein interactions. Here we examine several DDR pathways that highlight and provide prime examples of these emerging concepts. A combination of approaches including genetic, cellular, and structural biology have begun to reveal new insights into the molecular interactions that govern the DDR within chromatin. While many questions remain, a clearer picture has started to emerge for how DNA-templated processes including transcription, replication and DSB repair are coordinated. Multivalent interactions with several biomolecules serve as key signals to recruit and orientate proteins at DNA lesions, which is essential to integrate signaling events and coordinate the DDR within the milieu of the nucleus where competing genome functions take place. Genome architecture, chromatin structure and phase separation have emerged as additional vital regulatory mechanisms that also influence genome integrity pathways including DSB repair. Collectively, recent advancements in the field have not only provided a deeper understanding of these fundamental processes that maintain genome integrity and cellular homeostasis but have also started to identify new strategies to target deficiencies in these pathways that are prevalent in human diseases including cancer.

Keywords: DNA damage, chromatin, R-loops, DNA repair, protein domains, nucleic acids, genome integrity

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INTRODUCTION

DNA lesions trigger the rapid mobilization of numerous DNA damage response (DDR) proteins to the damage site where they function to not only repair the break but to also coordinate other DDR activities with additional ongoing cellular functions including transcription, replication, chromatin organization, cell cycle progression, and proliferation. Considering the vast number of proteins that assemble at breaks and the various DDR activities that they regulate, the importance of coordinating these interactions both physically and kinetically is clear. Cells use multivalent binding interactions with diverse biomolecules at the DNA lesion, whose environment is within chromatin, to control molecular signals that promote detection, processing, and repair of breaks (**Figure 1**). The various biomolecules that can be encountered at breaks include unmodified and modified nucleic acids of various structures (e.g., ssDNA and dsDNA, RNA and DNA, DNA and RNA methylation), nucleosomes (e.g., acidic patch region, core and variant histones), histone and protein modifications, as well as other DDR and chromatin factors (Ciccia and Elledge, 2010; Leung et al., 2014; Agarwal and Miller, 2016; Kim et al., 2019; Lanz et al., 2019; Bader et al., 2020; Skrajna et al., 2020; Sriraman et al., 2020; Tan and Huen, 2020; Fijen and Rothenberg, 2021; Klaric et al., 2021; Lee et al., 2021; Par et al., 2021). The nucleosome, which contains 147 bp of DNA wrapped around two copies of four core histones, constitutes a repetitive structure within cells that organizes the genome, while also playing an essential role in the processing of breaks. Core histone proteins that make up the nucleosome are highly modified by numerous post-translational modifications (PTM), including phosphorylation, methylation, ubiquitination, and acetylation. PTMs on histones regulate chromatin structure and function, playing essential roles in DNA-based processes including DNA repair (Thompson et al., 2013; Bowman and Poirier, 2015; Kim et al., 2019). Modified histones create a highly heterogeneous habitat in which damage can occur across the genome. Upon DNA damage, additional signaling events result in a cascade of PTM alterations within chromatin and associated repair proteins that attract DNA damage response factors to the break, where additional signaling events take place to create a repair-competent environment often at the expense of processes that were occurring pre-DNA damage. A prime example of this is transcription, which is repressed proximal to break sites to reduce conflicts between these pathways (Caron et al., 2019; Puget et al., 2019; Tan and Huen, 2020; Ui et al., 2020). Chromatin and DDR proteins contain diverse functional domains capable of interacting with the various signals that are found at the DNA break site. It is through the engagement of these multiple interactions that proteins recognize breaks within chromatin and mount a DNA damage response, which involves the transmission of signals both on chromatin and through the cell that ultimately coordinate DNA repair activities with other cellular actions (e.g., transcription, replication, and cell cycle progression). Some examples of the types of proteins that participate in the DDR include histone modifying enzymes, histone chaperones, chromatin remodelers, DNA and RNA binding and modifying enzymes, as well as DNA repair proteins themselves (Ciccia and Elledge, 2010; Polo and Jackson, 2011; Price and D'Andrea, 2013; Chiu et al., 2017; Klaric et al., 2021; Par et al., 2021). Thus, at sites of DNA lesions, these diverse sets of factors must interact with many different biomolecules to coordinate their response both in space and in time to initiate, promote and conclude DNA damage signaling and repair activities. Given the complex nature of these interactions, it is not surprising that defects in these pathways result in genome instability, a known contributor to human diseases including cancer, neurodegeneration, immunodeficiency and aging (Jackson and Bartek, 2009; McKinnon, 2017; Schumacher et al., 2021).

DNA double-strand breaks (DSBs), pose a serious threat to genomic integrity and therefore need to be repaired in an efficient and timely fashion. The repair of DSBs in mammalian cells typically proceeds through one of two main pathways, homologous recombination (HR) or non-homologous end-joining (NHEJ) (Scully et al., 2019). Repair of DSBs by homologous recombination is most-prevalent in S and G2 cell cycle phases due to the fact that this repair pathway is templated and uses a homologous sequence (i.e., sister chromatid) to complete error-free repair. The primary initiating event for HR repair is the recruitment of CtIP and the MRN complex to the DSB site where these factors generate a 3′ DNA overhang using endo-and exo-nuclease activities (Lamarche et al., 2010; Makharashvili and Paull, 2015; Keijzers et al., 2016). The 3′ overhang functions to both inhibit engagement of the NHEJ pathway and to also initiate additional activities that promote HR repair. In addition to HR, several other repair pathways can act on resected ends to promote their repair. These pathways include alternative end-joining and single-strand annealing. These pathways are less frequently engaged in HR-proficient cells but appear to play important functions in cells where HR is impaired. We refer readers to several recent reviews on this topic (Ceccaldi et al., 2016; Verma and Greenberg, 2016; Scully et al., 2019; Zhang and Gong, 2021). In HR, the single stranded DNA overhang is initially bound by RPA but is later replaced by the recombinase RAD51 through a pathway dependent on BRCA1, PALB2 and BRCA2 (Prakash et al., 2015; Kawale and Sung, 2020). RAD51 facilitates the invasion of the 3′ overhang into the homologues template *via* D-loop formation, where synthesis dependent repair occurs (Sung et al., 2003). DNA end resection is tightly regulated by various mechanisms including cell cycle and DDR factors. CDK-mediated end resection and HR regulation involves the phosphorylation of CtIP on Thr847 (Huertas et al., 2008; Huertas and Jackson, 2009). Given that CtIP interacts with and assists in promoting MRE11 function (Sartori et al., 2007; Anand et al., 2016), CtIP acts as a sensor for the cell cycle, as a CDK substrate, and transmits the information to start resection. BRCA1 also regulates DNA end resection, including through its ability to interact with CtIP in a phospho-specific manner (Yu and Chen, 2004; Yu et al., 2006; Chen et al., 2008; Yun and Hiom, 2009). Mutations of either BRCA1 or BRCA2 increase cancer risk in several different tumor types, including breast and ovarian, highlighting the importance of DSB repair factors in maintaining genome integrity and suppressing human diseases such as cancer (Li and Greenberg, 2012; Lord and Ashworth, 2016).

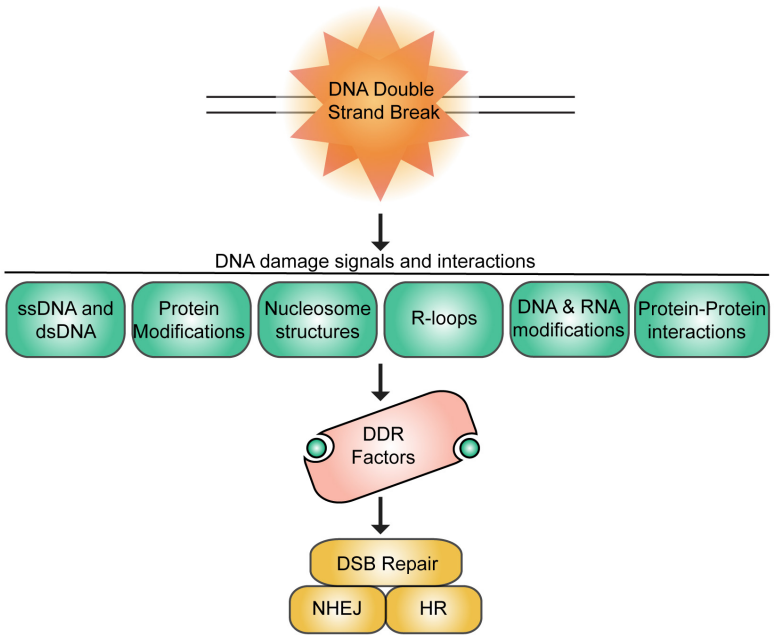

FIGURE 1 | Summary of DNA damage signals and interactions at DNA double-strand breaks. DSBs signal various chromatin associated signals that promote interactions and recruitment of DNA repair factors to breaks to engage DSB repair primarily by homologous recombination repair (HR) or non-homologous end-joining (NHEJ).

Unlike HR, NHEJ is a non-templated DSB repair pathway that engages the broken DNA ends and ligates them back together with little to no DNA end resection. Upon DSB formation, DNA ends are first recognized and protected from digestion by the KU70-KU80 complex (Doherty and Jackson, 2001; Chang et al., 2017). Depending on the physical features of the DNA ends, various additional NHEJ proteins are recruited including the kinase DNA-PKcs. Some breaks are re-ligated together with no end processing if the DNA ends are blunt and compatible. If incompatible ends are present, DNA-PKcs works with various other NHEJ proteins including the nuclease Artemis and polymerases to process the ends before ligation. The XRCC4-LIG4 complex is then recruited to the break to catalyze the re-ligation of the two broken DNA ends. PAralog of XRCC4 and XLF, (PAXX) interacts with KU70-KU80 to stabilize these complexes on damaged DNA to promote NHEJ in a manner independent of any apparent DNA binding activities (Ochi et al., 2015). Since some processing can occur to prepare the ends for joining and no template is used, some genetic material can be deleted or added into the break site (Rodgers and McVey, 2016). These properties of NHEJ make this DSB repair pathway more mutagenic and error-prone compared to HR.

An important question to consider is how DSB repair pathway choice is determined? Given that NHEJ is non-templated, this repair pathway occurs throughout all cell cycle phases and is believed to be the prominent repair pathway in mammalian cells (Chang et al., 2017). For HR, the resection machinery is active during S/G2 when a sister-chromatid template is present. However, in S/G2, it has been calculated that NHEJ is still the more actively engaged pathway compared to HR (Beucher et al., 2009). In addition to the cell cycle phase, other factors have also been proposed to regulate DSB pathway choice including transcription, replication, and chromatin modifications (Shrivastav et al., 2008; Marnef et al., 2017; Scully et al., 2019). The engagement of multivalent interactions also influences the pathways utilized to repair DSBs. For example, while the antagonistic relationship between the non-homologous end-joining promoting factor 53BP1 and the homologous recombination protein BRCA1 is well established, these factors utilize multiple signal recognition mechanisms within chromatin at damage sites to determine DSB repair pathway choice throughout the cell cycle (see below).

The integration of multiple interactions controls other non-DNA repair factors that influence DNA repair through their regulation of chromatin-related functions. Several factors, including the Polycomb repressive complex 1 and 2 (PRC1/2) and the nucleosome remodeling and deacetylase (NuRD) complex, can function in gene regulation through their ability to bind and alter chromatin structure and function (Lai and Wade, 2011; Basta and Rauchman, 2015; Yu et al., 2019; Piunti and Shilatifard, 2021), including in DNA break-induced transcriptional responses (reviewed here). Interactions of these complexes with breaks not only act at the level of regulating protein recruitment and activities but can also alter the biophysical properties of protein condensates themselves to create liquid-liquid phase separated compartments that have been shown to be important in both transcription and the DDR (Jiang et al., 2020; Peng et al., 2020; Fijen and Rothenberg, 2021). The chromatin environment proximal to DSBs can also contain diverse nucleic acid structures which can serve as an interface

for DDR factors; in particular, RNA:DNA hybrids (R-loops) have recently emerged as a source and consequence of DSBs (Crossley et al., 2019; Marnef and Legube, 2021). R-loops, DNA and RNA, as well as chemically modified nucleotides, have all been shown to serve to further coordinate the recruitment and function of factors within the DDR (Allison and Wang, 2019; Bader et al., 2020; Sriraman et al., 2020; Klaric et al., 2021; Lee et al., 2021; Par et al., 2021). Here we highlight several principal examples, illustrating how multifaceted interactions within proteins and protein complexes collaborate at DNA damage sites to coordinate the DDR and DSB repair within the chromatin environment through the engagement of diverse molecular signals.

DOUBLE-STRAND BREAK REPAIR PATHWAY CHOICE FACTOR 53BP1

53BP1 is a large, 1,972 amino acid protein that contains multiple domains capable of interactions with chromatin marks and diverse DSB effector molecules at DSBs [**Figure 2A**; reviewed in Panier and Boulton (2014) and Mirman and de Lange (2020)]. 53BP1 engages DSB sites *via* multivalent interactions where it acts to control DSB repair pathway choice. Several specific domains are contained within the 53BP1 minimal foci forming region (FFR) that function to localize 53BP1 to DNA lesions. The FFR region of 53BP1 is composed of the dynein light chain (LC8) binding domain, oligomerization domain (OD), a glycine/arginine-rich (GAR) domain, a tandem Tudor domain, and a ubiquitin-dependent recruitment (UDR) motif (**Figure 2A**). The function of the GAR domain found within this region of 53BP1 remains unclear.

Upon DNA damage, 53BP1 is translocated to damaged chromatin through multiple interactions with modified histones and the nucleosome. The localization of 53BP1 to DSBs is regulated by ATM mediated phosphorylation of the histone variant H2AX on Ser139; a modification that is read by twin BRCT domains within MDC1 which in turn promotes the accumulation of the ubiquitin E3 ligases RNF8 and RNF168 (Stucki et al., 2005; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007; Doil et al., 2009; Stewart et al., 2009; Bekker-Jensen and Mailand, 2010). The DDR is driven by many such phospho-binding events that are mediated by a host of phospho-epitope binding domains including BRCT, FHA, WD40 and others [reviewed extensively in Reinhardt and Yaffe (2013)]. RNF8 ubiquitinates linker histone H1 with K63-linked ubiquitin chains at DNA damage sites, which are bound by ubiquitin binding motifs (UIM and MIU) within RNF168 to localize this E3 ubiquitin ligase to DNA lesions (Thorslund et al., 2015). While RNF168 contains several defined ubiquitin binding domains (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009), many other DDR factors contain ubiquitin binding domains that bind to ubiquitin signals involved in signaling and repair DNA breaks [reviewed in Hofmann (2009) and Schwertman et al. (2016)]. The sequential recruitment of RNF8 followed by RNF168 has also been shown to involve L3MBTL2 through its recruitment by MDC1 and ubiquitination by RNF8, which serves to promote RNF168 accrual at DSBs (Nowsheen et al., 2018). Once localized to DSBs, these DDR factors regulate 53BP1 accumulation at damage sites in several ways. RNF8 and RNF168 mediated K48 poly-ubiquitin chains are placed onto L3MBTL1 and JMJD2D leading to their proteasome-mediated degradation. In undamaged conditions, L3MBTL1 and JMJD2A occupy H4K20me2 sites and prevent the recognition of this modification by 53BP1 (Acs et al., 2011; Mallette et al., 2012). At the same time, RNF168 catalyzes the mono-ubiquitination of H2A at lysine 15 (H2AK15ub) (Pinato et al., 2009; Stewart et al., 2009; Gatti et al., 2012; Mattiroli et al., 2012). This modification is recognized by the ubiquitin-dependent recruitment (UDR) domain of 53BP1 (Fradet-Turcotte et al., 2013). 53BP1 is retained at DSB through an additional recognition of H4K20me2, which is mediated by the tandem Tudor domains (Botuyan et al., 2006). These interactions between 53BP1 and modified histones have been further characterized using Cryo-EM (Wilson et al., 2016). Using H4K20me2 and H2AK15ub modified nucleosomes, this work revealed the structural details of 53BP1 bivalent interactions with these histone marks as well as identified an additional interaction surface between the nucleosome acidic patch and the 53BP1 UDR domain. The nucleosome acidic patch has emerged as a vital interaction hub on the nucleosome for many DDR factors in addition to 53BP1, including RNF168 (Leung et al., 2014; Mattiroli et al., 2014; Agarwal and Miller, 2016). In the case of 53BP1 and H2AK15ub recognition, it was found to be reliant on the presence of two arginine fingers in H2A and the 53BP1 UDR domain association with the nucleosome acidic patch [**Figure 2B**; (Wilson et al., 2016)]. Thus, these studies reveal the complex nature of 53BP1 regulation at break sites within chromatin, which utilizes multiple interactions to govern its recruitment and activities at breaks.

Recruitment and retention of 53BP1 by two different chromatin modifications likely provides a mechanism to ensure 53BP1 specifically associates with DNA damage sites by using a combination of signals that alone are not sufficient for binding but together tether 53BP1 to the break to elicit its response. In addition to the competing mechanisms with L3MBTL1 and JMJD2A (Min et al., 2007; Lee et al., 2008), 53BP1 is also regulated by histone acetylation. In response to DNA damage, the histone acetyltransferase TIP60 acetylates H2AK15 (H2AK15ac), which antagonizes RNF168-driven mono-ubiquitination (H2AK15ub) of the same site, a mark required for 53BP1 recruitment (Jacquet et al., 2016; **Figure 2B**). In this way, the mutually exclusive ubiquitination and acetylation on H2AK15 establishes a 53BP1 recruitment switching mechanism. TIP60 also acetylates H4K16, which is in close proximity to H4K20 (Tang et al., 2013). This acetylation sterically hinders the recognition of histone methylation on H4K20 by the Tudor domains of 53BP1 (**Figure 2C**). Thus, TIP60 acetylation on histones antagonizes both histone modification recruitment mechanisms for 53BP1, allowing for a robust attenuation of 53BP1 mediated repair by TIP60. The recruitment of 53BP1 to DNA damages sites is also controlled by the SUMOylation activity of PIAS4 as the expression and activity of this E3 SUMO ligase has been established as a requirement for 53BP1 recruitment to DSBs (Galanty et al., 2009). Interestingly, SUMOylation by PIAS4 was also found to be required for the DSB recruitment of RNF168;

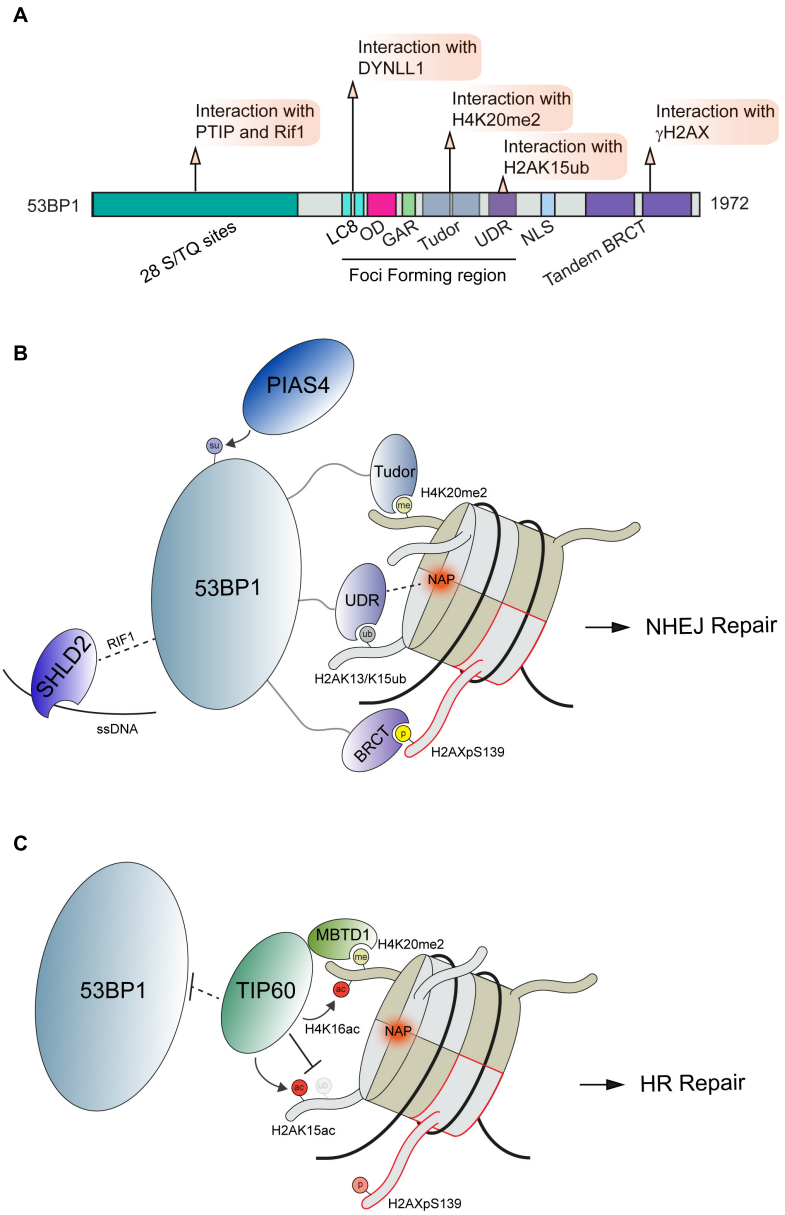

FIGURE 2 | Interactions and regulation of 53BP1 during DSB repair. **(A)** Domain map of 53BP1 and DDR related interactions. **(B)** Schematic interactions between 53BP1 and chromatin proximal to DNA damage sites. 53BP1 engages with chromatin through recognition of H4K20me2 by its tandem tudor domains, interacting with the NAP and H2AK13/K15ub *via* its UDR domain and binding to H2AXpS139 *via* its BRCT domain. These interactions along with the others shown position 53BP1 on nucleosomes. **(C)** Negative regulation of 53BP1 by the histone acetyltransferase TIP60. To antagonize 53BP1 function TIP60 prevents access to H4K20me through its association with MBTD1 while also acetylating H4K16 and H2AK15. PTIP, Pax Transactivation domain-Interacting Protein; RIF1, Replication Timing Regulatory Factor 1; DYNLL1, Dynein Light Chain LC8-Type 1; OD, Oligomerization Domain; GAR, Glycine/arginine Rich Domain; UDR, Ubiquitin-Dependent Recruitment Domain; NLS, Nuclear Localization Sequence; BRCT, BRCA1 C-terminus domain; PIAS4, Protein Inhibitor Of Activated STAT 4; SHLD2, SHieLDin complex subunit 2; TIP60, 60 kDa Tat-Interactive Protein; MBTD1, MBT Domain Containing 1; NAP, Nucleosome Acidic Patch.

raising the possibility that 53BP1 regulation by PIAS4 occurs at the level of RNF168. Although 53BP1 has been found to be SUMOylated, the direct effect of this modification on 53BP1 functions has yet to be fully elucidated (Garvin and Morris, 2017; **Figure 2B**). We note that histones, including H2AX, are SUMOylated by PIAS4 (Chen et al., 2013), so the potential for SUMOylation to regulate 53BP1 on chromatin is also possible yet unexplored. There are likely additional mechanisms whereby PTMs regulate 53BP1 function on chromatin in the DDR.

In addition to histone methylation and ubiquitination, 53BP1 also directly interacts with γH2AX *via* its C-terminal BRCT repeat domain (Kleiner et al., 2015). Although this interaction is dispensable for 53BP1 accumulation at DNA lesions, the BRCT domain is crucial for the repair of DSBs in heterochromatin

(Lee et al., 2010; Noon et al., 2010). In addition, ATM also directly phosphorylates the N-terminus of 53BP1 to allow recruitment of the effector protein Rif1, which acts in the 53BP1-Rif1-Rev7 axis to limit 5′ end resection and BRCA1 accumulation at DSB sites to facilitate NHEJ repair (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Zimmermann et al., 2013; Boersma et al., 2015; Xu et al., 2015). Phosphorylation of the 53BP1 N-terminus also serves to recruit PTIP through interactions between the PTIP BRCT domains and p-Ser25 on 53BP1 (Munoz et al., 2007; Callen et al., 2013). Once associated with 53BP1, PTIP promotes NHEJ and inhibits BRCA1 mediated HR repair through a mechanism that is still under investigation (Li and Greenberg, 2012; Callen et al., 2013; Escribano-Diaz and Durocher, 2013). In 2018, numerous labs converged on the identification of the Shieldin complex, a 3 protein complex consisting of SHLD1 (C20orf196, RINN3), SHLD2 (FAM35A,RINN2) and SHLD3 (CTC-534A2.2, RINN1) that forms a stable complex with REV7 [Dev et al., 2018; Findlay et al., 2018; Gao et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018; Noordermeer et al., 2018; Tomida et al., 2018); reviewed in Setiaputra and Durocher (2019)]. FAM35A (SHLD2), a component of the Shieldin complex, was reported to act downstream of 53BP1 (Dev et al., 2018; Gupta et al., 2018; Mirman et al., 2018). The association of 53BP1 with Shieldin is regulated by phosphorylation of the 53BP1 N-terminal region containing S/TQ repeats. Phosphorylated 53BP1 associates with the effector proteins Rif1, Shieldin, and PTIP (Munoz et al., 2007; Chapman et al., 2013). Functioning in concert with 53BP1, the Shieldin complex counteracts DNA end resection to support NHEJ. This is believed to occur in part through the ability of 53BP1-Shieldin complex to recruit CTC1-STN1-TEN1 (CST) to DSBs that together with Polα-primase act to counteract end resection by filling in resected DSBs (Mirman et al., 2018). In addition to the interaction with the 53BP1-Rif1 complex, the Shieldin complex can also bind to ssDNA *via* the SHLD2 oligonucleotide/oligosaccharide binding fold domain (Dev et al., 2018; Noordermeer et al., 2018). The ssDNA binding activity is believed to play a crucial role in tethering this complex to DNA repair intermediates to recruit the 53BP1-Rif1-Shieldin pathway to inhibit HR and promote NHEJ. In addition to the histone modifications described above, protein-protein interactions also impact 53BP1 recruitment to DSB sites. The self-dimerization of 53BP1 occurs through its OD domain independently of DNA damage; however, this domain is reported to stimulate 53BP1 accumulation at DSBs (Ward et al., 2006; Zgheib et al., 2009). An interaction between 53BP1 and dynein light chain (DYNLL1) *via* its LC8 binding domain has been reported, with this interaction promoting the retention of 53BP1 at damaged chromatin [**Figure 2A**; (Becker et al., 2018; West et al., 2019)]. DYNLL1 also interacts directly with MRE11 to limit its resection activity (He et al., 2018), which provides another example of how multiple protein interactions impinge on a pathway, which in this case acts to limit DNA end resection.

These studies highlight how 53BP1 promotes DNA repair as a consequence of multivalent interactions with chromatin and other proteins. The interactions with nucleosomes along with 53BP1 self-dimerization have recently been identified as mediators of 53BP1 phase separation (Kilic et al., 2019; Piccinno et al., 2019). 53BP1 nuclear bodies were found to exhibit hallmarks of liquid-like behavior when localized to DSBs. Of note, it was found that the protein AHNAK interacts with 53BP1 in its oligomerization domain, thereby regulating multimerization and phase separation (Ghodke et al., 2021). In AHNAK deficient cells, 53BP1 displays augmented phase separation that alters cellular responses to DNA damage. It has been demonstrated that several upstream DDR factors, including MDC1 and γH2AX, do not exhibit liquid-like behavior (Kilic et al., 2019). This raises the question of how the molecular interactions governing the association and dissociation of DDR factors regulate liquid condensates. One could envision that defects in this pathway could result in aberrant repair signaling and reactions resulting in mutations or inappropriate function of these protein complexes that must be tightly regulated to channel their activities to the correct genome location at the appropriate time. It is worth speculating that additional interactions among 53BP1, including proteins and other biomolecules, are likely to regulate and drive these interactions that are essential for recognizing and repairing breaks within chromatin.

REGULATION OF BRCA1 BY CHROMATIN INTERACTIONS

The well-established DNA repair factor BRCA1 is known to form several distinct complexes including BRCA1-A, BRCA1-B, and BRCA1-C through alternative interactions (Chen et al., 2006; Savage and Harkin, 2015). Through these binding partners, BRCA1 serves as an integration point for several essential cellular processes and DNA repair (Ciccia and Elledge, 2010; Venkitaraman, 2014; Prakash et al., 2015; Gorodetska et al., 2019). Perturbations of BRCA1 function can act as a potent driver of cancer progression and can impact therapeutic responses to chemotherapies including platinum drugs and PARP inhibitors (Farmer et al., 2005; Li and Greenberg, 2012; Venkitaraman, 2014; Lord and Ashworth, 2016, 2017; Mylavarapu et al., 2018). Here we focus on the interactions regulating BRCA1 functions in DNA repair in chromatin; in particular the BRCA1-A complex. This complex consists of BRCA1, RAP80, BRCC36 and BRCC45, MERIT 40, and Abraxas (ABRA1) and is essential for controlling DSB repair efficiency by HR (Harris and Khanna, 2011; Wang, 2012; Rabl, 2020). The zinc finger ZMYM3 is also reported to be associated with the RAP80, ABRA1, and BRE components of the BRCA1-A complex that fine-tunes BRCA1 loading at DNA lesions (Leung et al., 2017). ZMYM3 is a member of the myeloproliferative and mental retardation (MYM)-type zinc finger protein family, which share conserved repeats of MYM-type zinc finger motifs (van der Maarel et al., 1996; Popovici et al., 1998; Smedley et al., 1999). ZMYM3 is comprised of several domains including a MYM-type zinc finger, TRASH, H2A/H2AX interacting region, a BRCA1-A complex binding area and a domain of unknown function (DUF) (**Figure 3A**). Collectively, these domains play an important role in regulating ZMYM3 functions at damage sites as the deletion of each motif results in impaired HR

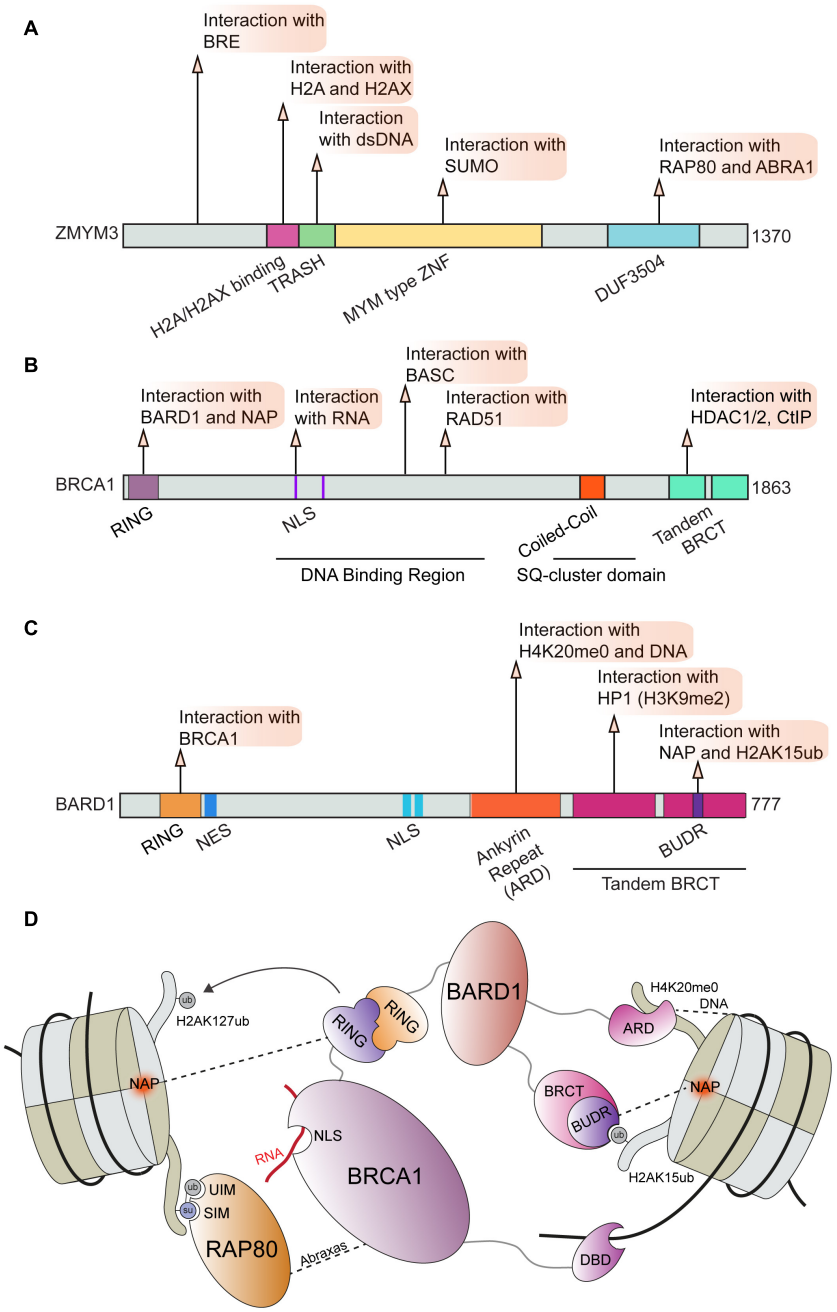

FIGURE 3 | BRCA1 and associated factors in DNA repair. Domain map and DDR interactions of **(A)** ZMYM3; **(B)** BRCA1; and **(C)** BARD1. **(D)** Schematic of interactions between the BRCA1-BARD1 complex and chromatin that facilitate BRCA1 mediated DNA repair. BRCA1 can directly bind to RNA *via* its NLS region and to DNA *via* its DBD. BRCA1 binding to BARD1 through its RING domain and association with RAP80 through direct interactions with Abraxas are essential for BRCA1 function during DDR. BARD1 and RAP80 interact directly with several chromatin marks and serve to correctly position this complex at damage sites. BRE, Brain and Reproductive Organ-Expressed; RAP80, Receptor-Associated Protein 80; ABRA1, Abraxas; TRASH, Trafficking, Resistance, And Sensing Heavy Metals Domain; MYM, MYeloproliferative and Mental Retardation; DUF3504, Domain of Unknown Function; BASC, BRCA1-associated genome surveillance complex; HDAC1/2, Histone Deacetylase 1/2; CtIP, CtBP-interacting protein; RING, Really Interesting New Gene; NLS, Nuclear Localization Sequence; BRCT, BRCA1 C-terminus domain; NES, Nuclear Export Sequence; HP1, Heterochromatin Protein 1; BUDR, BARD1 ubiquitin (Ub)-dependent recruitment and BRCT-associated ubiquitin-dependent recruitment; NAP, Nucleosome Acidic Patch; UIM, Ubiquitin Interacting Motif; SIM, SUMO Interacting Motif; DBD, DNA Binding Domain.

repair and genome instability (Leung et al., 2017). ZMYM3 interacts with H2A and the H2A variant H2AX, as well as double-stranded DNA *via* its H2A/H2AX binding region and TRASH domain, respectively (Leung et al., 2017). Loss of ZMYM3 results in defective BRCA1 foci at damage sites and reduced HR although how these multiple interactions within

chromatin drive the function of this zinc finger protein remains poorly understood.

The ZMYM3 MYM-type zinc finger motif is also required for ZMYM3 chromatin association and efficient HR repair (Leung et al., 2017). Interactions between MYM-type zinc finger motifs and SUMO have been reported (Guzzo et al., 2014; Garvin and Morris, 2017); however, the functional consequences of the ZMYM3-SUMO interactions in regulating HR remain unknown. Given that many DDR factors involved in DSB repair are SUMOylated (Garvin and Morris, 2017), ZMYM3 may interact with SUMOylated substrates to coordinate and impact HR repair. Regulation of the BRCA1-A complex by SUMO may also occur through SUMO binding by RAP80 *via* its SUMO-interacting motif (SIM) (Anamika and Spyracopoulos, 2016; Lombardi et al., 2017). Interestingly, both SUMO binding and ubiquitin binding domains are required for RAP80 localization to DSBs; this dual recognition may fine-tune BRCA1-A complex recruitment to damage sites (Hu et al., 2012). The contribution of ubiquitination by RNF8 and RNF168 to RAP80 recognition of ubiquitin-SUMO mixed-chains still requires further investigation as the dual marks recognized by RAP80 may be conjugated by RNF4, a SUMO-targeted ubiquitin ligase (STUbL) (Guzzo et al., 2012; Chang Y. C. et al., 2021). The regulation of the BRCA1-A complex by RAP80 may also occur through interactions between ZMYM3 and RAP80. ZMYM3 directly interacts with ABRA1 and RAP80 *via* its C-terminus, and also associates with BRE through an N-terminal region (Leung et al., 2017). Interactions between ZMYM3 and RAP80, as well as ABRA1, appear to be required for the DDR function of ZMYM3 as deletion of ZMYM3 C-terminus abolishes its translocation to DNA damage sites. Even though the interaction of ZMYM3 with RAP80 and ABRA is needed for ZMYM3 damage accumulation, ZMYM3 counteracts the BRCA1 suppressive regulatory activity of RAP80 and ABRA1. Indeed, RAP80 deficiency in ZMYM3 KO cells rescues HR defects, suggesting that ZMYM3 helps antagonize RAP80 and other BRCA1A complex members to modulate HR efficiency at breaks. This finding adds new layers of regulation to the previously reported roles of RAP80 as a suppressor of BRCA1 promoted HR (Coleman and Greenberg, 2011; Hu et al., 2011). Given that all these molecules are recruited at DSB sites, ZMYM3 may balance the HR prohibitory role of BRCA1-A complex members to control BRCA1 accumulation and therefore HR at breaks, likely through its ability to interact with DNA, histones and SUMO.

ZMYM3 is only one of several chromatin factors that influence the recruitment of BRCA1 to DSBs. For example, BRCA1 and its obligate binding partner BARD1 were shown to be retained at damaged DNA sites through H3K9me2, which is mediated by the interaction between the BRCT domain of BARD1 and HP1 (Wu et al., 2015). In addition, the BRCA1 and BARD1 complex was reported to be recruited to damaged DNA sites in S-phase through an interaction with unmodified histone H4 lysine 20 (H4K20me0) (Nakamura et al., 2019; **Figure 3D**). In this mechanism, the ankyrin repeat domain (ARD) of BARD1 recognizes H4K20me0, a result solidified by the finding that a mutation in the ankyrin repeats disabling H4K20me0 recognition leads to a failure of BRCA1 to accumulate at DSBs (Nakamura et al., 2019). As previously described, H4K20me2 is a major binding site of 53BP1 that targets its recruitment to DNA lesions (Svobodova Kovarikova et al., 2018). In turn, dilution of methylated histones, including H4K20me2, after replication facilitates BRCA1 recruitment to promote HR repair in S-phase until the balance between unmodified and methylated H4K20 is reached in which case 53BP1-dependent NHEJ can also occur (Pellegrino et al., 2017; Simonetta et al., 2018). It is possible that *de novo* methylation of H4K20me0 at breaks in S-phase could convert this mark to H4K20me2 thereby allowing 53BP1-dependent DDR processes to occur, an option that has been observed (Tuzon et al., 2014). Regardless, these observations point to the methylation status on H4K20 as an important mechanism directing DSB repair pathway choice through 53BP1 engagement.

Recent studies have also identified additional regulatory interactions of BRCA1-BARD1 through contact with the nucleosome core particle (NCP) and various histone marks. Using a combination of biochemistry and Cryo-EM structural studies, it was found that BARD1 binds to H2AK15ub, H4K20me0, DNA and the nucleosome acidic patch (Becker et al., 2021; Dai et al., 2021; Hu et al., 2021). The Cryo-EM structures of BARD1 bound to a ubiquitinated NCP also provided new insights on the established interaction between BARD1 and H4K20me0, where it was observed that several residues on the H4 tail interact with the ARD domain of BARD1 (Dai et al., 2021; Hu et al., 2021). These results are in agreement with the predicted model for the BARD1-H4K20me0 binding interface (Nakamura et al., 2019). The ARD domain of BARD1 was also observed to bind DNA, which participated in the affinity of BARD1 to the NCP (Dai et al., 2021; Hu et al., 2021). One of the structures revealed that BARD1 interacts with the nucleosome acidic patch through the BUDR motif contained within one of the twin BRCT domains of BARD1 (**Figures 3C,D**; Hu et al., 2021). BRCA1 was also observed to interact with the nucleosome acidic patch (Hu et al., 2021), which is consistent with previous studies (McGinty et al., 2014; Witus et al., 2021). Finally, three investigative teams reported that a BRCT domain within BARD1, termed BUDR by two independent groups [BUDR–BARD1 ubiquitin (Ub)-dependent recruitment motif (Dai et al., 2021); BUDR–BRCT-associated ubiquitin-dependent recruitment motif (Becker et al., 2021)] binds to H2AK15ub (Becker et al., 2021; Dai et al., 2021; Hu et al., 2021). This is significant as this mark is catalyzed by RNF168, which promotes BRCA1 recruitment and this mark is also recognized by 53BP1 (Doil et al., 2009; Stewart et al., 2009; Mattiroli et al., 2012; Fradet-Turcotte et al., 2013). These findings instantly furnish a mechanism by which BRCA1-BARD1 can antagonize 53BP1 chromatin binding to promote HR through an ability to bind both H2AK15ub and H4K20me0, a mark and a histone region also recognized by 53BP1. Thus, these studies demonstrate how multivalent interactions of the BRCA1-BARD1 complex, which are summarized in **Figure 3**, regulate the association of this complex with damaged-containing chromatin. These interactions highlight once again the concept whereby multiple low affinity interactions cooperate to target complexes to their sites of action, which in this case is chromatin where the coordination of DSB repair pathway choice and the promotion of HR by the BRCA1-BARD1 complex takes place.

We speculate that these multivalent interactions may provide additional control points for dictating how DNA repair proceeds and which BRCA1 containing complexes are recruited to sites of damage in a controlled fashion (**Figure 3**).

BRCA1 also interacts with other proteins at damage sites to regulate its functions. For example, the involvement of the BRCA1 coiled-coil (cc) domain in mediating interactions essential for DNA repair has recently gained attention. Coiled-coil domains are comprised of bundled alpha helices, these can be positioned in parallel or anti-parallel orientations and are established mediators of protein-protein interactions (Strauss and Keller, 2008; Truebestein and Leonard, 2016; Mier et al., 2017). The BRCA1 cc domain is known to mediate its association with PALB2 through interactions with the PALB2 cc domain. The association of BRCA1 and PALB2 is essential for BRCA1 functions in HR repair as this interaction promotes the association of BRCA1 and BRCA2 (Sy et al., 2009). The complex of PALB2 with BRCA1 is inhibited in the G1 phase as PALB2 undergoes proteasome-mediated degradation in the G1 phase which further constrains DSB repair by HR to the S and G2 cell cycle phases (Orthwein et al., 2015). Interestingly, the PALB2 cc domain was recently found to be capable of mediating PALB2 homodimerization, which may regulate the efficiency of BRCA1 mediated HR repair (Song et al., 2018). The function of PALB2 independent of BRCA1 in promoting DNA repair can impact the clinical outcome for cancer patients undergoing treatment with PARP1 inhibitors as it has been recently shown that restoring the function of PALB2 in BRCA1 null cancers also devoid of 53BP1 function can overcome resistance to PARP inhibitors (Belotserkovskaya et al., 2020). BRCA1 also associates with CtIP through interactions mediated by its cc domain (Yu et al., 2006), an interaction that has been found to facilitate replication fork stability but is dispensable for HR repair in mammalian cells (Reczek et al., 2013; Przetocka et al., 2018). The cc domain of CtIP has also been shown to mediate the dimerization of CtIP. Upon dimerization, the CtIP cc domains form a compact 4-helix bundle structure which is distinct from the CtIP-BRCA1 interaction (Dubin et al., 2004). Work remains to fully characterize BRCA1 dependent and independent functions of CtIP. Given that BRCA1 interacting partners may have functions in DNA repair independent of BRCA1 containing complexes, advancing our understanding of how these binding events are regulated will provide new insight into how DNA repair is fine-tuned. In addition to regulation *via* protein-protein interactions, BRCA1 can also impose regulation of DNA repair through its E3 ubiquitin ligase activity when in complex with BARD1 (Kalb et al., 2014). The ubiquitination of H2A on lysines 127 and 129 by the BRCA1-BARD1 complex has been identified as a prerequisite for SMARCAD1 mediated chromatin remodeling, which facilitates HR repair (Densham et al., 2016). Considering this effect on chromatin structure and DNA accessibility by BRCA1 catalyzed ubiquitination, it is not unreasonable to consider that this modification may have additional roles in regulating BRCA1 effectors in HR repair. Further work is needed to fully characterize the contribution of BRCA1 interactors and modifications mediated by BRCA1 in the regulation of DNA repair. A more complete understanding of these multivalent interactions may provide new avenues for therapeutic intervention in cancer types driven by BRCA1 dysfunction (Na et al., 2014).

REGULATION OF TRANSCRIPTION AT DOUBLE-STRAND BREAK SITES

Active transcription through chromatin presents a complex physical structure containing newly synthesized RNA, the separated DNA strands, histones, and the transcription machinery (Li et al., 2007; Barnes et al., 2015; Venkatesh and Workman, 2015). This diverse environment requires regulatory factors capable of recognizing these various structures and proteins engaged at DNA lesions within sites of active transcription. This idea is exemplified by the PRC1 complex, which recognizes multiple histone marks and nucleosome features in order to regulate transcription at DSBs in addition to its roles in transcription during development (Leeb and Wutz, 2007; Aranda et al., 2015). The PRC1 complex is comprised of several subunits which can mediate distinct interactions with chromatin (**Figure 4**). All described variants of the PRC1 complex contain the core components Ring1B (RNF2) and a PCGF protein (most commonly BMI1) however, multiple distinct forms of PRC1 are expressed in human cells, which further contributes to the diverse interactions that this complex can accommodate (Chittock et al., 2017). The Ring1B component of PRC1 acts as an E3 ubiquitin ligase that mono-ubiquitinates histone H2A and H2AX at lysine 119 proximal to DSB sites, with this signal being associated with the repression of transcription (Tamburri et al., 2020) and promotion of the DDR (Shanbhag et al., 2010; Kim et al., 2019). However, some ubiquitination independent transcriptionally repressive functions of PRC1 have been described during normal transcriptional regulation (Pengelly et al., 2015; Tsuboi et al., 2018). Whether or not these functions also contribute to DNA damage activities of the PRC1 complex is not yet defined.

Several interactions with histones and DNA are required to correctly position the PRC1 complex so that it specifically ubiquitinates H2A or H2AX on lysine 119. The activity of Ring1B on H2A and H2AX was shown to require the nucleosome acidic patch in both biochemical and cell-based systems (Leung et al., 2014). X-ray crystallography provided structural details on how Ring1B interacts with the nucleosome; in particular, Ring1B Arg98 inserts into an H2A acidic patch by making hydrogen bonds with H2A side chain carboxylates (McGinty et al., 2014; **Figures 4A,C**). BMI1 participates in polar interactions with H3 and H4, however, the effect of this interaction on PRC1 activity remains undefined (Barbour et al., 2020). The positioning of the PRC1 complex on nucleosomes is further directed by interactions between the associated E2 enzyme, UBCH5C, and DNA (Bentley et al., 2011; McGinty et al., 2014). The catalytic activity of the PRC1 complex is enhanced by the contact between UBCH5C and the DNA. The multivariant binding exhibited by PRC1 may serve to promote specific functions or recruit specific PRC1 variant complexes to chromatin. We note that PRC1 has been shown to be positioned on chromatin in proximity

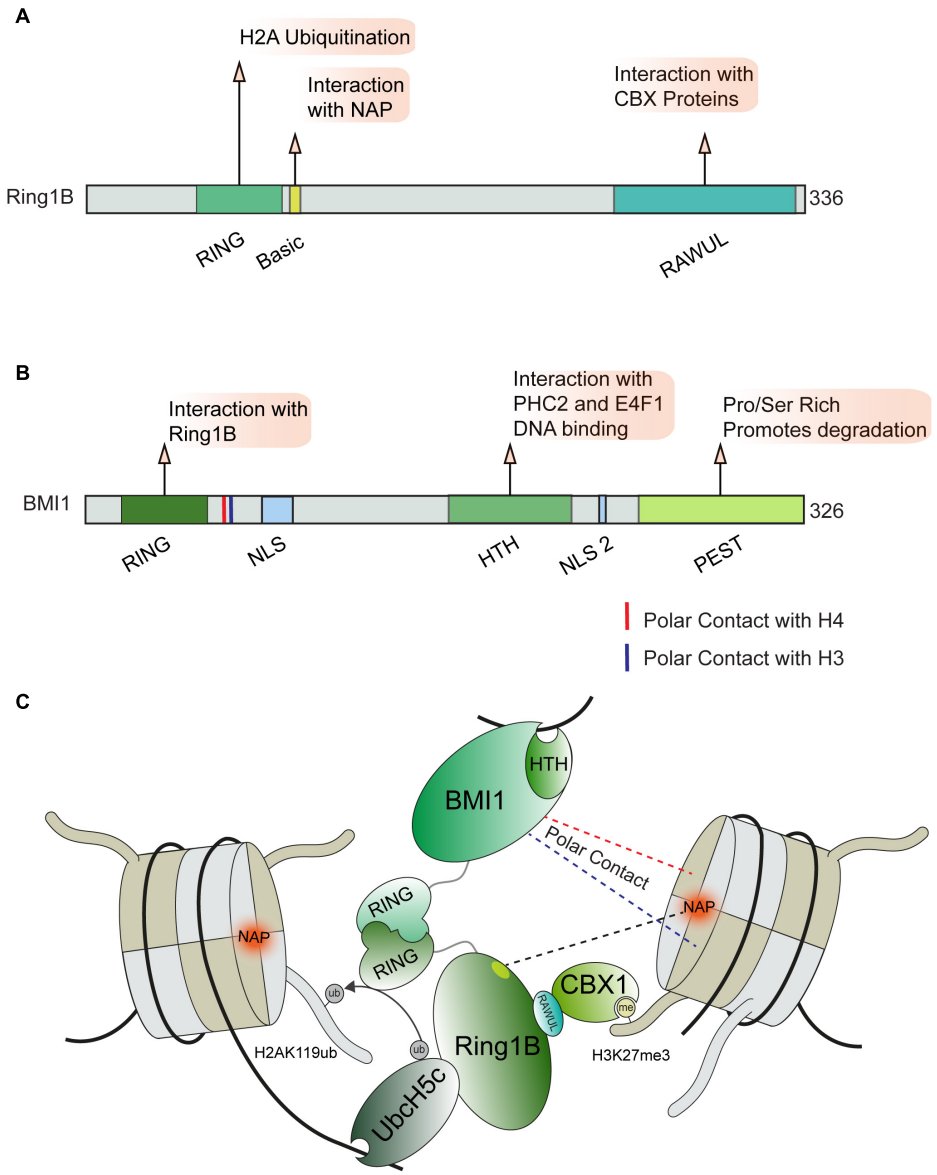

FIGURE 4 | PRC1 complex and chromatin interactions involved in DNA repair. **(A)** Ring1b and **(B)** BMI1 domain maps and DDR related interactions. **(C)** Depiction of chromatin interactions exhibited by core PRC1 members BMI1 and Ring1B involved in transcriptional repression and DNA repair. BMI1 and Ring1B associate *via* their RING domains, Ring1B directly interacts with CBX1 and the E2 enzyme Ubch5c which help position the PRC1 complex for ubiquitin conjugation on H2AK119. Polar contact between BMI1 and H3 or H4 are indicated by blue and red dotted lines, respectively. BMI1, B lymphoma Mo-MLV insertion region 1 homolog; RING, Really Interesting New Gene; CBX, Chromobox Homolog; PHC2, Polyhomeotic-like protein 2; NAP, Nucleosome Acidic Patch; RAWUL, Ring-finger and WD40 associated Ubiquitin-like; NLS, Nuclear Localization Sequence; HTH, Helix Turn Helix; PEST, rich in proline (P), glutamic acid (E), serine (S), and threonine (T).

to areas of active replication, which raises the possibility of additional interactions between PRC1 and the replisome or aberrant nucleic acid structures (e.g., R-loops), which warrants further investigations. While this localization could be attributed to known PRC1 interactions, recent reports have identified PRC1 as essential for the progression of the replication fork, processing of R-Loop structures, and the integrity of common fragile sites which may indicate a more direct role in these processes (Klusmann et al., 2018; Sanchez et al., 2020). In addition to the PRC1 complex, several other pathways that regulate H2AK119ub at break sites have been identified including PRC2, PBAF, ENL, and FRRUC complexes, for which we refer readers to recent in-depth reviews that have covered the extensive involvement of multiple complexes in repressing transcription at DNA breaks, including through the regulation of H2A ubiquitination (Caron et al., 2019; Puget et al., 2019; Tan and Huen, 2020).

The importance of transcriptional regulation at DNA damage sites is supported by the fact that this process is controlled through multiple pathways which rely on diverse interactions

with chromatin and DNA. As an *exemplum primi*, the KDM5A-ZMYND8-NuRD pathway forms multiple contacts with chromatin and modifications, which are critical for this complex to function at DNA breaks [**Figure 5**; (Gong et al., 2015, 2017; Savitsky et al., 2016; Spruijt et al., 2016; Gong and Miller, 2019)]. Mechanistically, KDM5A promotes transcriptional repression and DNA damage repair at DSB sites through the demethylation of histone H3 at lysine 4 (H3K4me3), which allows for the subsequent stable recruitment of ZMYND8-NuRD *via* recognition of TIP60 mediated acetylation of H4 by the ZMYND8 BRD domain (Gong et al., 2015). The association between the ZMYND8 MYND domain and the PPPLΦ domain of the NuRD complex GATAD2A subunit localizes the NuRD complex to DSB sites where it can promote DNA repair through transcriptional repression *via* nucleosome remodeling mediated by the CHD4 subunit [**Figure 5B**; (Gong et al., 2015; Spruijt et al., 2016)]. ZMYND8 can also engage with nucleosomes through interactions with H3K15ac and H3K14me1, which are mediated by the ZMYND8 "reader domain" (containing tandem PHD, BRD, and PWWP domains, [**Figures 5B,C**; (Savitsky et al., 2016)]. This domain within ZMYND8 also binds DNA (Savitsky et al., 2016). In order to support ZMYND8-NuRD recruitment to DSBs, KDM5A relies on multiple interactions on chromatin to correctly position its catalytic Jumonji C (JmjC) domain on H3. KDM5A binding to H3 is made by two plant homeodomain zinc fingers (PHD), PHD1 that recognizes the unmodified N-terminal tail of H3 (Torres et al., 2015) and PHD3 that specifically interacts with H3K4me3 (Wang et al., 2009). The interactions between KDM5A PHD1 domain and the unmodified H3 N-terminal tail also regulates KDM5A activity through induced conformational changes (Longbotham et al., 2021; **Figures 5A,C**). For recruitment to DNA damage sites, PHD1 but not PHD3 was required to support KDM5A translocation to breaks (Gong et al., 2017). Recently, the localization of KDM5A to sites of DNA damage was also found to be dependent on the presence of the histone variant macro H2A1.2 (mH2A1.2) and PARP1 activity (Kumbhar et al., 2021). Depletion of either mH2A1.2 or PARP1 disrupted the localization of KDM5A to DSBs and perturbed the ability of KDM5A to promote DNA repair and transcriptional repression. Interestingly, the association between KDM5A and PAR chains was found to be mediated by a previously unidentified coiled-coil domain (cc domain) within the C-terminus of KDM5A spanning residues 1,501–1,562. The presence of this domain was also found to be required to support KDM5A localization and function at break sites (Kumbhar et al., 2021). Further analysis uncovered that KDM5A exhibits preferential binding to extended PAR chains (ex. 27mer) compared to chains of shorter lengths (**Figure 5C**). This specificity may provide an additional layer of regulation to dictate KDM5A functions at sites of DNA damage. Importantly, cc domains have not been previously identified as a PAR binding domain (Teloni and Altmeyer, 2016) yet this domain within KDM5A binds PAR chains with an apparent affinity in the range of established PAR binding domains involved in the DDR including PBM, PBZ, and macro domains [reviewed in Teloni and Altmeyer (2016)]. This finding raises several intriguing questions about PAR and chromatin mediated interactions at DSBs. Given that approximately 10% of all proteins are predicted to contain coiled-coil domains, further explorations are warranted to characterize the role of cc domains in facilitating interactions with PAR. The role of phase separation in DNA damage response factors has gained attention recently (Pessina et al., 2019) and regions of intrinsic disorder and cc domains are known to contribute to the process of liquid-liquid phase separation (LLPS) (Anurag et al., 2012; Schuster et al., 2020). The potential for interactions between cc domains and PAR to regulate functions mediated by LLPS should be considered and may be determined by PAR binding/chain lengths and the activity of PARPs during DNA break repair.

RNA:DNA HYBRIDS IN DOUBLE-STRAND BREAK REPAIR

R-Loops are 3-stranded RNA:DNA hybrid molecules that form when RNA transcripts hybridize with the template DNA, which poses a substantial obstacle for the replication machinery and causes genomic instability and replication associated DNA breaks (Puget et al., 2019; Brambati et al., 2020; Marnef and Legube, 2021). Structurally, R-loops consist of a region of base-paired RNA:DNA, a displaced single strand of DNA and RNA overhangs (both 3′ and 5′); these distinct nucleic acid structures can be bound by a growing number of factors to catalyze their resolution (Cristini et al., 2018; Allison and Wang, 2019). The structure of R-loops can also directly promote mutagenesis as it has been proposed that the exposed ssDNA strand is vulnerable to nucleases or DNA damaging agents (Huertas and Aguilera, 2003; Makharashvili et al., 2018). The role of RNA nucleases and helicases, including RNaseH1/2 and Senataxin, respectively, in resolving R-loop structures is now well described (Fedoroff et al., 1993; Cerritelli and Crouch, 2009; Hatchi et al., 2015; Groh et al., 2017; Parajuli et al., 2017; Cohen et al., 2018; Lockhart et al., 2019). DSB repair factors also have also been shown to directly bind to R-loops. For example, BRCA1 and the BRCA1/BARD1 complex was shown to preferentially bind R-loops over dsDNA *in vitro* and BRCA1 colocalized with R-loops in IR-treated cells, which was detected using super-resolution fluorescence microscopy (D'Alessandro et al., 2018). Interestingly, this study showed that expression of RNaseH1 in IR-treated cells impaired BRCA1 recruitment to damage sites. An association between TERRA R-loops and BRCA1 was also recently described at telomeres and it was found that BRCA1 can associate directly with TERRA RNA through interactions mediated by the BRCA1 N-terminal NLS region (Vohhodina et al., 2021). Binding of RNA *via* NLS sequences has been identified in other factors including the ribonuclease Dicer, which can be attributed to the density of positive charged amino acids in these regions that can facilitate binding to the ribonucleotide backbone (LaCasse and Lefebvre, 1995). The binding of TERRA by BRCA1 results in the suppression of TERRA transcription and promotes the repair or R-loop associated DNA damage at telomeres (Vohhodina et al., 2021). The association of BRCA1 and R-loops at sites of DNA damage may also occur through NLS

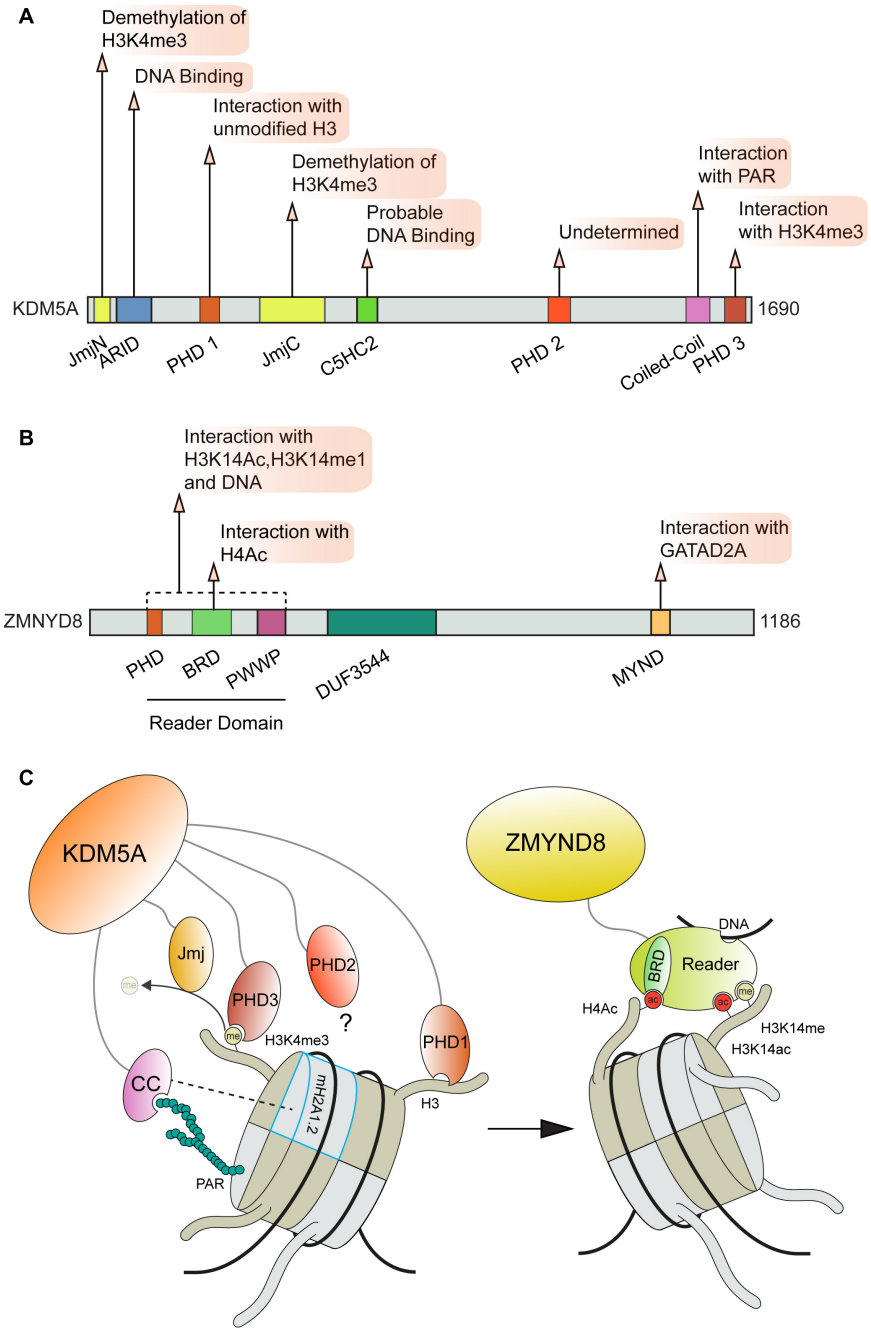

FIGURE 5 | Involvement of ZMYND8 and KDM5A domains and interactions in DNA repair. **(A)** KDM5A and **(B)** ZMYN8 protein domain maps and interactions. **(C)** KDM5A interactions that facilitate its function at breaks and recruitment of ZMYND8 to damage sites. KDM5A interacts with nucleosomes through recognition of unmodified H3 tails and H3K4me3 *via* its PHD1 and PHD3 domains, respectively. KDM5A also recognizes PAR chains and macroH2A1.2 through a coiled-coil (cc) domain. ZMYND8 binding to nucleosomes is facilitated primarily through its Reader domain which recognizes H4ac, H3K14me and H3K14ac histone marks. KDM5A, Lysine-specific Demethylase 5A; Jmj, Jumonji domain; ARID, A–T Rich Interaction Domain; PHD, Plant HomeoDomain; ZMYND8, Zinc Finger MYND-Type Containing 8; BRD, Bromodomain; PWWP, "Pro-Trp-Trp-Pro" domain; DUF, Domain of Unknown Function; MYND, Myeloid, Nervy, and DEAF-1 domain; CC, Coiled Coil; PAR, Poly ADP-Ribose.

mediated interactions (San Martin Alonso and Noordermeer, 2021). In addition to BRCA1, BRCA2 also promotes R-loop processing, which has been shown to be regulated by the helicase DDX5 and RNaseH2 (D'Alessandro et al., 2018; Sessa et al., 2021). In the case of DDX5, BRCA2 was shown to stimulate its helicase activity (Sessa et al., 2021). Using only the N-terminal 250 amino acids of BRCA2, which was shown to encompass the DDX5-interaction region, this fragment of BRCA2 retained

the ability to stimulate DDX5 unwinding of R-loops. These results suggest that BRCA2 itself does not directly bind to R-loops but rather regulates these structures through protein interaction partners that themselves can recognize and act on R-loops. PALB2, which is found in complex with BRCA1 and BRCA2, contains strand exchange activity involving its N-terminal DNA binding domain that can also bind RNA (Deveryshetty et al., 2019). Thus, all three of these HR proteins have been shown to interact with R-loops either directly or through interaction partners.

ssDNA binding proteins involved in DSB repair have also been linked to R-loops. The role of RPA in regulating R-loop formation and resolution has been of interest for some time and RPA co-localizes with RNaseH1 (Nguyen et al., 2017). It was proposed that RPA association with R-loops was through interactions between RPA and the displaced single stranded DNA (Pokhrel et al., 2019) but more recently it has been found that RPA can directly engage with R-loops and bind to RNA with moderate affinity (Mazina et al., 2020). Finally, the most downstream factor involved in HR-mediate repair of DSBs is the recombinase RAD51, which replaces RPA on ssDNA through the activities of BRCA1, PALB2, and BRCA2. Evidence in yeast has suggested that in addition to DNA-DNA strand exchange, RAD51 can also promote DNA-RNA strand exchange that could be involved in R-loop biogenesis (Wahba et al., 2013) although another study obtained results showing that R-loops involved in genome instability form independently of RAD51 (Lafuente-Barquero et al., 2020). The RAD51 interacting protein RAD51AP1 generates R-loops *in vitro* and surprisingly was shown to generate a new recombination intermediate termed a DR-loop, which contains an R-loop within a D-loop. Like several other factors including PALB2, the ssRNA binding activity and R-loop forming ability were dependent on the DNA binding domain of RAD51AP1, suggesting that nucleic acid binding regions can multitask on various structures that form at breaks and during the repair process. It is worth noting that RAD51 in human cells has been reported to promote telomeric recruitment of TERRA *in trans* and formation of telomeric R-loops (Feretzaki et al., 2020) and this activity was found to promote telomere elongation in telomerase negative ALT positive cells. Taken together, these studies highlight the interplay between R-loops and genomic features including DSBs and telomeres. Given the prevalence of both DNA and RNA binding activities in several core HR proteins, it is tempting to speculate that regulatory mechanisms must exist through multiple binding events that function to orientate these complexes at the DNA lesion and ensure their engagement with the requisite structure intermediate rather it be of DNA or RNA origin. It is likely that the deployment of reconstituted biochemical and single molecule systems, structural studies and *in vivo* techniques including super resolution microscopy and Cryo-EM Tomography will be needed to address the challenging questions that remain for how these multi-protein molecular machines and complexes function within chromatin to sense, process, and repair DNA breaks.

The formation of stable R-loops in the genome can give rise to a unique situation where transcription and replication complexes are competing for occupancy of the same DNA template. A growing body of evidence supports a model where a significant source of R-Loop associated DNA damage results from transcription-replication conflicts (TRC), which ultimately can also lead to DSBs (Helmrich et al., 2011; Garcia-Muse and Aguilera, 2019; Puget et al., 2019; Sanchez et al., 2020). The use of novel reporter systems has demonstrated that in both bacterial and human systems, TRCs are most detrimental to cells when they occur in a "head-on" orientation, meaning that the replication and transcription complexes are moving toward each other on the DNA (Hamperl et al., 2017; Lang et al., 2017). R-loops induce DSBs through replication stalling and breakage, which is supported by their increased frequency in close proximity to DSBs (Marnef and Legube, 2021). Thus, DNA stress response pathways involved in DSB repair and replication involve R-loops. To understand mechanistically how R-loops and the proteins that regulate them are involved in these pathways, it will be helpful to identify the DNA and/or RNA binding factors, their modes of binding to various nucleic acid structures and the activities used to regulate R-Loops. This information can inform working models and insights into how these transactions work and are regulated in cells to maintain genome integrity. The speed at which this field is moving is rapid, with future studies expected to reveal the inner workings of how R-loops impact genome integrity through their functions in repair, replication and transcription.

Several reports have elucidated the involvement of DNA damage associated helicases in resolving R-loops. For example, the Fanconi Anemia (FA) helicase FANCM, as well as FANCD2 and FANCI, have all been implicated in R-Loop resolution (Okamoto et al., 2019). Interestingly, the association of FANCI-FANCD2 (ID2) with R-loops appears to be specific to the displaced ssDNA region or the free RNA overhangs and not the RNA:DNA hybrid region of the R-Loop (Liang et al., 2019). Binding of the ID2 dimer to R-loops was found to promote FANCD2 mono-ubiquitination by the FA core complex; however, the functional consequence of this event for R-Loop resolution remains poorly defined. The recognition of ssDNA and RNA overhangs by FANCD2 raises some exciting possibilities and areas for further exploration. Foremost, the identification of the region on FANCD2 capable of interacting with R-loop structures would provide more insight into how R-loop resolving factors may function. The recently identified DNA binding motifs within FANCD2 present one intriguing possibility for how FANCD2 may recognize structural features present within R-Loops (Niraj et al., 2017). FANCM has also been shown to resolve telomeric R-loops through its ATPase activity (Silva et al., 2019) and/or the interaction with the BLM-TOP3A-RMI complex (Lu et al., 2019). These findings further support a multifaceted role for canonical DDR factors which engage R-loops at structurally and potentially functionally diverse areas of the genome to promote genome integrity mechanisms. Additionally, in mutant cells where these pathways are defective, the contribution of unresolved R-loops to FA and genome instability, including through the production of TRCs and DSBs are not fully elucidated.

The role of nucleosome remodeling complexes in resolving R-loops has also recently been investigated. As a case in point,

the INO80 complex was identified as a R-loop resolving factor. The INO80 complex has well established functions during replication and transcription, during which INO80 positions histones at transcription start sites and interacts with the transcription complexes RNAPII and PAF1 (Poli et al., 2017). During replication, INO80 is required for replication restart after fork stalling (Lee et al., 2014) and is necessary for replication fork progression through nucleosome bound DNA (Kurat et al., 2017). New findings now indicate that the effect of INO80 on replication fork progression may be in part due to its R-loop resolving functions. Defects in replication fork progression in INO80 deficient cells can be rescued by overexpression of RNaseH1, providing strong evidence for this idea (Prendergast et al., 2020). Strikingly, it was found that INO80 can locally resolve R-loops within chromatin at stalled forks using the LacO-LacR array system (Prendergast et al., 2020). Although the structure of the INO80 complex bound to nucleosomes has been determined by Cryo-EM (Ayala et al., 2018), it is not yet clear which subunit, activity or binding substrate is required for R-loop resolution. Nucleosome remodeling by the SWI/SNF complex has also been implicated in the resolution of R-Loops (Bayona-Feliu et al., 2021). It was found that depletion of the BRG1 subunit resulted in increased R-Loop associated damage and increased transcription-replication conflicts, indicating that SWI/SNF remodeling activity is required for resolving R-Loops resulting from head on collisions between the replisome and RNAPII. Interestingly, BRG1 co-localizes with FANCD2 at R-loops and co-depletion of these factors has an epistatic effect on cellular R-Loop formation; indicating that these complexes may work together to resolve R-Loops. This association between SWI/SNF and FA proteins is consistent with previous work describing a direct interaction between BRG1 and FANCA (Otsuki et al., 2001). As replication associated factors continue to be explored in the resolution of R-loops, the role of these additional interaction interfaces in regulating R-loop metabolism and the consequences for genome stability and DNA break formation will be an essential area of inquiry. As remodelers impact transcription, it cannot be ruled out that these activities

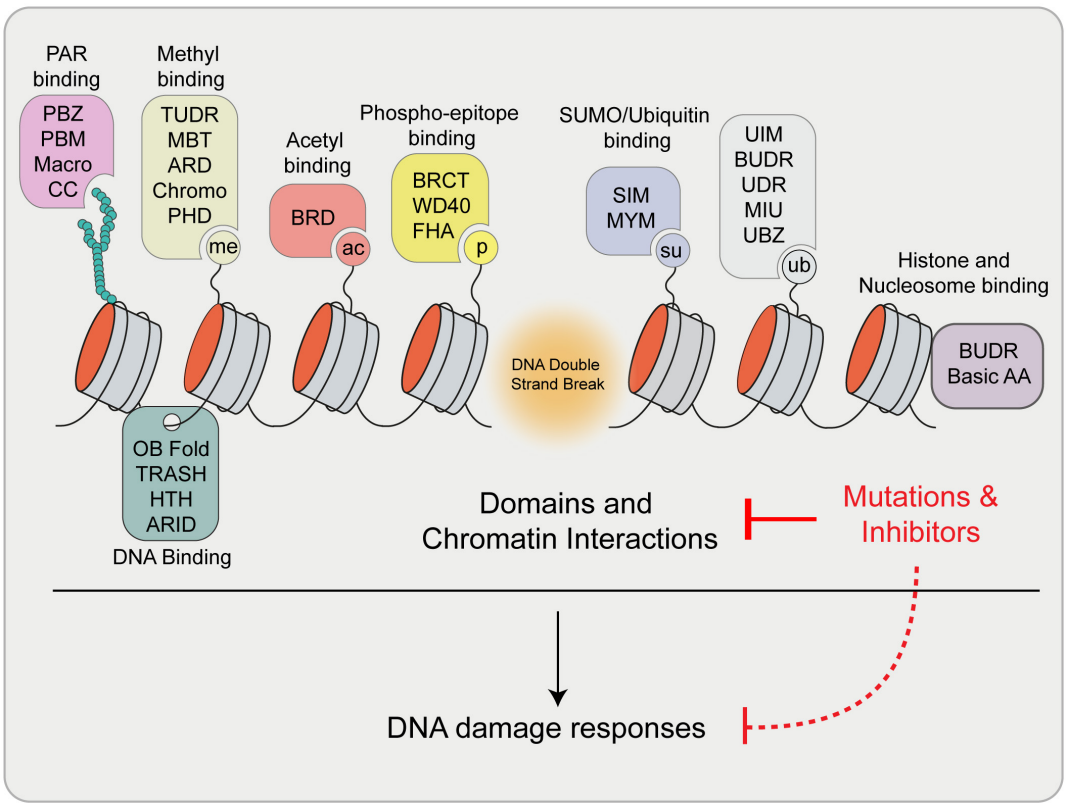

FIGURE 6 | Summary of reviewed and highlighted interaction domains involved in the DDR. Upon DSB formation, many signals are generated that are recognized by specific domains within proteins involved in DNA damage signaling and repair. These proteins engage break sites and chromatin through these interactions to exert their function. Mutations and/or the use of inhibitors of these domains have the potential to disrupt these interaction and pathways, which may impact downstream DDR processes. Definitions: PBZ, PAR Binding Zinc finger; PBM, PAR Binding Motif; CC, Coiled coil; ARD, Ankyrin Repeat Domain; MBT, Malignant Brain Tumor Domain; PHD, Plant Homeodomain; BRD, Bromodomain; BRCT, BRCA1 C-terminus domain; FHA, Forkhead-associated domain; SIM, SUMO Interacting Motif; MYM, MYeloproliferative and Mental retardation; UIM, Ubiquitin Interaction Motif; BUDR, BARD1 ubiquitin (Ub)-dependent recruitment and BRCT-associated ubiquitin-dependent recruitment; UDR, Ubiquitin-Dependent Recruitment domain; MIU, Motif Interacting with Ubiquitin; UBZ, Ubiquitin Binding Zinc finger; OB Fold, Oligosaccharide-Binding Fold; TRASH, Trafficking, Resistance, And Sensing Heavy metals domain; HTH, Helix Turn Helix; ARID, A–T Rich Interaction Domain; Basic AA, Arginine Anchor.

are linked to both replication and DNA damage associated R-loop functions of these large molecular machines that interact with and function within chromatin.

Proteomic approaches have shed light on the factors that respond to R-loops (Cristini et al., 2018). For instance, the Gromak group identified over 450 R-loop interacting proteins by utilizing the S9.6 RNA:DNA hybrid antibody coupled with IP/MS analysis. Another study obtained complementary results using a biotin tagged *BAMBI* promoter and *DPP9* 3′UTR sequences, which are known sites of R-loop accumulation (Wang et al., 2018). Although these studies were performed in different cell types, there were 197 common R-loop interacting factors identified between them including known R-loop resolving factors, helicases, and proteins capable of interacting with RNA and DNA. In addition to recognizing R-loop structural features, factors responding to R-loops may also interact with chemically modified DNA or RNA present at the R-Loop (Al-Hadid and Yang, 2016; Lee et al., 2021). In support of this notion, a recent study utilizing DRIP-Seq found that the majority of R-loops throughout the genome contain N6-methyladenosine (m^6A) RNA modifications (Abakir et al., 2020). Of note, this modification was found to be recognized by the m^6A reader YTHDF2, which in turn promoted the degradation of the R-loop. The RNA strand of an R-loop may also contain 5-methylcytosine (m^5C) modifications, which occurs in response to DNA damage and is catalyzed by the TRDMT1 RNA methyltransferase (Chen et al., 2020). The expression of TRDMT1 and the m^5C modification were both found to aid the recruitment of HR repair factors RAD51 and RAD52, further highlighting the potential importance of this RNA modification in DNA repair. RAD52 has been found to bind R-loops to promote XPG mediated repair, which is involved in transcription-associated homologous recombination repair (Yasuhara et al., 2018). The contribution of m^5C to RAD52 R-loop binding was assessed and interestingly, it was found that RAD52 binds to m^5C modified R-loops with a higher affinity than unmodified hybrids (Chen et al., 2020). Further work is needed to determine the mode of binding to the m^5C modification in RAD52 as currently the region binding this modification has not been determined. It is still a matter of debate about the function of R-loops at DSBs and their origins, including whether or not these structures promote or inhibit DNA repair [reviewed in Skourti-Stathaki and Proudfoot (2014); Crossley et al. (2019); Puget et al. (2019); and Marnef and Legube (2021)]. Regardless, to understand mechanistically how DDR proteins recognize and promote R-loop formation and/or resolution, it will be paramount to determine how these factors recognize and interact with R-loops, including at DNA lesions. The presence of DNA damage specific R-loop modifications presents yet another additional layer of complexity requiring further inquiries.

CONCLUSION AND PERSPECTIVES

As highlighted here, essential factors involved in DNA repair exhibit diverse binding and interactions within chromatin, which can control specific functions during the DDR and influence how DNA lesions are managed. These factors utilize a wide range of specific protein domains that are used to bind various biomolecules at sites of damage (summarized in **Figure 6**). The view of DNA repair is evolving and now constitutes consideration of not only protein and protein interactions and DNA binding at breaks but also the involvement of RNA structures including R-loops, modified proteins and nucleic acids, as well as other interactive signals that drive DNA repair processes. These multivariant interactions may also present potential vulnerabilities for controlling the activity of these factors. Considering the number of structurally unique protein domains required to coordinate DNA repair on chromatin, it is not surprising that many small molecule inhibitors are available that are potentially capable of disrupting the functions of these domains (Arrowsmith and Schapira, 2019; Mio et al., 2019). Chemical or peptide based inhibitors have recently been developed to target several domains that are found within DDR proteins including tandem tudor domains (Chang L. et al., 2021), PRC1 chromodomains (Stuckey et al., 2016), PHD zinc fingers (Wagner et al., 2012), bromodomains (Filippakopoulos et al., 2010) and ubiquitin interacting motifs (UIM) (Manczyk et al., 2019). In addition to their potential clinical applications, the development and availability to researchers of specific inhibitors of these repair-chromatin interactions will be advantageous for untangling and defining the specific contribution of individual contacts within these proteins and their ability to mediate DNA repair.

Altering the physical properties of chromatin bound complexes also provides a potential avenue for specifically controlling the action of repair factors. The formation of membrane-less condensates has been found to facilitate transcription (Boija et al., 2018) and promote DNA repair (Oshidari et al., 2020). With this in mind, the prospect of specifically targeting the function of specific DNA factors by inhibiting their ability to undergo phase separation emerges (Klein et al., 2020). The potential to target phase separation therapeutically also benefits from the fact that many chromatin bound factors can be found localized within distinct phase separated-complexes under different cellular conditions; an example being the multiple forms of PRC1 complexes (Chittock et al., 2017). Under specific conditions during development PRC1 can function independently of its catalytic activity and Polycomb body formation (Tsuboi et al., 2018); however, recent evidence supports enhanced functions of PRC1 through phase separation mediated by the Ph-SAM subunit of PRC1 (Seif et al., 2020). This type of movement between phase separated states may provide a method to target the localization and function of DNA repair factors at specific genomic locations. More work is needed to understand the precise regulation and function of DNA repair associated condensates and how these can be manipulated specifically without altering other biological processes that utilize these pathways. For example, transcription and repair events are intimately linked in both DSB repair and in engaging phase separation as a regulatory mechanism. It may be challenging then to uncouple one process from the other, which has always been difficult for multi-functional proteins unless separation of function mutations can be generated. The use of comprehensive

CRISPR-Cas9 protein domain screens or CRISPR-dependent cytosine base editing screens that can generate protein variants can provide powerful unbiased separation of function screens to address the specific contribution of domains within proteins involved in multiple interactions and biological processes (Shi et al., 2015; Cuella-Martin et al., 2021). In addition, sequences from tumor genomes (e.g., TCGA) may provide additional insights into the function of these domains in cancer, which often exhibit defects in DDR pathways (Helleday et al., 2008; Mio et al., 2019). Determining the functional domains within DDR factors and their potential druggability and/or mutation status in cancer will likely be a valuable endeavor. This information can improve our mechanistic understanding of how DNA repair occurs on chromatin templates in cells and ultimately identify vulnerabilities and/or drug targets for therapeutic interventions in human diseases including cancer.

Given the clear connection between DNA repair and chromatin that occurs at the level of interactions between DNA repair factors and nucleic acids, nucleosomes and modified histones, the physical state of chromatin itself should be considered and may also have dramatic effects on how DNA repair proceeds (Aymard et al., 2014; Marnef et al., 2017; Fortuny and Polo, 2018; Fortuny et al., 2021). The compaction or decompaction of chromatin into heterochromatin and euchromatin largely depends on pathways that engage DNA (i.e., replication and transcription). When damage occurs at regions of DNA that are being replicated or transcribed, the coordination of these events relies heavily on the multivalency of the regulatory factors involved. Interestingly, a growing body of evidence supports a model where ongoing transcription can promote DSB repair through homologous recombination (Ouyang et al., 2017). In a compact, heterochromatic environment, DNA repair is challenged by a high density of repetitive sequences and hindered access to the damaged DNA. The density of heterochromatin is dramatic enough that it was recently found to behave as a solid structure (Strickfaden et al., 2020). The accessibility of damaged DNA to repair factors can be enhanced by specific signals that regulate the transition between different chromatin states, with histone acetylation being a prime example due to its impact on chromatin folding, as well as DNA repair (Eberharter and Becker, 2002; Rodriguez and Miller, 2014; Kim et al., 2019; Chen et al., 2021). However, histone acetylation alone was found to not be sufficient to induce liquid-like properties in DNA. It is likely that in order to repair DSBs within regions of the genome that are difficult to access (e.g., heterochromatin, replicating and or transcribing DNA), repair factors will need to overcome these barriers to access the DNA lesion. Given the diverse nature of chromatin and the activities of the genome within not only the same cell but between different cell types, we speculate that the mechanisms utilized by these factors may differ depending on chromatin states and genome location/process in which the DNA damage occurs. Taken as a whole, the diverse interactions of DNA repair factors on chromatin that are highlighted here provide a framework for considering the complexity of repairing a lesion within a chromatinized and functioning genome. It is fascinating to consider the diverse nature of these interactions that drive repair within chromatin and consider future studies aimed at refining our view of the regulatory mechanisms that ensure proper engagement of these signals by the DDR and chromatin factors to govern the maintenance of genome integrity.

AUTHOR CONTRIBUTIONS

AS and KM wrote the manuscript with assistance from DL and DK. AS and KM constructed the figures. All authors reviewed and finalized the final version of the manuscript.

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REVIEW
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DNA Double Strand Break Repair Pathways in Response to Different Types of Ionizing Radiation

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The superior dose distribution of particle radiation compared to photon radiation makes it a promising therapy for the treatment of tumors. However, the cellular responses to particle therapy and especially the DNA damage response (DDR) is not well characterized. Compared to photons, particles are thought to induce more closely spaced DNA lesions instead of isolated lesions. How this different spatial configuration of the DNA damage directs DNA repair pathway usage, is subject of current investigations. In this review, we describe recent insights into induction of DNA damage by particle radiation and how this shapes DNA end processing and subsequent DNA repair mechanisms. Additionally, we give an overview of promising DDR targets to improve particle therapy.

Keywords: linear energy transfer, double strand break repair, combination therapy, DNA repair pathway, end resection

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INTRODUCTION

Over the past decades, the interest in particle radiotherapy for the treatment of tumors has been on the increase. This is illustrated by the fact that, as of 2021, about 100 proton therapy centers are operational world-wide (Paganetti et al., 2021). In addition, a few carbon ion irradiation centers have been established and are used for the treatment of patients (Tinganelli and Durante, 2020). The central rationale for particle therapy is its superior spatial dose distribution in tissue in comparison to conventional X-ray therapy. Photons deposit the maximum dose at the entrance of the tissue, followed by a gradually decline of dose throughout the remaining tissue. In contrast, protons and other particles deposit a relatively low dose at the entrance while at a certain depth the dose sharply increases, forming the so-called Bragg peak (Newhauser and Zhang, 2015). In this way the major dose is delivered to the tumor and the dose delivered to the surrounding tissue is minimized. The main advantage of this dose distribution is the sparing of so-called organs at risk, resulting in less irradiation-induced side-effects.

Both relative biological effectiveness (RBE) and linear energy transfer (LET) are used to describe differences between particle radiation and X-ray radiation. The RBE is defined as the ratio of the reference radiation type absorbed dose to the absorbed dose of a radiation type that induces the same biological endpoint, for example cell survival (Gulliford and Prise, 2019). X-rays with a defined energy or cobalt-60 γ-rays are often used as reference radiation type. The LET is defined as the amount of energy that a particle transfers to the material traversed per unit distance (Gulliford and Prise, 2019). Radiation types are usually divided into low LET radiation and high LET radiation. Examples of low LET radiation are X-rays or γ-rays and examples of high LET radiation are

α-particles and carbon ions. Protons have a relatively low LET compared to α-particles and carbon ions. However, the LET varies throughout the Bragg curve and is, compared to photons, especially higher in the Bragg peak.

Currently, the RBE in clinical proton therapy is taken to be 1.1 (Paganetti et al., 2002; Paganetti, 2014). In comparison, the RBE of, for example, α- particles is significantly higher, with reported values ranging from 3 to 15 (Durante et al., 1995; Thomas et al., 2003; Franken et al., 2012). The use of a standard RBE of 1.1 in clinical proton therapy is based on measured RBE values *in vivo* from some of the very first proton studies (Dalrymple et al., 1966; Urano et al., 1980). However, there are several uncertainties regarding the RBE of the proton beam, since there are differences in LET throughout the Bragg curve. Especially, in the Bragg peak and at the distal edge of the Bragg peak the LET is significantly higher compared to photon radiation. Additionally, there is a substantial variability in results from both *in vitro* and *in vivo* studies studying the RBE of protons (Paganetti et al., 2002; Paganetti, 2014). This variability can be explained by the fact that the RBE is not only dependent on the LET, but also on other physical factors, such as energy and dose rate of the proton beam, and biological factors, such as type of tumor, cell cycle stage and oxygenation level (Vitti and Parsons, 2019; McNamara et al., 2020). To get insight in the effective RBE of protons and which factors determine the effective RBE, more studies directly comparing the proton versus the photon response in defined cell and *in vivo* models, using defined beam characteristics have to be performed (Durante et al., 2019). In addition, studying cellular responses after high LET irradiation in defined models gives insight into the effect of LET on the RBE and sheds light on the possible added value of high-LET irradiation therapy, such as carbon ion therapy, in comparison to proton therapy (Ma et al., 2015; Nagle et al., 2016; Tinganelli and Durante, 2020).

An important determinant of the effectiveness of radiotherapy is the repair of the DNA damage that is induced by the radiation. In particular, DNA double strand breaks (DSBs) are considered to be a determinant of cell survival since they can lead to cell death if left unrepaired. In this review we provide an update on recent insights into the repair of DNA damage induced by different radiation types. To this aim we provide an overview of the DNA damage response (DDR) upon ionizing radiation (IR), the determinants of the different options between DNA end protection and resection and how the arising substrate can undergo subsequent DNA repair. Additionally, we will give an overview of combination therapies that can potentially be implemented to exploit the properties of particle therapy.

INDUCTION OF DNA DAMAGE BY DIFFERENT TYPES OF RADIATION

Photon radiation induces mainly isolated lesions including single strand breaks (SSBs), base damage and DSBs. In contrast, particle radiation with high LET, such as α-particles and carbon ions are thought to induce a more highly localized and clustered DNA damage (CDD).The LET of protons varies throughout the Bragg curve and therefore the spatial distribution of the induced lesions by protons might be different throughout the Bragg curve. Usually, CDD is defined as two or more lesions formed within one or two helical turns of the DNA. However, this definition does not indicate anything about the type of lesions. For example, DNA damage clusters can consist of non-DSB lesions, such as SSBs and base damage or a DSB with nearby non-DSB lesions or a cluster of DSBs, containing multiple DSBs in close proximity (Nickoloff et al., 2020). It is important to have a clear definition and characterization of CDD, since different DNA lesions could have different effects on DNA repair mechanisms.

It is widely appreciated that the complexity and yield of radiation-induced CDD increases with increasing LET. However, this view might be oversimplified, since particles are physically different from each other and from photons in, for example, energy, charge and diameter (Jezkova et al., 2018).To get insight into the induction of DNA damage by different particles in comparison to photons, the spatial configurations of the induced DNA damage has to be determined. Monte Carlo simulations of the induction of clustered DNA lesions by IR are, at present, the only means to predict the spatial configurations of individual lesions per cluster (Georgakilas et al., 2013). Visualizing DNA damage clusters in cells by use of (immuno)fluorescence microscopy is challenging, since a high resolution is needed to separate individual lesions (Natale et al., 2017). Several studies have shown that the use of electron microscopy (EM) can overcome this resolution barrier (Lorat et al., 2015; Timm et al., 2018). However, EM has certain disadvantages compared to (immuno)fluorescence microscopy, such as the infeasibility to do live-cell imaging and the limited options for labeling DNA repair proteins, which hamper the systemic and thorough understanding of radiation-induced cellular responses.

One of the first events after induction of DSBs by IR is the phosphorylation of histone H2A.X, also referred to as γH2A.X, and the accumulation of the DDR protein 53BP1 which forms so-called IR induced foci (IRIF) at the site of the break (Panier and Boulton, 2014). Immunostaining and fluorescent microscopy imaging of these foci has revealed that γH2A.X and 53BP1 foci are larger after proton irradiation compared to photon irradiation (Szymonowicz et al., 2020). High-resolution stimulated emission depletion (STED) microscopy has shown that these larger foci consist of several individual sub-foci (Szymonowicz et al., 2020). This suggests that either the foci observed after proton irradiation consist of multiple lesions or that the chromatin condensation is different around the induced breaks (Lopez Perez et al., 2016; Natale et al., 2017). Additionally, foci induced by protons remain longer compared to those induced by photons, indicating that they are repaired less efficiently (Oeck et al., 2018; Szymonowicz et al., 2020). Similar observations have been made in cells irradiated with other particles, such as α-particles and carbon ions (Nagle et al., 2016; Roobol et al., 2020). 53BP1 foci induced by α-particle are bigger than foci induced by photons (Roobol et al., 2019). Following live dynamics of GFP-tagged 53BP1 foci in α-particle irradiated cells has shown that the repair of DSBs is slower after high LET IR compared to low LET IR. This indicates that these lesions are different from each other and are also repaired differently.

The production of closely spaced lesions rather than individual lesions by particle irradiation is considered crucial for mutagenesis, genomic instability and cell death (Georgakilas et al., 2013). However, how the type of DNA damage caused by different irradiation types correlates with DNA repair mechanisms and subsequent mutagenesis or cell death is not fully understood. Therefore, more studies characterizing the configurations of particle-induced DNA damage and studying subsequent DNA repair and cellular responses are needed.

DNA DAMAGE RESPONSE

The induction of DNA lesions by IR triggers a cascade of cellular responses, called the DDR, that includes localization and recognition of the lesions which ultimately leads to the repair of the induced damage. DNA damage often triggers cell cycle arrest, and when not properly repaired, apoptosis and cellular senescence. DSBs initiate a cell cycle arrest through checkpoints in G1 and G2 phase of the cell cycle (Shaltiel et al., 2015). These checkpoints prevent the replication and segregation of the damaged DNA, which is crucial for maintenance of genomic integrity. The induction of either cell cycle arrest, apoptosis or cellular senescence can be mediated by the transcriptional regulator p53 which is phosphorylated and activated by ATM. After induction of DSBs variations in p53 protein levels regulate the induction and duration of cell cycle arrest and apoptosis by controlling the expression of a wide variety of target genes (Loewer et al., 2013; Reyes et al., 2018).

Upon DSB induction, the Mre11/Nbs1/Rad50 (MRN) complex accumulates at the DNA damage, bridges the two DNA ends and activates ATM (Reginato and Cejka, 2020). One of the first events after the induction of a DSB is the phosphorylation of histone H2A.X on Serine 139 of its C-terminal tail by the DDR kinases ATM, ATR and DNA-PKcs up to megabases flanking the DNA damage site (Scully and Xie, 2013). MDC1 binds directly to γH2A.X and functions as a scaffolding protein that is thought to mediate most of γH2A.X functions (Stucki et al., 2005). However, MDC1 can also bind to chromatin in a γH2A.X independent manner, indicating that MDC1 might have additional γH2A.X-independent functions (Salguero et al., 2019). MDC1 mediates chromatin methylation and ubiquitination by functioning as a docking site for RNF8 which subsequently leads to the recruitment of RNF168. Ubiquitination of histones by RNF8 and RNF168 initiates a downstream cascade that is crucial for the localization of downstream DNA repair factors. Several proteins, which are involved in different DNA repair pathways, such as the BRCA1-BARD1 complex, 53BP1, and the MRN complex are localized to the site of DNA damage and mediate resection or protection of the DNA ends.

END PROCESSING

DNA repair pathways that can act on DNA ends are homologous recombination (HR), single strand annealing (SSA), non-homologous end joining (NHEJ) and theta-mediated end joining (TMEJ).Which pathway is used for the repair of DSBs follows from the enzymes that act at the DNA end. The DNA repair pathways will be discussed in further detail in the next section of this review. This section will focus on processing of the DNA end and how this is influenced by the cell cycle stage, differentiation stage, and complexity of the DNA damage (**Figure 1**).

The first short-range end resection of DNA ends is performed by the endonuclease Mre11 in complex with Rad50 and Nbs1 (**Figure 1**). Subsequently, long range 5′-3′ end resection can occur, which is executed by the nucleases EXO1 and DNA2 in collaboration with BLM or WRN helicase (**Figure 1**). The single strand DNA (ssDNA) strands that arise during resection are bound by RPA to protect them from degradation and forming secondary structures. While the nucleases have a direct role in resecting the DNA ends, BRCA1 and 53BP1 have indirect roles in regulating resection. The unique interaction between 53BP1 and BRCA1 is illustrated by the fact that loss of 53BP1 can reverse lethality of BRCA1-deficient cells and mice (Bouwman et al., 2010). This rescue is, at least in part, mediated by restoration of HR through an increase in end resection due to the loss of 53BP1 and BRCA1-independent, RNF168-mediated localization of Rad51 (Bunting et al., 2010; Zong et al., 2019). Although it is widely appreciated that 53BP1 inhibits end resection in G1 phase of the cell cycle, it is also shown that 53BP1 in S and G2 phase of the cell cycle plays a role in inhibiting hyperresection, allowing limited resection and repair by HR rather than SSA (Ochs et al., 2016).

When 53BP1 binds to the site of DNA damage, it is phosphorylated by ATM. This phosphorylation leads to accumulation of PTIP and RIF1. Loss of RIF1 or its effector protein, Shieldin, are epistatic with 53BP1 deletion in sensitization to DSB-inducing therapies, increase of end resection after induction of DSBs and rescue of HR in BRCA1-deficient cells (Setiaputra and Durocher, 2019). The inhibition of resection by Shieldin is mediated by CST-Polα-mediated fill-in synthesis, since CST prevents end resection, interacts with Shieldin and accumulates at DNA damage sites in complex with pol α in a 53BP1- and Shieldin-dependent manner (**Figure 1**; Barazas et al., 2018; Mirman et al., 2018). However, this might not be the only way by which end resection and subsequent repair is influenced by the presence of Shieldin at DNA ends. Disruption of the 53BP1-PTIP interaction in BRCA1-deficient cells rescues end resection, but not Rad51 loading (Callen et al., 2020). Rad51 loading in these cells is restored when the Shieldin subunit, Shld3, is depleted (Callen et al., 2020). This study also shows that PTIP prevents long-range end resection by DNA2, while Shieldin prevents long-range end resection by EXO1 (Callen et al., 2020). In summary, this shows that the 53BP1 effector axes, RIF1-Shieldin and PTIP are both important for protection of DNA ends and might have differential effects on DNA repair.

Binding of BRCA1 to the DNA ends results in release of RIF1 from the site of DNA damage, as resection in BRCA1-deficient cells is rescued by RIF1 depletion. Upon irradiation there is an increased amount of RPA foci in these cells as the result of restored resection. Additional depletion of the phosphatase PP4C does not increase the amount of RPA foci, while RPA foci increase in cells containing 53BP1 phosphorylation mutants. This

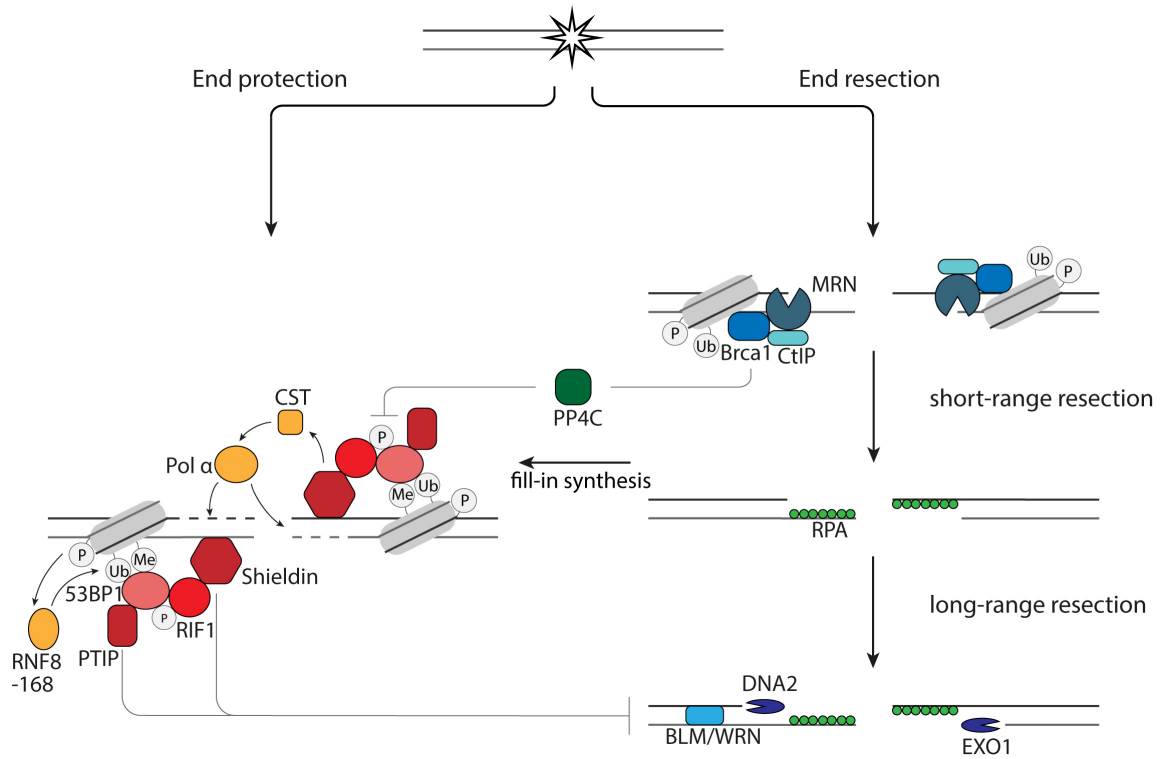

FIGURE 1 | End resection versus end protection. 53BP1 protects DNA ends via its effector proteins, PTIP, RIF1, and Shieldin via (1) inhibition of EXO1 and DNA2 and (2) fill-in synthesis by the CST-Pol α. End protection is counteracted by BRCA1 via dephosphorylation of 53BP1 by PP4C.

shows that the release of RIF1 is the result of dephosphorylation of 53BP1 by PP4C (Isono et al., 2017). Whether a similar mechanism is applicable for PTIP is not known. An important regulator of BRCA1 and thereby end resection is CtIP. CtIP can be post-translationally modified on different sites. Post-translational modifications of CtIP are important for the bridging of DNA ends, stimulation of Mre11 activity, interaction with BRCA1, localization of BLM and EXO1 at DNA ends and enhancement of DNA2-mediated long-range end resection (Wang et al., 2013; Anand et al., 2016; Daley et al., 2017; Ceppi et al., 2020; Öz et al., 2020).

One of the factors that influences the processing of DNA ends is the cell cycle stage. End processing in different cell cycle stages is controlled by a number of factors, including CDKs and cyclins (Hustedt and Durocher, 2017). The level of CDKs is low in G1 phase, but rises during S and G2 phases. In S/G2 phase, CDKs promote end resection by phosphorylating CtIP, EXO1, DNA2, and Nbs1 (Chen et al., 2011; Mirman et al., 2018; Öz et al., 2020). Additionally, factors involved in either end processing are differentially expressed in the different cell cycle stages. For example, the expression of some of the proteins involved in end resection, such as CtIP and Mre11, is higher in S and G2-phase than in G1-phase (Kanakkanthara et al., 2016). The chromatin surrounding the DSB is another cell cycle regulated factor that influences end resection. 53BP1 binding to γH2A.X domains is dependent on the additional chromatin marks H4K20me2 and H2AK15ub. The ubiquitination of H2A on lysine 15 is mediated by RNF168 and thus the direct result of DSB induction (Mattiroli et al., 2012). In contrast, H4K20me2 is present throughout the cell cycle. However, upon replication in S-phase this histone mark is diluted by incorporation of H4K20me0 histones, which allows accumulation of the BRCA1-BARD1 complex, displacement of 53BP1 and end resection (Simonetta et al., 2018; Nakamura et al., 2019).

Additionally, end processing is influenced by the differentiation stage of the cells. Embryonic stem (ES) cells proliferate fast and have a relative short G1 phase (Vitale et al., 2017). Despite their fast proliferation, ES cells repair DNA damage with high fidelity and have a low mutation accumulation rate. This high fidelity repair is mainly attributed to the fact that repair of DSBs in ES cells is less dependent on NHEJ compared to differentiated cells (Bañuelos et al., 2008; Tichy, Pillai et al., 2010). Higher expression levels of proteins that promote end resection such as BLM, WRN, and BRCA1 in ES cells in comparison to differentiated cells probably contribute to this phenomenon by enhancing end resection and directing the substrate into homology directed repair (Maynard et al., 2008). End protection of telomeres is mediated by the Shelterin complex. TRF2 is an essential protein of this complex and prevents the end-to-end fusion of telomeres by NHEJ. However, TRF2 is not essential for end protection of telomeres in ES cells (Ruis et al., 2021). This example illustrates that differentiated and non-differentiated cells might use different mechanisms to safeguard the integrity of their genome.

Another factor that influences the processing of DNA ends, is the complexity of the DNA damage. As described before particles are thought to induce more CDD compared to photons. Therefore, they can be used as a tool to study the effect of complexity of DNA damage on DNA repair. Specifics about the radiation sources used and biological models in the studies that are cited throughout this review can be found in **Table 1**. Several studies compared particle-induced and photon-induced end resection. For example, the percentage of RPA foci positive cells is increased in cells irradiated with high-LET particles compared to photon-irradiated cells (Averbeck et al., 2014)Additionally, iron and carbon ions induce more RPA and CtIP phosphorylation compared to γ- and X-rays (Yajima et al., 2013). Moreover, CtIP depletion impairs repair of carbon ion induced DNA damage, but not of X-ray induced DNA damage, indicating that resection is an essential step in the repair of carbon ion induced lesions (Yajima et al., 2013; Averbeck et al., 2014). A recent study shows that α-particle-induced foci contain multiple RPA foci (Roobol et al., 2020). These findings suggest that DNA ends of DNA damage induced by high LET radiation are more prone to end processing compared to DNA ends of DNA damage induced by low LET radiation.

DOUBLE STRAND BREAK REPAIR PATHWAYS CONTRIBUTION IS INFLUENCED BY RESECTION RANGE

In this section the DNA repair mechanisms NHEJ, HR, SSA and TMEJ and their activity upon (particle) radiation-induced DSBs will be discussed (as illustrated in **Figure 2**).

The first major pathway of DSB repair following X-ray irradiation is NHEJ. NHEJ acts mainly on DSB ends protected from resection by 53BP1, as described above. First, the DSB end is bound by the Ku70/80 heterodimer. Ku70/80 forms a ring structure which interacts with the sugar phosphate backbone in a sequence-independent manner (Walker et al., 2001). It has a high affinity for dsDNA ends, including blunt ends and 5′ and 3′ overhangs, but has a significantly lower affinity for long stretches of ssDNA (Mimori and Hardin, 1986; Ono et al., 1994; Fell and Schild-Poulter, 2015). DNA-PKcs locates to Ku70/80 and orchestrates the repair by phosphorylating other downstream factors, such as Artemis and XRCC4. DSB ends produced by IR are often not directly ligatable due to mismatching overhangs, damaged nucleotides or bulky adducts, but need processing first (Liao et al., 2016; Pannunzio et al., 2018). This task is mainly performed by Artemis, a versatile endonuclease, and three DNA polymerases, namely pol μ, pol λ and terminal deoxynucleotidyl transferase (TdT). After processing, the DSB ends can be ligated by the DNA ligase IV/XRCC4 dimer, which is enhanced by XLF and PAXX (Wyman and Kanaar, 2006; Pannunzio et al., 2018; Zhao B. et al., 2020). In contradiction to its name, NHEJ can make use of limited sequence homology (<4 bp) between the overhangs of the DSB ends, however, this does not require end resection. While NHEJ does usually not restore the original sequence, it does restore the structural integrity of the DNA quickly. Hereby it prevents inappropriate end joining and translocations that could lead to loss of genetic material, chromosome aberrations, and carcinogenesis (Zhao B. et al., 2020; Zhao L. et al., 2020).

A second major pathway for DSB repair is HR. In contrast to NHEJ, HR acts upon long-range resected ends and uses more than 100 bp of homology. HR is initiated by loading of Rad51 onto the ssDNA (Wyman and Kanaar, 2006; Wright et al., 2018). Rad51 forms helical filaments on ssDNA that acts as a scaffold for itself and for other interacting proteins. Rad51 filament formation is hindered by RPA which is bound to the ssDNA. BRCA2 mediates the loading of Rad51 by displacing RPA and acting as nucleation platform for Rad51 (Wright et al., 2018). BRCA2 sequestration of Rad51 is suggested to prevent inappropriate Rad51-DNA interactions (Reuter et al., 2014). The Rad51 filament is not a static structure but changes filamental pitch based on ATP hydrolysis. These local changes in filament pitch promote nucleoprotein filament movement (Wright et al., 2018). The formation of the nucleoprotein filament, also called the presynaptic complex (PSC), potentiates recognition of a homologous donor. Homology recognition and strand invasion are mediated by binding of Rad54 to Rad51 (Crickard et al., 2020). This interaction is dependent on bromodomain containing protein, BRD9 (Zhou et al., 2020). Upon induction of DNA damage, Rad54 is acetylated on Lysine 515 (K515-Ac). BRD9 binds K515-Ac on Rad54 and facilitates Rad54's interaction with Rad51, which is essential for HR (Zhou et al., 2020). Recent single-molecule studies have shown that Rad54 changes the homology search from a diffusion based search to an ATP dependent motor-driven mechanism. The current hypothesis is that Rad54 reinforces the binding of the PSC to a dsDNA donor, after which it can be scanned thoroughly for homology. Upon binding of the PSC to a donor strand, the dsDNA is transiently separated to allow Rad51 to probe for homologous sequences. This transient melting of the DNA is most likely mediated by RPA and the Rad54 motor activity, influencing the DNA topology. It was also shown that both donor DNA strands can be sampled for homology in the presence of RPA, revealing a new role for RPA in homology search (Crickard et al., 2020).

Once a complementary sequence has been located, the synaptic complex is formed. The 3′ end of the invading strand can intertwine with the donor DNA, forming heteroduplex DNA (hDNA) suitable for DNA synthesis. The formed structure is called a D-loop. Rad54 motor activity again plays an important role in this process, likely by altering the topological state of the DNA (Wright et al., 2018). This hDNA structure is bound by DNA polymerase, mainly polymerase δ, after which DNA synthesis can commence. The D-loop can be processed by two pathways, synthesis-dependent strand annealing (SDSA) or by formation of a double Holliday junction (dHJ). In somatic cells, a D-loop is more likely to be processed by SDSA then by dHJ formation. This probably has evolved because dHJs can result in crossover products, which can lead to loss of heterozygosity of critical genes such as tumor suppressors (Wyman and Kanaar, 2006; Wright et al., 2018).

In SDSA, DNA synthesis extends the invading strand such that it has sufficient overlap with the other DSB end once the D-loop is disrupted. After disruption of the D-loop by

TABLE 1 | Overview of studies investigating DNA repair after particle irradiation.

Process	Study model	Radiaton type	LET (keV/μm)	Energy	Read out	References
End resection	U2OS (siRNA Ctip, Mre11, and Exo1)	X-ray	2	250 keV	Clonogenic survival	Averbeck et al., 2014
	AG1522D	12C ions	90	100 MeV/nucleon	RPA foci	
	MEFs (H2AX−/−, Ku80−/−)		170	11.4 MeV/nucleon	γH2A.X foci	
	NFFhTERT (siRNA Ctip)	40Ca ions	200	186 MeV/nucleon		
		59Ni ions	350	265 MeV/nucleon		
		14N ions	400	11.4 MeV/nucleon		
		48Ti ions	2180	11.4 MeV/nucleon		
		59Ni ions	3430	11.4 MeV/nucleon		
		119Sn ions	7880	11.4 MeV/nucleon		
		197Au ions	13000	11.4 MeV/nucleon		
		207Pb ions	13500	11.4 MeV/nucleon		
		238U ions	15000	11.4 MeV/nucleon		
End resection	U2OS	X-rays			RPA tracks/foci	Yajima et al., 2013
	HeLa	γ-rays			γH2A.X tracks/foci	
	U251	12C ions	70	290 MeV/nucleon	Western blot (p-RPA, p-ATM,	
	MEFs	Fe ions	200	500 MeV/nucleon	p-Chk1, γH2A.X)	
	1BR-hTERT					
	48BR					
End resection	U2OS	X-ray		195 kV	53BP1 foci	Roobol et al., 2020
		Alpha particles	115 ± 10	5.486 MeV	RPA foci	
	MEF LIG4−/−	X-ray			Clonogenic survival	
	MEF RAD54−/−	Proton	Entrance plateau		γH2A.X foci	
	MEF LIG4−/− RAD54−/−		protons			
NHEJ and HR	BxPC3 (BRCA2-proficient)		Center SOBP	100-109.9 MeV		Szymonowicz et al., 2020
	Capan-1 (BRCA2-deficient)					
	M059J (DNA-PKcs −/−)					
	M059K (DNA-PKcs+/+)					
NHEJ and HR	M059J (DNA-PKcs −/−)	X-ray		6 MeV	Clonogenic survival	Bright et al., 2019
	M059K (DNA-PKcs+/+)	Proton	1.1, 2.5, and 7.3	100 MeV	γH2A.X foci	
	HCC1937 (BRCA1 −/−)					
	HCC1937-BRCA1 (BRCA1 complemented)					
	HT1080 (wt, shRad51, shDNA-PKcs)					
NHEJ and HR	A549	X-ray		200 keV	Clonogenic survival	Fontana et al., 2015
	A549-DNA-Pkcs inhibitor NU7026	Proton	Center SOBP	138 MeV	γH2A.X foci	
	A549-siRNA DNA-PKcs					
	A549-siRNA Rad51					
	M059J (DNA-PKcs −/−)					
	M059K (DNA-PKcs+/+)					
NHEJ and HR	AA8 (WT and siRad51)	X-ray		200 keV	Clonogenic survival	Grosse et al., 2014
	Irs1sf (HR deficient XRCC3 −/−)	Proton	Center SOBP	138 MeV	γH2A.X foci	
	CH09 (WT)				Chromosomal abberations	
	UV5 (ERCC5 −/−)					
	XR-C1 (DNA-PKcs−/−)					
NHEJ and HR	H1299	X-ray		150/200 keV	Clonogenic survival	Ma et al., 2015
	H1299 + DNA PK inhibitor NU7026	12C ions	50, Center SOBP	290 MeV/nucleon	γH2A.X flow cytometry	
	H1299 + Rad51 inhibitor B02					
NHEJ and HR	3D PDAC tumors + inhibitors (B02, NU7026)	X-ray		200 keV	3D tumoroid formation	Görte et al., 2020
	BxPC3	Proton	3.7, Center SOBP	150 MeV		
	MiaPaCa2					
	Panc-1					
	Patu8902					
	Capan-1					
	COLO357					
NHEJ and HR	AA8 (WT)	γ-rays (137 Cs)		662 keV	Clonogenic survival	Carter et al., 2018
	V79 (WT)	Proton	2.2, Center SOBP	200 MeV	γH2A.X foci	
	Irs1sf (HR deficient XRCC3 −/−)	12C ions	50, Center SOBP	290 MeV/nucleon	Chromosomal abberations	
	Irs1 (HR deficient XRCC2 −/−)					
	XR1 (XRCC4 −/−)					
	V3 (DNA-PKcs −/−)					

This table provides an overview of the radiation parameters and biological models used in the cited studies: the specific DNA repair process studied, cell model used (including information about protein knock-out or knockdown), radiation type, LET, energy of the particles, and the read-out that was used to study the indicated process.

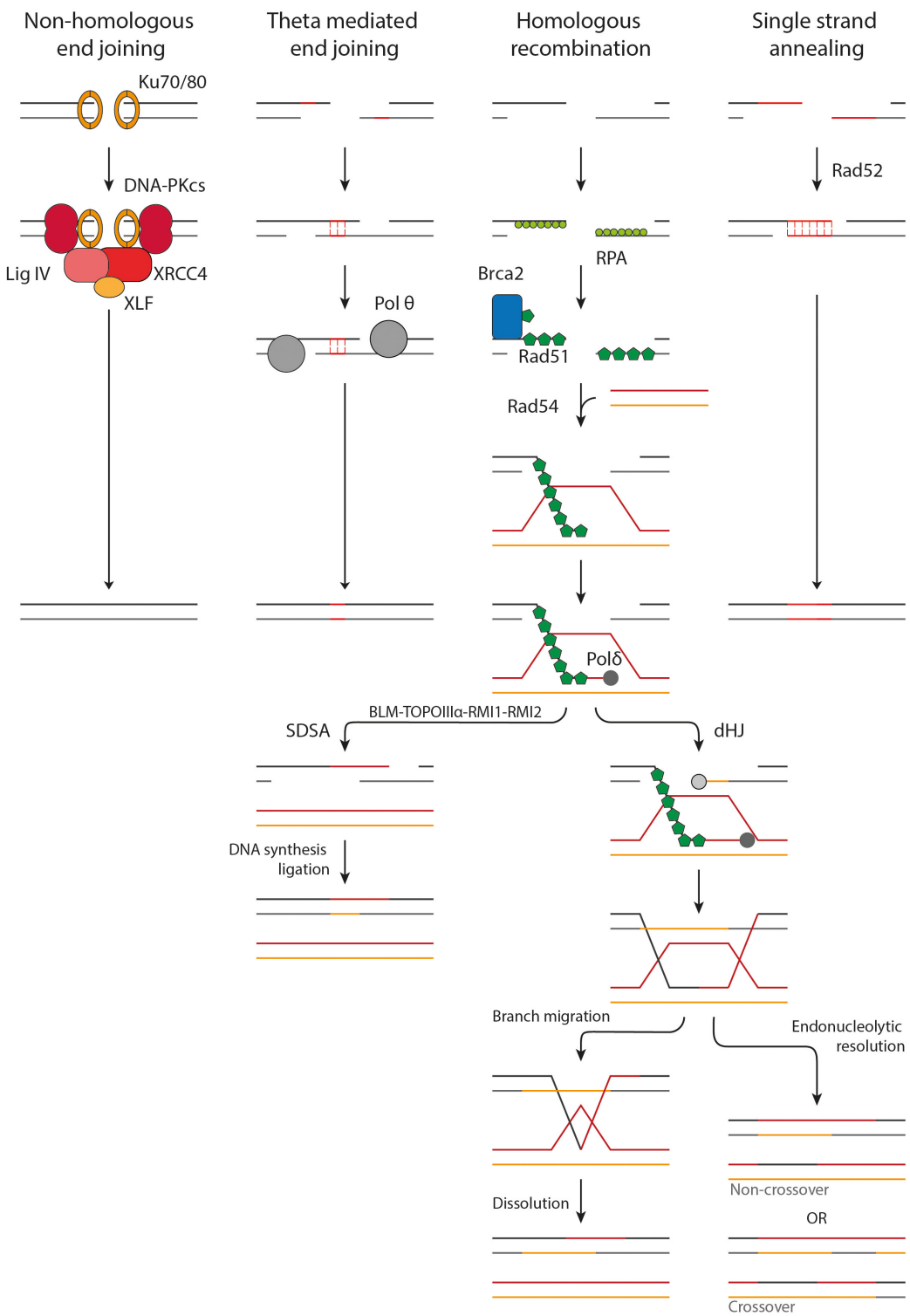

FIGURE 2 | Overview of the DSB repair pathways. NHEJ acts upon protected DSB ends. Ku70/80 binds to the DSB ends followed by the accumulation of DNA-PKcs, XRCC4, XLF, and LigIV. The DSB ends are processed and ligated. TMEJ acts on short-range resected ends. Microhomology of 2–20 bp is required to transiently align the DSB ends, after which the gaps are filled in by Pol θ. HR requires long-range resection and makes use of a homologous donor sequence for repair. After resection Rad51-loading onto the ssDNA is mediated by BRCA2. Homology search is facilitated by Rad54. Once a homologous donor strand has been found a D-loop is formed and DNA synthesis is started. In SDSA the D-loop is disrupted, followed by DNA synthesis and ligation to repair the DSB. A D-loop can also progress into the formation of a dHJ, which can be resolved by branch migration and dissolution or endonucleolytic resolution. Endonucleolytic resolution can result in a non-crossover or crossover product.

the BLM-TOPOIIIα-RMI1-RMI2 complex, the ssDNA ends can anneal to each other and DNA synthesis can commence from the other DSB end by a still unidentified polymerase. After a final ligation of the remaining nicks, the DSB is repaired and the DNA is restored. In dHJ formation the remaining 3′ ssDNA end not associated with the homologous donor also invades the D-loop. DNA synthesis occurs on both donor strands followed by ligation of the nicks, resulting in the formation of a dHJ. These HJs can be processed by either dissolution or endonucleolytic resolution. In dissolution, the HJs are brought together via branch migration (Wright et al., 2018). Recently, it has been shown that branch migration is mediated by the N-terminal domain of Rad54 (Goyal et al., 2018). When the two HJs meet they can be resolved, leading strictly to non-crossover. With endonucleolytic resolution the phosphodiester backbone is cleaved across the HJ. If the cleavage is in the same plane it results in non-crossover but if the HJs are cleaved in different planes a crossover product is formed (Wyman and Kanaar, 2006; Wright et al., 2018). HR is usually error-free because it makes use of a sister chromatid as a template for repair. This dependence on a template limits its activity to late S and G2 phase (Wyman and Kanaar, 2006; Wright et al., 2018).

A third pathway that acts on resected DNA ends is TMEJ. This pathway is considered as an alternative end joining pathway, since it only makes use of microhomologies of 2-20 bp. Because of this, TMEJ is often referred to as alternative end joining (alt-EJ) or microhomology-mediated end joining (MMEJ). Here, we will use the term TMEJ to distinguish between the theta-mediated end joining pathway and alternative end joining in general, which may encompass other end joining activity. If a homologous sequence is present, TMEJ can act upon ssDNA revealed by short range end-resection (∼20 bp) (Zhao B. et al., 2020; Xue and Greene, 2021). Whether TMEJ can also act upon long-range resected ends remains to be proven (Truong et al., 2013). During TMEJ the DSB ends are transiently aligned using the revealed microhomologies. An important protein in this process is polymerase θ (pol θ). Pol θ is an A-family DNA polymerase with helicase activity. It can displace RPA from ssDNA and it plays an important role in searching and aligning the microhomologies (Mateos-Gomez et al., 2017; Sallmyr and Tomkinson, 2018). The non-homologous 3′ tails that remain after annealing of the microhomologies are removed, presumably by the structure specific nuclease complex XPF/ERCC1. Subsequently, the remaining gaps can be filled in by pol θ (Seol et al., 2018). Finally, ligation is performed by DNA ligase IIIα-XRCC1 (Sallmyr and Tomkinson, 2018). TMEJ is intrinsically mutagenic due to the deletion of a copy of the microhomology the sequence in between the microhomology sites.

The fourth form of DSB repair is SSA, which is also considered an alternative repair pathway and is less frequently activated. SSA acts upon long-range resected DNA ends (>1000 bp), utilizing homologies of more than 50 bp to anneal homologous sequences (Zhao B. et al., 2020; Xue and Greene, 2021). The key player in SSA is Rad52 which interacts with the RPA coated ssDNA and aligns the complementary regions. The 3′ tails are cleaved off by XPF/ERCC1, after which the gaps can be filled in by an unidentified polymerase. The final ligation is performed by DNA ligase I (Sallmyr and Tomkinson, 2018; Zhao B. et al., 2020). SSA is intrinsically mutagenic and is associated with larger deletions due to the deletion of one of the copies of the annealed repeat and the large sequence in between the complementary sites (Zhao B. et al., 2020).

The used DSB repair pathway to repair radiation-induced DSBs is not a choice as such, but rather it is dictated by the amount of resection, as well as the available resources, such as microhomologies, sister chromatids and repair proteins. Although the DSB repair pathways are often described conceptually as isolated pathways, flexible, and reversible interactions between the various DSB repair factors occur, eventually leading to repair of the DSB. As described in the previous sections, high LET particle-induced damage is thought to have a differential configuration than X-ray induced damage, usually termed CDD. By which pathway this different type of DNA damage is repaired, is still subject of current investigation. However, some studies studying DNA repair in cells depleted of key DNA repair proteins by CRISPR/Cas9 or siRNAs have been performed. Generally, NHEJ deficiency sensitizes to all types of radiation (Grosse et al., 2014; Fontana et al., 2015; Gerelchuluun et al., 2015; Ma et al., 2015; Bright et al., 2019; Görte et al., 2020), although the effect is less pronounced with high LET radiation (Ma et al., 2015; Bright et al., 2019). Various studies show that DSBs induced by low LET protons are mostly repaired by HR while DSBs induced by X-ray are predominately repaired by NHEJ. However, others do not observe this radiosensitization by HR knockdown for low LET protons (Gerelchuluun et al., 2015; Görte et al., 2020) or it is only observed for high LET protons (Bright et al., 2019) or carbon ion radiation (Gerelchuluun et al., 2015; Ma et al., 2015) (see **Table 1** for details on the experimental setup). Unfortunately, these studies are difficult to compare due to differences in radiation energy, type, dose and the used biological model.

There are several possible explanations for why particle-induced DNA damage is more likely to be repaired by HR rather than NHEJ. High LET proton irradiation induces CDD consisting of DSBs, SSBs and abasic sites, while this is not the case for X-ray or low LET proton irradiation (Carter et al., 2018). Long ssDNA tails or ssDNA gaps near the DSB end can block Ku70/80 binding, hereby channeling the DSB into resection (Zhao L. et al., 2020). There might also be steric hindrance at the site of the CDD with multiple repair proteins competing to repair the different types of lesions. Resection is not hampered by the presence of abasic sites or SSBs, hereby making resection more favorable. Another possibility is that with higher LET more lesions are created, leading to exhaustion of the DNA repair protein pool, preventing end-protection (Roobol et al., 2020). 53BP1 can protect DSB ends from resection up to 20–40 simultaneous DSBs. If the DSB load exceeds this maximum capacity, the 53PB1 pool is exhausted, leading to resection and RPA loading (Ochs et al., 2016; Roobol et al., 2020). Interestingly, 53BP1 exhaustion, i.e., all available 53BP1 in the nucleus is chromatin bound, does not lead to upregulation of HR. At high doses, Rad51 focus formation is decreased and recombination efficiency is reduced (Ochs et al., 2016; Mladenov et al., 2020). This effect is not induced due to

exhaustion of the Rad51 pool since only 20% of available Rad51 is chromatin bound at maximum level of foci observed (Mladenov et al., 2020). Instead, high doses of IR induce hyperresection of breaks, which promotes SSA (Ochs et al., 2016; Mladenov et al., 2020). It is hypothesized that 53BP1 does not prevent resection entirely but instead fosters HR rather than the mutagenic SSA pathway (Ochs et al., 2016). Interestingly, evidence also exist that suggests that Rad51 focus formation and, by inference, HR is upregulated after knock-out of 53BP1 (Mladenov et al., 2020). Additional research studying repair pathway choice and the underlying mechanisms after induction of DSBs by particle-radiation is needed to fully unravel the differential contribution of the different repair pathways. This knowledge will provide rationales for combining particle radiation therapies with DDR targeting therapies.

COMBINATION THERAPIES

Particle radiotherapy is a promising treatment modality for the treatment of cancer, especially due to their superior spatial dose distribution in comparison to conventional X-ray therapy. However, the efficacy of particle radiotherapy could be further increased by combining it with inhibitors of DDR pathways. A potential strategy would be to exploit the difference in induced damage by the low LET entrance dose in healthy tissue, and the higher LET Bragg peak in the tumor. Another promising strategy is the induction of synthetic lethality, whereby a genetic defect in a DDR pathway is exploited using pharmacological inhibitors of compensatory DDR pathways. This can lead to cell death and genomic instability in the tumor, while the healthy tissue is spared, since it does not carry this DDR mutations (Pilié et al., 2019; Reuvers et al., 2020). This has sparked a great interest in the development of small molecule inhibitors of components of DDR pathways (**Figure 3**).

Poly (Adp-Ribose) Polymerase Inhibition

The poly (ADP-ribose) polymerase (PARP) family comprises a group of DDR proteins that mainly function to detect SSBs and DSBs, localize DNA repair proteins and stabilize replication forks during DNA repair (Pilié et al., 2019). Synthetic lethality can be achieved with PARP inhibitors (**Figure 3A**). Upon inhibition of PARP1, repair of SSBs is attenuated, and unrepaired breaks can be converted to one-ended DSBs during replication. This leads to replication fork collapse which requires HR for repair (Bryant et al., 2005; Wyman and Kanaar, 2006; Nonnekens et al., 2016). Tumors which are HR deficient (HRD), such as with BRCA1/2 mutations, cannot efficiently repair this damage, leading to an anti-tumor effect (Farmer et al., 2005; Pilié et al., 2019). Multiple PARP inhibitors, such as Olaparib, have been developed, some of which have reached clinical trials and FDA approval for treatment of cancers with germline BRCA1/2 mutations (Pilié et al., 2019). PARP inhibitors can also be used in combination with radiotherapy. Next to inducing DSBs, radiation also induces other types of DNA damage such as SSBs. By PARP1 inhibition these SSBs are converted to DSBs, increasing the DSB burden after irradiation (Nonnekens et al., 2016). Hereby, the efficacy of low LET radiation is increased. High-LET radiation induces CDD of which a large part is SSBs (Carter et al., 2018). PARP inhibition could effectively transform high-LET induced CDD with SSBs and DSBs to a very complex DSB cluster, which in turn could increase the effectiveness of particle therapy. It was recently shown that Olaparib decreases survival of HeLa cells after relatively high LET proton irradiation, which is due to a deficiency in CDD repair. PARP inhibition had no impact on cell survival after low LET proton irradiation (Carter et al., 2019).

Non-Homologous End Joining Inhibition

Non-homologous end joining reduces the efficacy of cancer treatment modalities such as radiation therapy, which rely on introducing DSBs. Inhibition of NHEJ greatly sensitizes tumor cells to radiotherapy. The radiosensitization effect is seen with multiple radiation modalities such as X-ray, proton and carbon-ion irradiation, across various cell lines, and in 3D tumor models (Grosse et al., 2014; Fontana et al., 2015; Gerelchuluun et al., 2015; Ma et al., 2015; Bright et al., 2019; Görte et al., 2020). The strongest radiosensitization is observed for low LET radiation (see **Table 1** for details on the experimental setup) (Fontana et al., 2015; Gerelchuluun et al., 2015; Ma et al., 2015; Bright et al., 2019).

Small molecule inhibitors have been developed that target the first step in the NHEJ pathway, namely binding of Ku70/80 dimer to DNA (**Figure 3B**; Weterings et al., 2016; Gavande et al., 2020). The most promising inhibitors target a ligand binding pocket in close proximity to the DNA-binding region, interfacing with both the Ku70 and Ku80 subunit. By blocking the DNA binding capacity of the Ku heterodimer, the downstream catalytic activity of DNA-PKcs is inhibited, hereby preventing DSB repair (Weterings et al., 2016; Gavande et al., 2020). The inhibitors induce increased sensitivity to DSB inducing agents, such as IR, in *in vitro* experiments. Further validation of the specificity and potency in *in vivo* experiments is necessary before the Ku inhibitors can progress into clinical studies.

The next protein in the NHEJ pathway, DNA-PKcs, has had great research interest as a target for inhibition of NHEJ. However, it has been challenging to develop a DNA-PKcs inhibitor that selectively inhibits DNA-PKcs without affecting the structurally related PI3 lipid and PI3K protein kinase (Goldberg et al., 2020). Recently, a new DNA-PK inhibitor, AZD8748, was identified, which shows only weak activity against PI3K lipid kinases and no significant off-target effects. Furthermore, AZD7648 is a potent DNA-PKcs inhibitor and an efficient sensitizer to radiation- and doxorubicin-induced DNA damage in models of non-small-cell lung cancer (NSCLC) cells and xenografts, as well as patient derived xenograft models. These promising results have led to the progression of AZD7648 to clinical studies (NCT03907969) (Fok et al., 2019; Goldberg et al., 2020).

Artemis is an important nuclease in NHEJ, responsible for processing the DSB ends. Without it, processing of

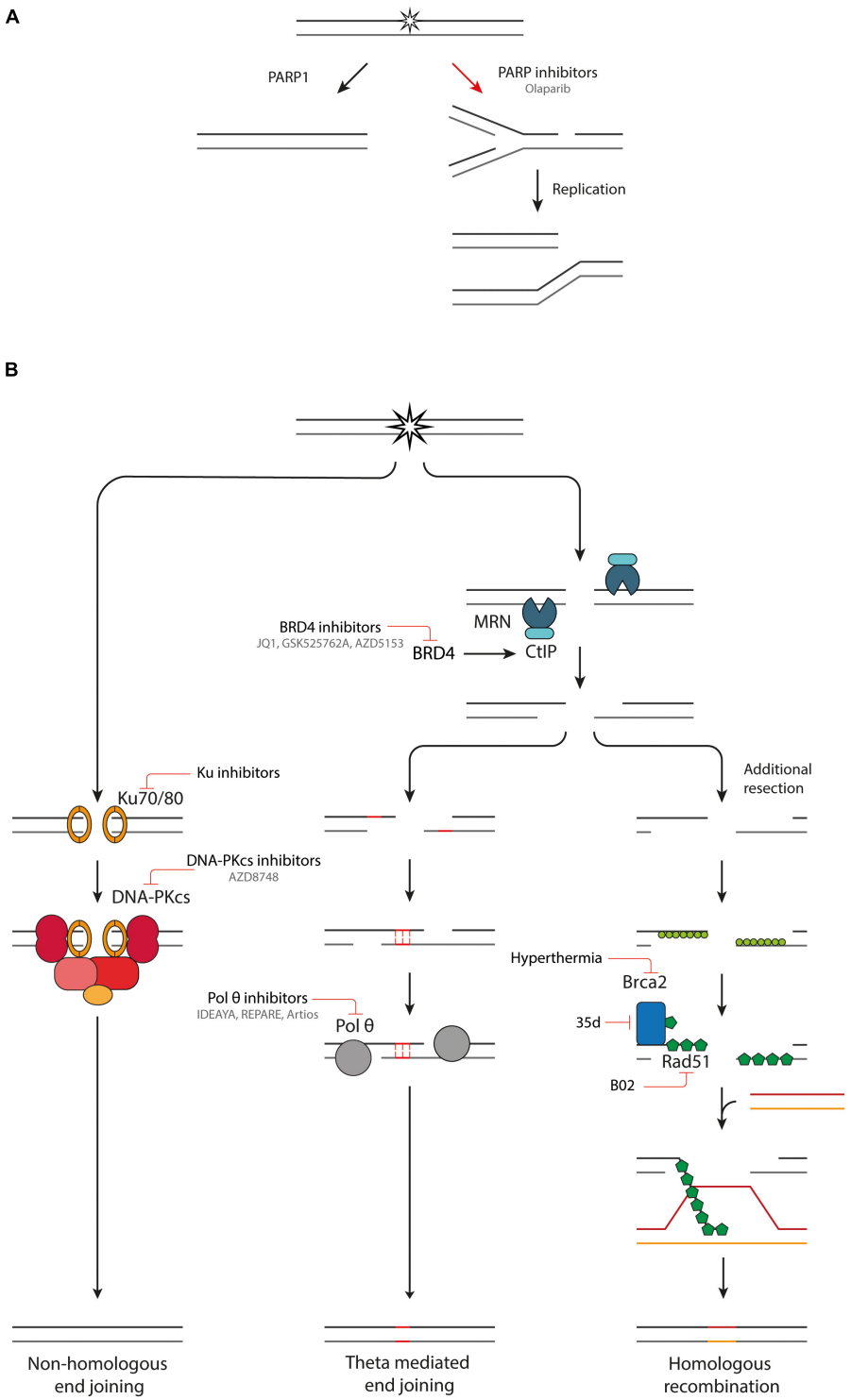

FIGURE 3 | Combination therapies targeting DDR pathways. **(A)** Inhibition of PARP1 leads to the conversion of SSBs into DSBs upon DNA replication. **(B)** An overview of NHEJ, TMEJ and HR and inhibitors targeting various components of these pathways.

'dirty' DSBs, which are commonly produced by radiation, is hampered (Kanaar et al., 2008; Zhao B. et al., 2020). Artemis inhibition could influence break structure and thus affect which downstream enzymes can further act on the break. Structure-based research into small molecule inhibitors have been hampered by a lack of crystal structure.

However, the recent publication of the crystal structure of the catalytic domain of Artemis opens up new opportunities for structure-based design of selective Artemis inhibitors (Karim et al., 2020).

Polymerase θ Inhibition

Inhibition of pol θ is another promising avenue for induction of synthetic lethality. Cells with defective HR machinery are reliant on TMEJ for DSB repair (Feng et al., 2019; Kamp et al., 2020; Schrempf et al., 2021). Using a CRISPR genetic screen, Pol θ inhibition is synthetically lethal with many proteins important in replication associated DSB repair (Feng et al., 2019). Currently there are two described mechanisms for the synthetic lethality between pol θ and HR inhibition. First, TMEJ is important for the repair of one-ended DSBs from collapsed replication forks, which would normally be repaired by HR. In the absence of effective HR machinery, cells become reliant on TMEJ to repair these lesions. This idea is supported by the fact that TMEJ inhibition synergizes with PARP inhibitors (Schrempf et al., 2021). The second mechanism is the anti-recombinase activity of pol θ. Pol θ contains Rad51 binding motifs and antagonizes Rad51-mediated recombinase. When the HR machinery is defective, pol θ is necessary to remove the Rad51 and to allow repair by other means. There is evidence that without pol θ there is accumulation of toxic Rad51 complexes preventing further repair (Ceccaldi et al., 2015; Cleary et al., 2020; Schrempf et al., 2021). TMEJ is especially interesting as target since it is unlikely to have a large effect on the survival of healthy tissues proficient in NHEJ and HR. However, a sensitization will be observed in cancer cells with defective DDR machinery, which are more reliant on TMEJ for repair of DSBs (Sallmyr and Tomkinson, 2018). Pol θ inhibitors are being developed by three independent biotech companies: IDEAYA, REPARE therapeutics and Artios Pharma (**Figure 3B**). The first clinical trials with pol θ inhibitors are expected to already start in 2021 (Schrempf et al., 2021).

Homologous Recombination Inhibition

As mentioned above, PARP inhibitor treatment can greatly increase tumor response in HRD tumors. The last two decades, work has been focused at expanding the utility of PARP inhibitor treatment, by looking into the possibility of inducing a HRD state in HR proficient tumors, to induce synthetic lethality. This has led to the development of small molecule inhibitors for various HR proteins (**Figure 3B**; Carvalho and Kanaar, 2014). Next to their utility in combination with PARP inhibition, HR small molecule inhibitors can also sensitize tumor cells to radiation. The radiosensitization of HR inhibition has been studied in the context of low and high LET irradiation (see **Table 1** for details on the experimental setup) (Grosse et al., 2014; Fontana et al., 2015; Gerelchuluun et al., 2015; Ma et al., 2015; Bright et al., 2019; Görte et al., 2020). So far, the results have been contradictory on whether HR inhibition sensitizes cells to proton irradiation. HR inhibition-induced radiosensitization has been observed with low LET protons (Grosse et al., 2014; Fontana et al., 2015). However, others only observe the radiosensitization effect for high LET protons (Bright et al., 2019) or only with carbon ions with an even higher LET (Gerelchuluun et al., 2015; Ma et al., 2015). A common trend in these studies is that HR inhibition radiosensitization increases with LET. This could make HR inhibitor treatment especially promising in combination with particle therapy. When administering a DDR inhibitor systemically, both healthy, and tumor tissue will be affected. Combined with radiotherapy this could lead to severe side effects. Although there are some contradicting results, evidence shows that HR inhibition radiosensitizes cells predominantly to high LET radiation, only present in the Bragg peak targeted at the tumor, and not to low LET radiation which hits the surrounding healthy tissue. Hereby toxicity to healthy tissue could be reduced, increasing the therapeutic window.

Several pharmaceutical inhibitors of HR are currently under development. A promising target for inducing HRD is bromodomain containing 4 (BRD4) inhibition. BRD4 is a member of the bromodomain and extraterminal (BET) protein family and facilitates oncogene transcription. Multiple small molecule inhibitors can selectively target BRD4 such as JQ1, GSK525762A and AZD5153. There are multiple ongoing clinical trials with BRD4 inhibitors or more general BET inhibitors (NCT01587703 and NCT03059147) (Sun et al., 2018). The mechanism by which BRD4 inhibition treatment induces HRD has been resolved recently. Next to variably affecting expression of many DDR proteins, a consistent downregulation of CtIP is observed with four different BRD4 inhibitors. CtIP interacts with the MRN complex, promoting end resection of DSB breaks and inducing nuclease activity of the MRN complex. Downregulation of CtIP impairs HR. Furthermore, BRD4i works synergistically with PARP inhibition, hereby inducing synthetic lethality (Sun et al., 2018).

Small molecule inhibitors that directly interfere with Rad51 have also been developed. A particularly interesting target is the disruption of Rad51-BRCA2 binding. BRCA2 is an important mediator in Rad51 loading onto ssDNA (Wright et al., 2018). Without this interaction Rad51 loading is greatly reduced. A new series dihydroquinolone pyrazoline derivatives have been designed that target the LDFE binding pocket of Rad51 (Bagnolini et al., 2020). The compound 35d inhibits the protein-protein interaction between Rad51 and BRCA2, by binding to Rad51 and is capable of reducing HR efficiency. Furthermore, in combination with PARP inhibition it induces synthetic lethality in pancreatic cancer cells. Unfortunately, its low solubility currently prevents it from further studies in *in vivo* models.

B02 is a small molecule inhibitor that interferes with the DNA binding capacity of Rad51, hereby inhibiting DNA strand exchange and branch migration (Huang et al., 2012). B02 sensitizes breast cancer cells to various types of chemotherapy *in vitro* and in a xenograft model (Huang and Mazin, 2014). In combination with radiotherapy B02 shows a radiosensitizing effect to photon and proton irradiation in NSCLC and pancreatic cancer cells. This effect was even further increased in combination with PARP inhibition treatment (Wéra et al., 2019).

Apart from chemical inhibition of repair proteins, physical procedures can also influence pathway effectivity such as temperature and oxygenation status (Krawczyk et al., 2011; Luoto et al., 2013).

Chronic tumor hypoxia downregulates expression of key HR proteins (Chan et al., 2009). Rad51 is downregulated by hypoxia in multiple tumors by transcriptional repression (Bindra et al., 2004). BRCA1 expression is also downregulated in hypoxic cells which could alter the resection of DSBs and shunt them into NHEJ (Bindra et al., 2005). Hypoxia-mediated downregulation of RNA expression is not only observed for HR genes (e.g., Rad51, Rad52, Rad54, BRCA1, and BRCA2), but some NHEJ genes are also affected (e.g., Ku70, DNA-PKcs, DNA Ligase IV, and XRCC4). However, this downregulation of NHEJ-related RNA expression does not appear to result in an altered protein level (Meng et al., 2005). This hypoxia-mediated downregulation of HR is also reflected in a decreased recombination efficiency and increased sensitivity to DNA cross-linking agents (Chan et al., 2008). The above mentioned studies were performed under moderate (0.1–0.5%) to severe (<0.1%) tumor hypoxia, however, under mild hypoxia (0.5–2.5%) these effects might be less pronounced or absent. Furthermore, the duration of hypoxia influences the effects as well since these effects are only observed under chronic hypoxia (>48 h) and not under acute hypoxia.

Tumor hypoxia hampers effective radiotherapy by an increased radioresistance of hypoxic cells, due to a decreased level of free oxygen radicals during irradiation (Bindra et al., 2004). The use of high LET particle radiation is promising for the eradication of hypoxic cells. The oxygen enhancement ratio (OER), defined as the ratio of doses given under hypoxic and normoxic conditions to produce the same biological effect, decreases with increasing LET. However, the benefit of using carbon-ions instead of protons was shown to be relatively moderate (1–15%) at clinically relevant oxygen levels (Wenzl and Wilkens, 2011). However, by exploiting the HRD in the hypoxic cell population, novel therapies could be used to selectively target these cells.

Hyperthermia is considered to be one of the most potent radiosensitizers. During hyperthermia treatment, the tumor region is heated locally to temperatures in the range of 40–44°C, using specialized equipment (Horsman and Overgaard, 2007; Van Den Tempel et al., 2017). Hyperthermic radiosensitization can be attributed to many macroscopic and microscopic biological effects in the tumor such as improved tumor oxygenation and DDR modulation (Van Den Tempel et al., 2017; Elming et al., 2019). One of the more recently described effects is hyperthermia-induced HRD (Krawczyk et al., 2011; Van Den Tempel et al., 2017). Upon subjecting cells to hyperthermia, BRCA2 is degraded, hereby inhibiting Rad51 loading onto resected 3′ends and preventing HR. It has been established that optimal HR inhibition is reached by subjecting cells to hyperthermia at 41-43°C for 30 to 60 min (Van Den Tempel et al., 2017), and that BRCA2 degradation is mediated by the proteasome (Krawczyk et al., 2011; Van Den Tempel et al., 2019). In both cultured cells and fresh patient material, Rad51 focus formation is abolished after hyperthermia application (Krawczyk et al., 2011). Because of the reduced HR, tumor cell are dependent on other, more error-prone, and DSB repair pathways. This results in a higher number of translocations after irradiation (Bergs et al., 2013). Furthermore, a synergistic effect is reached by combining PARP inhibitor treatment with hyperthermia (Krawczyk et al., 2011). With the help of hyperthermia, a HRD status can be induced in innately HR proficient tumor cells, hereby inducing synthetic lethality. The main advantage of hyperthermia over small molecule inhibitors is the targeting possibility of hyperthermia. By locally applying hyperthermia, HRD is only induced in the tumor region, hereby preventing systemic effects (Krawczyk et al., 2011; Van Den Tempel et al., 2019).

CONCLUDING REMARKS

There is rising interest in particle radiotherapy for the treatment of tumors. This is mainly based on its superior dose distribution in comparison to photons. However, there is insufficient understanding on how cells and tumors engage the DDR in response to particle irradiation, which is a crucial process in determining the effectiveness of the therapy. DNA damage induced by high LET radiationis currently collectively referred to as CDD which reflects the fact that high LET radiation induces different types of DNA lesions compared to photons and that different particles have different lesion spectra. More research using markers for DSBs and other types of lesions will improve the understanding of this CDD. Furthermore, mechanistic understanding of whether and how this damage induces an altered DDR is still lacking. Studies comparing photon and particle induced DNA repair will shed light on the differential DNA repair mechanisms of particle-induced DNA damage and photon-induced DNA damage. Moreover, these studies will reveal fundamental molecular knowledge about factors that can be involved in differential end resection or protection and subsequent DNA repair pathways. This knowledge is crucial for further improvement of radiotherapy, since it opens up new possibilities for the rational design of combination therapies with DDR inhibitors that could potentially further increase the efficacy and applicability of radiotherapy.

AUTHOR CONTRIBUTIONS

GK, TH, and JE: conceptualization. GK, TH, JE, and RK: writing, reviewing, and editing. All authors contributed to the article and approved the submitted version.

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The Effect of Atypical Nucleic Acids Structures in DNA Double Strand Break Repair: A Tale of R-loops and G-Quadruplexes

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The fine tuning of the DNA double strand break repair pathway choice relies on different regulatory layers that respond to environmental and local cues. Among them, the presence of non-canonical nucleic acids structures seems to create challenges for the repair of nearby DNA double strand breaks. In this review, we focus on the recently published effects of G-quadruplexes and R-loops on DNA end resection and homologous recombination. Finally, we hypothesized a connection between those two atypical DNA structures in inhibiting the DNA end resection step of HR.

Keywords: R-loops, DNA double strand break repair, homologous recombination (HR), DNA end resection, G-quadruplex

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NON-CANONICAL NUCLEIC ACIDS STRUCTURES

In recent years it has been well established that DNA does not always adopt the canonical right-handed B-DNA configuration that is depicted in textbooks (**Figure 1A**). This DNA structure is based in two linear antiparallel DNA strands that twist together around the same axis forming a double helix that contains a major groove and a minor groove. Albeit this is the form that the most DNA acquires in the cell, there are other non-canonical conformations of DNA. Such structures have been shown to exist *in vitro* and *in vivo,* and to be related to many biological processes although their roles remain to be fully characterised. There are many non-canonical DNA structures, and exhaustive recent reviews can be found at (Kaushik et al., 2016; Saini et al., 2013). Among these alternative structures we will focus on G-quadruplexes (G4s, secondary structures arising in repetitive guanine rich areas of either DNA or RNA) (**Figure 1B**) (Bochman et al., 2012; Kaushik et al., 2016) and R-loops (three-stranded structures that harbour a DNA-RNA hybrid) (**Figure 1C**).

G-QUADRUPLEXES

As previously stated, G4s are non-canonical nucleic acid secondary structures formed in guanine-rich areas. The connection of four guanines by Hoogsteen hydrogen bonding generates a square planar arrangement known as G-quartet (**Figure 1B**). These planar G-quartets can stack on top of each other generating four-stranded helical structures. G4 structures can adopt a variety of conformations resulting from different arrangements of strand directions. Thus, G4 structures can be intramolecular (formed within one strand) or intermolecular (formed from multiple strands), parallel or antiparallel (**Figure 1B**) (Spiegel et al., 2020). The human genome contains thousands sequences with the potential to form such structure, known as PQS (Putative Quadruplex Sequence)

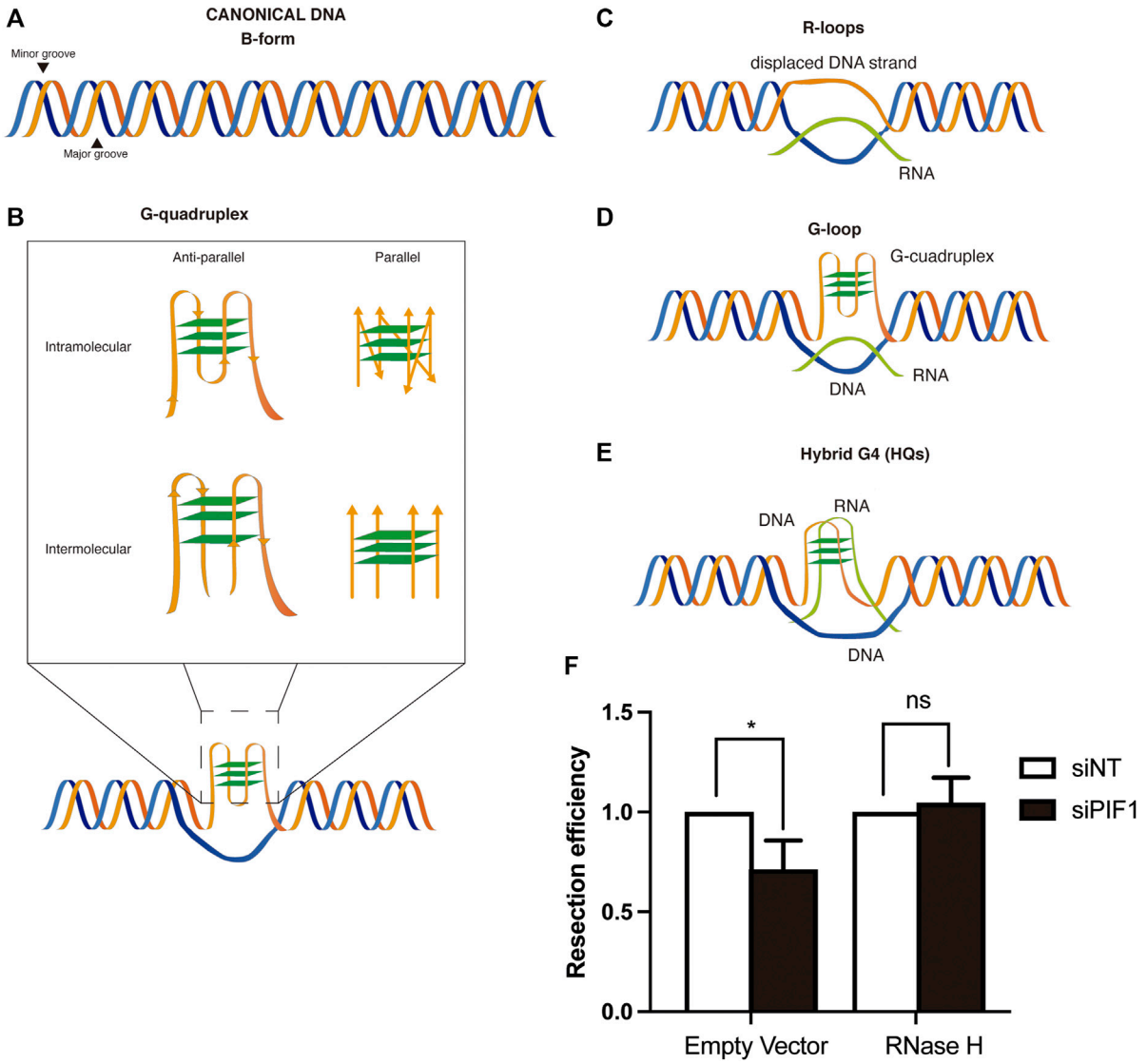

FIGURE 1 | DSB repair and non-canonical DNA structures. **(A)** Canonical B-form duplex DNA structure. **(B)** Different conformations of G-quadruplexes. Inset: G-quadruplex might be intramolecular (generated on one strand of the DNA) or intermolecular (generated by several strands of DNA). In each case, they can be parallel or anti-parallel depending on the orientation of the strands. **(C)** R-loops structures are formed by the base-paired annealing of an RNA molecule with a DNA strand and the consequent displacement its complementary one. **(D)** G-loops structures arise from the formation of a G4 in the displaced ssDNA strand of an R-loop. **(E)** Hybrid G4s are chimeric structures in which the G-quadruplex is formed by the interaction of G at both a ssDNA and RNA molecules of an R-loop, displacing the other strand of the DNA **(F)** RNase H overexpression rescues the resection defect observed after PIF1 depletion. DNA resection proficiency measured as the percentage of RPA-foci-positive U2OS cells in cells expressing FLAG-RNase H or a FLAG empty vector and with either an siRNA against PIF1 (Dharmacon, CAUAUCUGCUAAAGCGAAU) or control siNT. Briefly, cells were seeded and grown for 24 h on coverslips. The day of transfection, medium was replaced by fresh DMEM without antibiotics and cells were incubated with a mix of siRNA and Lipofectamine diluted for 6 h in Opti-MEM before transfection with the plasmids with FuGENE six Transfection Reagent (Promega). 48 h after siRNA transfection, cells were irradiated (10 Gy) and incubated at 37°C for 1 h. Coverslips were then washed once with PBS followed by treatment with pre-extraction buffer (25 mMTris-HCl, pH 7.5, 50 mMNaCl, 1 mM EDTA, 3 mM MgCl2, 300 mM sucrose and 0.2% Triton X-100) for 5 min on ice. Cells were fixed with 4% paraformaldehyde (w/v) in PBS for 15 min. Following two washes with PBS, cells were blocked for 1 h with 5% FBS in PBS, co-stained with anti-RPA (Abcam ab2175) and anti-ϒH2AX (Cell Signaling 2,577) antibodies in blocking solution overnight at 4°C, washed again with PBS and then co-immunostained with the appropriate secondary antibodies (Alexa Fluor 594 goat anti-mouse (Invitrogen A-11032), Alexa Fluor 488 goat anti-rabbit (Invitrogen A-11034) in blocking buffer. After washing with PBS and dried with ethanol 70 and 100% washes, coverslips were mounted into glass slides using Vectashield mounting medium with DAPI (Vector Laboratories). RPA foci immunofluorescences were analyzed using a Leica Fluorescence microscope with a HCX PL APO 63x/1.4 OIL objective. In all cases, at least 200 cells were analysed per condition and the experiments were replicated independently at least three times. Significance was determined by Student's t test comparing each condition to siNT cells. *$p < 0.05$.

(Chambers et al., 2015; Hänsel-Hertsch et al., 2016; Zheng et al., 2020). G4s are formed naturally *in vivo* (Kouzine et al., 2019; Lipps and Rhodes, 2009) but their presence can be stabilized by different drugs, known as G4 ligands (**Table 1**; more ligands can be found at www.g4ldb.com) (Drygin et al., 2009; Xu et al., 2017). Although G4s are widely distributed across the genome,

TABLE 1 | G-quadruplexes ligands and their main characteristics.

G-quadruplex ligand	Clinical trial	Relevant characteristics	References paper
Telomestatin	—	Telomerase inhibition High G4s selectivity over duplex DNA	Kim et al. (2002)
TMPyP4	—	Telomerase inhibition Poor G4s selectivity over duplex DNA	Izbicka et al. (1999)
BRACO-19	—	Reduce telomerase activity High G4s selectivity over duplex DNA	Gowan et al. (2002)
RHSP4	—	Telomerase inhibition High G4 affinity	Gowan et al. (2001)
CX3543/Quarfloxin	Phase I	RNA G4 affinity Binds ribosomal DNA G4	Drygin et al. (2009)
CX5461	Phase I	RNA G4 affinity Binds ribosomal DNA G4	Xu et al. (2017)
MM41	—	High selectivity for G4s in *BCL2* promoter	Ohnmacht et al. (2015)
Pyridostatin	—	High G4 selectivity over duplex DNA	Rodriguez et al. (2008)

More G4s ligands can be found at www.g4ldb.com

their appearance seems to be enriched in promoters, telomeres, 5′ UTRs and splicing sites (Chambers et al., 2015). The presence of these structures in such pivotal areas for gene regulation has implicated them in a variety of biological processes.

In many species, telomeric DNA consists of repetitive short G-rich sequences that fold into G4s. These structures have been implicated in the maintenance of telomeres by protecting their degradation and regulating telomere length (Zahler et al., 1991). Indeed, the use of G4 stabilizing compounds inhibits telomerase activity leading to telomere shortening (Sun et al., 1997), although not every G4 ligand is able to inhibit telomerase since this enzyme seems to be able to elongate through parallel G4s, but not antiparallel ones (Moye et al., 2015; Paudel et al., 2020; Zhang et al., 2010). The enriched presence of PQS in the promoters of transcriptionally active genes seems to highlight G4 implication in regulating gene expression (Fleming et al., 2018; Lee et al., 2020). In addition, the use of G4-ligands, such as pyridostatin (PYR) (**Table 1**), modulates transcription of the *BRCA1* gene in neurons (Moruno-Manchon et al., 2017). Furthermore, the deficiency of the helicase RTEL1, involved in G4 dissolution (see below), leads to an altered transcription of genes possessing potential G4 sequences in their promoters (Kotsantis et al., 2020). Also, the presence of PQS have been confirmed genome-wide at mapped replication origins of higher eukaryotes (Besnard et al., 2012; Langley et al., 2016). Notably, G-quadruplexes are of functional importance for replication origin activity since the deletion of a G-rich element known as Origin G-rich Repeated Element (OGRE) strongly reduced origin activity in mouse cells and the introduction in an ectopic origin-free area stimulated the re-establishment of a new functional origin (Prorok et al., 2019). Conversely, G4 formation also show negative effects in the normal cellular metabolism. For example, the presence of these structures might block DNA polymerases progression and, in consequence, collapse the replication fork (Sarkies et al., 2010). Also, as discussed below, G4s are known to promote genomic instability. Due to these negative effects, several G4 ligands (**Table 1**) are currently in clinical trial for cancer treatment.

DISSOLUTION OF G4S

G4s are dynamic structures, so cells have evolved different mechanisms to resolve these structures. The main proteins associated are helicases (Mendoza et al., 2016) (see **Table 2**). Several helicases have been reported to be involved in G4-resolution. The main ones can be divided in several superfamilies (SF), including PIF1, BLM, WRN, RecQ, FANCJ, DNA2 and the aforementioned RTEL1 (Mendoza et al., 2016). Precisely, RTEL1 is important for the maintenance of telomeres integrity helping dismantling telomeric DNA secondary structures to allow efficient telomere replication (Vannier et al., 2012). Although those are the most studied helicases involved in this process, there has been a growth in the number of helicases with a role in G4 processing. The knock out of the helicase DHX36, for example, increases the stress response due to the stabilization of an RNA G4 (Sauer et al., 2019). Indeed, these helicases usually collaborate with other proteins to unwind G4s. That is the case of DDX1 that has been lately reported to be recruited by Timeless to the replication fork to ensure processive replication nearby G4 structures (Lerner et al., 2020).

R-LOOPS

Another non-canonical secondary structure that can be formed on DNA is the R-loop. R-loops are three-stranded structures in which a DNA-RNA hybrid is formed in a dsDNA context, thus creating a displaced ssDNA region (**Figure 1C**). They arise usually as a consequence of negative supercoiling of the DNA template behind the transcription complex, especially in highly transcribed regions. As well as G-quadruplexes, R-loops are both implicated in physiological DNA transactions such as class switch recombination of immunoglobulins (Yu et al., 2003), telomere maintenance (Tan et al., 2020a), gene regulation (Sun et al., 2013; Grunseich et al., 2018) or in double-strand breaks (DSBs) repair (Liu et al., 2021) but also are associated with negative effects such an increase of DNA damage.

TABLE 2 | Helicases involved in G-quadruplexes resolution and their connection with R-loop biology.

Factor	Role in G4 biology	Mutant phenotype	R-loop related?	References
PIF1	5′-to-3′ DNA helicase, unwinds G4s and regulates telomere maintenance	Increased cancer risk	Yes	Paeschke et al. (2013)
RecQ helicases	5′-to-3′ DNA helicase, participates in DNA replication, repair and telomere maintenance	Bloom's syndrome (BLM), Werner syndrome (WRN), Rothmund-Thomson syndrome (RECQ4)	Yes	Wu et al. (2015)
FANCJ	5′-to-3 DNA helicase, required for the repair of DNA crosslinks	Fanconi Anemia	Not determined	Wu and Spies. (2016)
DNA2	5′-to-3′ DNA helicase with nuclease activity, involved in DNA replication and DNA repair in nucleus and mitochondria	Sensitivity to DNA replication stress, genome instability, mitochondrial myopathy, and Seckel syndrome	Not determined	Masuda-Sasa et al. (2008)
RTEL1	ATP-dependent DNA helicase involved in telomere-length regulation, DNA repair and the maintenance of genomic stability	Dyskeratosis congenita and Hoyerall-Hreidarsson syndrome, telomere-related pulmonary fibrosis and/or bone marrow failure	Yes	Wu et al. (2020)
DHX36	RNA helicase, involved in genomic integrity, gene expression regulations and as a sensor to initiate antiviral responses	Aicardi-Goutieres Syndrome and Fanconi Anemia	Not determined	Sauer et al. (2019)
CHD7	Chromatin remodeling protein with DNA helicase activity	CHARGE syndrome	Not determined	Zhang et al. (2018)
EXO1	5′ → 3′ exonuclease, mediates resection at stalled forks due to G4	Telomere defects, increased fork stalling	Not determined	Stroik et al. (2020)
DDX11	5–3′ Fe–S DNA helicase, involved in DNA replication, DNA repair, heterochromatin organization and ribosomal RNA synthesis	Warsaw breakage syndrome	Not determined	Lerner et al. (2020)
DHX9	3′-to-5′ RNA helicase involved in DNA replication, transcriptional activation, post-transcriptional RNA regulation, mRNA translation and RNA-mediated gene silencing	Werner Syndrome and Abnormal Retinal Correspondence	Yes	Chakraborty and Grosse, (2011)
XPB/XPD	DNA helicase that functions in nucleotide excision repair	Xeroderma pigmentosum B/D, Cockayne's syndrome, and trichothiodystrophy	Not determined	Gray et al. (2014)

The most studied pathway in which "scheduled" R-loops have been implicated is in regulating gene expression. The enriched presence of these structures at promoters or termination regions might facilitate the modulation of this process at different steps. First, R-loops have been described to affect chromatin dynamics principally by preventing methylation of CpG islands (Ginno et al., 2012) which would favour transcription. In addition, they can also act in gene silencing since their presence correlates with higher levels of the chromatin condensation mark phosphorylated histone H3 S10 (H3S10P) (Castellano-Pozo et al., 2013). On a second level, R-loops generated from long non-coding RNA can also recruit or displace transcription regulators to the gene promoter modulating gene expression (Boque-Sastre et al., 2015; Arab et al., 2019). Finally, the presence of R-loops at the 3′end of some genes can modulate transcription termination by stalling RNAPII until resolution by RNA-DNA helicases and the nascent RNA is release and degraded (Skourti-Stathaki et al., 2014).

Although R-loops have usually been conceived as drivers of DNA damage, a new associated role in double-strand breaks (DSBs) repair is emerging for these structures. Indeed, the presence of DNA-RNA hybrids in transcriptionally active regions seems to be important for the recruitment of RAD52 and the later RAD52-dependent activity of BRCA1 in antagonizing RIF1-53BP1 blockade of DNA end resection (Yasuhara et al., 2018). The correct processing of R-loops is a key step in determining if their presence may hamper DNA repair or, on the contrary, favour the process. To regulate this, BRCA2 seems to be the responsible protein by recruiting factors involved in R-loop degradation, i. e RNase H2, and, in consequence, ensuring proper repair of the damage by Homologous Recombination (HR) (D'Alessandro et al., 2018).

The above-described beneficial roles of R-loops in DNA metabolism imply that it is not the structure *per se* but the deregulation of their processing what causes genome instability. Consistent with this idea, the mutation or loss of factors involved in R-loops resolution, such as SETX, RNase H or Fanconi Anemia (FA) factors, leads to persistent R-loops that may result in DNA damage (Wimberly et al., 2013; Herrera-Moyano et al., 2014; Cohen et al., 2018). One of the best-known pathways hampered by non-regulatory R-loops is DNA replication. The "unscheduled" presence of these structures can block replication fork progression which could result in transcription-replication collisions and, eventually, in DNA breakage (Gan et al., 2011). Furthermore, this increased rate of DNA breaks caused by R-loops has been associated with the hyper-recombination phenotype observed in a transcription impaired background that might lead to genomic instability (Huertas and Aguilera, 2003).

G4S AND R-LOOPS ARE CONNECTED

The similarity between both structures resides not only in their function but also in their genome distribution. In a recent genome-wide mapping, an enrichment of G4 rich regions was

reported in potential R-loop forming regions (Kuznetsov et al., 2018) supporting the possibility that G4s and R-loops may act synergistically in their biological function (Lee et al., 2020; Shrestha et al., 2014), in some cases creating a novel structure that combines both and it is known as a G-loop (Duquette et al., 2004) (**Figure 1D**). Indeed, in a recent study, it has been observed that the formation of an R-loop at the 5' UTR of a gene leads to the G4 folding that, in turn, stabilizes the R-loop promoting transcription (Lee et al., 2020). The current view is that the formation of an R-loop facilitates the formation of G4s in the now free ssDNA strand. Then, the formation of the G4 in turn stabilizes the R-loop structure in a positive feedback loop (Lim and Hohng, 2020). Along this lines, it has been shown that stabilization of G4s using ligands increases R-loop formation and promotes R-loop-mediated replication stress (Chappidi et al., 2020; De Magis et al., 2019; Kotsantis et al., 2020). These G-loops have been shown to have different outcomes in transcription levels depending on where is the G4 located, having a positive impact in mRNA production if it is in the non-template strand and a negative one when it is located in the template strand (Lee et al., 2020). Additionally, the possibility of the existence of hybrid G4s (HQs) formed between the nascent RNA and the coding DNA strand (that was first described in the mitochondrial R-loop (**Figure 1E**; Wanrooij et al., 2012)) might also be taken into account. Indeed, the co-transcriptional formation of those structures has been reported to occur and to have a role in transcription acting as a cis element of control (Zheng et al., 2013). However, and despite the inference that G-loops and HQ structures may exist *in vivo*, it has been proven technically challenging to demonstrate that they do as opposed to a simply cohabitation of G4s and R-loops in close proximity.

G4S, R-LOOPS AND GENOMIC INTEGRITY

The Repair of DNA Double Strand Breaks

DNA is constantly confronted by different DNA damaging sources that endanger its integrity. The most cytotoxic type of lesion are the DNA double-strand breaks (DSBs). This is because when both strands get simultaneously broken, there is not an intact template from which the DNA sequence can be restored (Bennett et al., 1993; Kawashima et al., 2017). In order to preserve genomic stability, cells have developed a well-regulated signalling cascade, known as the DNA Damage Response (DDR), to detect and repair these DNA alterations (Hosoya and Miyagawa, 2014; Majidinia and Yousefi, 2017).

In human cells, there are two main mechanisms to repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). On the one hand, NHEJ directly ligates the DNA ends with little processing or none at all, and functions throughout the cell cycle (Scully et al., 2019). On the other hand, HR can use an undamaged homologous DNA sequence as a template to faithfully restore the DNA sequence involved in the break (Ranjha et al., 2018; Wright et al., 2018). This pathway is only available during S and G2 phases due to the need of an identical sister chromatid to repair the broken ends.

G4S AND R-LOOPS FORMATION INCREASE GENOMIC INSTABILITY

As previously stated, R-loops are well known stimulators of genomic instability, as its presence caused an increase in replication problems and hyper-recombination (further reviewed in (García-Muse and Aguilera, 2019; Rondón and Aguilera, 2019)).

On the other hand, recently it has been described that the use of G4 ligands (**Table 1**) is linked to an increase in DNA damage due to the induction of replication stress at PQS (Rodriguez et al., 2012). Moreover, an increase in cell cytotoxicity is observed when G4 ligands are used in a cell background where HR-factors are impaired (Zimmer et al., 2016; Xu et al., 2017). Although their evolutionary conservation supports a physiological role of the G4-forming sequences in these DNA processes, G4 structures weaken the genome and render it prone to accumulate DNA damage when they are not efficiently regulated. Indeed, the mapping of DSBs at the genome, using the technology DSB capture, showed an enrichment of these structures nearby the DNA breaking point (Lensing et al., 2016). Also, the association of G4s with DNA damage has been usually linked to their tendency to stall replication forks and cause chromosome breakage when not properly resolved (Paeschke et al., 2011).

Additionally, when the mechanisms responsible for G4 resolution fail to do so, replication forks that encounter these structures may stall or collapse leading to DNA DSBs (Lopes et al., 2011). This situation poses an extra challenge to the cell that might employ the pathways necessary to resolve it. Since the most faithful pathway to deal with this kind of breaks is HR, it is not a surprise that this process is also implicated in G4 induced damage resolution. Notably, the stabilization of G4s in cells deficient for HR factors, such as BRCA1/2 and EXO1, leads to an increased lethality (Zimmer et al., 2016; Xu et al., 2017; Stroik et al., 2020). Furthermore, several proteins with G4 unwinding capacity are recruited to DNA damage and facilitate DNA repair. This includes various helicases involved in G4 resolution such as BLM, WRN (Bernstein et al., 2010; Croteau et al., 2014), FANCJ (Wu et al., 2008; Sarkies et al., 2012) or PIF1 (Bochman et al., 2010; Muñoz-Galván et al., 2017). Indeed, we recently demonstrated that the 5′-3′ helicase PIF1, is required for the correct resection processivity by both unwinding G4 and recruiting BRCA1 to the break (Jimeno et al., 2018).

Finally, the role of G4s in DNA repair might not be limited only to their damage inducing capacity. Indeed, in (De Magis et al., 2020), the authors showed that G4s are also positively implicated in the repair of DNA damage. This observation was supported by the fact that the stabilization of G4s by Zuo1 in *Saccharomyces cerevisiae* stimulated the recruitment of Nucleotide Excision Repair machinery acting as a loading platform which led to a more efficient repair of the damage.

The Connection of G4s, R-Loops and Topoisomerases I and II

Recently, several studies have emerged pointing to a role of Topoisomerases in the homeostasis and function of G4s in

human cells. On the one hand in a genetic CRISPR/Cas9 based multi-screen, it has been shown that both PYR and CX5461 cytotoxicity is mediated by Topoisomerase II trapping (Olivieri et al., 2020). In this study they show that DSBs produced by PYR are mainly repaired by NHEJ. This would be in accordance with the fact that this molecule negatively affects DNA end resection, a fact that would immediately bias the repair towards NHEJ (Jimeno et al., 2018). Indeed, they demonstrate that although PYR behaves different to etoposide, both increase TOP2 cleavage complexes (Olivieri et al., 2020). On the other hand, with another genetic approach, topoisomerase II-α has also been found as a major effector of the toxicity of PYR and CX-5461 clastogenic agents (Bossaert et al., 2021); they also show that, despite the stabilization of G4s after PYR treatment, DSBs accumulation needs on-going transcription. Also, a reduction in PYR-mediated DSBs is observed when RNase H1 was overexpressed. This observation is in the same line than our own data showing that RNase H1 overexpression suppresses the DNA resection phenotype observed in PIF1 mutants (**Figure 1F**). Both results indicate a possible role of R-loops in the DNA damage generated by G4s stabilization due to PYR. They also show that DSBs caused by G4s stabilizing agents need transcription elongation to be active in order to be formed, pointing to a role of the supercoiling produced by active transcription in the production of those DSBs. Also, in another study spontaneous DSBs have been shown to be more prone to accumulate in regions in the genome with the capacity to form stable DNA secondary structures, including G4 structures, those regions being also prone to Top2-mediated cleavage (Szlachta et al., 2020).

Indeed, a connection between G4s inducing or stabilizing agents and topoisomerases could be inferred from previous studies. For instance, topoisomerase I Inhibitors Indenoisoquinolines are able to bind and stabilize the G4 present in the promoter *of MYC* oncogene lowering its expression (Wang et al., 2019). In another study Shuai et al. describe several short chains that can form G-quadruplex to have certain level of inhibition to topoisomerase I (Shuai et al., 2010). Also, quinolino [3,4-b] quinoxalines and pyridazino [4,3-c]quinoline derivatives showed a high activity as Topo IIα inhibitors and G-quadruplex stabilizers and also showed cytotoxic properties against two human cancer cell lines (Palluotto et al., 2016).

R-Loops and G4s, Two Sides of the Same Coin?

As the data for a related activity between these non-canonical DNA structures arose, new evidence linking the damage induced by G4s with R-loops presence appeared. Indeed, the use of G4 stabilizers also produced an increase in R-loop formation and the suppression of these structures by RNase H1 overexpression avoided the G4-associated formation of DNA damage markers (De Magis et al., 2019). In addition, several helicases involved in G4 unwinding are also implicated in R-loop resolution (see **Table 2**). That is the case for BLM helicase whose deletion delayed both the clearance of G4 and R-loops with the subsequent delay in γH2AX foci, a DNA damage marker, fading (Tan et al., 2020b). Another example is the helicase DHX9 that has been shown to resolve not only R-loops but also G4s (Chakraborty and Grosse, 2011). Indeed, depletion of DHX9 reduced the levels of DNA resection (Chakraborty et al., 2018; Prados-Carvajal et al., 2018). This fact has also been demonstrated by others, since, it has been recently shown, that USP42 and DHX9 promote DSB repair, through R-loop resolution and have a role in DNA resection, as well (Matsui et al., 2020). Along the same lines, in yeast Sen1 suppresses R-loop formation at DSBs in order to promote resection and increase repair fidelity (Rawal et al., 2020). Finally, the G4 unwinding helicase PIF1, recently implicated in DNA resection (Jimeno et al., 2018), has also been reported to act on DNA-RNA hybrids by complementing RNase H activity (for review see (Pohl and Zakian, 2019)).

Interestingly, both structures are known to accumulate close to sites of DNA breaks (Bader and Bushell, 2020; Cohen et al., 2018; D'Alessandro et al., 2018; Lensing et al., 2016; Rodriguez et al., 2012). This is, in part, due to an increase propensity of DNA to break close to both structures, explaining why stimulation of their presence increase genomic instability, but at least for R-loops they seem to be formed also as a consequence of the breaks. Furthermore, treatment with some G4 ligands in cancer cell stimulate R-loop formation (Amato et al., 2020). Moreover, the increase in G4s formation or stabilization, either using PYR or by depleting PIF1, (Koirala et al., 2011; Paeschke et al., 2013; Dahan et al., 2018), or the increase of R-loops accumulation by different genetic means (García-Pichardo et al., 2017; Zhang et al., 2020; Tsai et al., 2021) both generates road-blocks for DNA resection that naturally require, in both cases at least partially, the pro-resection factor BRCA1 (Hatchi et al., 2015; Zimmer et al., 2016; Xu et al., 2017). Thus, a tantalizing hypothesis is that these two structures are connected in their effect as resection impediments, and that G4s might stimulate R-loops and/or vice versa and block resection. Along those lines, we have been able to show that RNase H1 overexpression, indeed, rescue the resection impairment phenotype observed when PIF1 is depleted (**Figure 1F**). Further work will be needed to clarify this hypothesis in the future.

The implication of these non-canonical secondary DNA structures in DNA damage, in the control of important processes for the biology of cancer cells and their enrichment in cancer-promoting genes has raised the possibility of using them as novel therapeutic targets. As mentioned, several G4 stabilizing compounds (**Table 1**) have been studied for their therapeutic potential in cancer cells deficient in proteins involved in DNA repair pathways (Xu et al., 2017; Zimmer et al., 2016). Both PYR and CX-3542 (**Table 1**), for example, induce an increased lethality BRCA1/2 deficient cells (Zimmer et al., 2016). Basically, when these proteins are absent, there is a deficiency in HR-based repair that leads to a failing in repairing the DSBs induced by these G4 stabilizers. In consequence, through a synthetic lethality mechanism, cells undergo apoptotic cell death. Another pathway that could be

exploited for cancer therapy is the downregulation of proto-oncogenes, like c-MYC, by the stabilization of the G4s present in their promoters as studied in (Jiang et al., 2020) with the use of benzoxazinone derivatives. The effect of stabilizing these non-canonical DNA structures should also be considered in the context of a concomitant R-loop stabilization (Amato et al., 2020). This evidence clearly opens a new appealing target to discover promising new approaches in drug design for cancer chemotherapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All three authors discussed and planned the paper. RC found information and performed the experiment shown. SJ and PH wrote the paper.

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REVIEW
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Encounters in Three Dimensions: How Nuclear Topology Shapes Genome Integrity

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Almost 25 years ago, the phosphorylation of a chromatin component, histone H2AX, was discovered as an integral part of the DNA damage response in eukaryotes. Much has been learned since then about the control of DNA repair in the context of chromatin. Recent technical and computational advances in imaging, biophysics and deep sequencing have led to unprecedented insight into nuclear organization, highlighting the impact of three-dimensional (3D) chromatin structure and nuclear topology on DNA repair. In this review, we will describe how DNA repair processes have adjusted to and in many cases adopted these organizational features to ensure accurate lesion repair. We focus on new findings that highlight the importance of chromatin context, topologically associated domains, phase separation and DNA break mobility for the establishment of repair-conducive nuclear environments. Finally, we address the consequences of aberrant 3D genome maintenance for genome instability and disease.

Keywords: genome integrity, nuclear organization, replication stress, Topologically Associated Domain, chromatin, DNA double-strand break repair, phase separation

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INTRODUCTION

Eukaryotic genomes are exposed to numerous sources of DNA damage, of which DNA double-strand breaks (DSBs) are arguably the most deleterious. DSBs can arise from exposure to genotoxic agents, many of which are used in cancer therapy, but they can also be the result of endogenous processes such as oxidative metabolism and DNA replication. Aberrant repair of DSBs can cause chromosomal translocations, genomic duplications or deletions, as well as DNA mutations, all of which may result in defective cell function, cell death or malignant transformation.

Three main pathways exist to repair DSBs in mammalian cells: i) non-homologous end-joining (NHEJ), a fast but error-prone re-ligation of broken DNA ends; ii) microhomology-mediated end joining (MMEJ, also known as alternative end joining or alt-EJ), a process that relies on moderate DNA end resection and frequently results in small insertions or deletions (indels); and iii) homologous recombination (HR), which is a templated process and therefore considered error-free, but generally restricted to S/G2 phases of the cell cycle (Jasin and Rothstein, 2013; Chang et al., 2017). While both error-free and potentially error-prone repair pathways play essential roles in genome maintenance, inappropriate repair pathway choice can have detrimental consequences for genome integrity (Scully et al., 2019). The latter can be the result of DNA repair factor mutations, as often observed in cancer cells (e.g., breast and ovarian cancers with defective BRCA genes), but further depends on a more complex set of temporal and local factors, most notably cell cycle phase and the DSB-surrounding nuclear environment. Defects in a given repair pathway or inappropriate repair pathway choice can be exploited for synthetic lethal cancer therapy approaches, such as

poly(ADP-ribose) polymerase 1 (PARP1) inhibition, which selectively kills HR-deficient cancers (Lord and Ashworth, 2017).

Recent advances in biochemical, biophysical, imaging and deep sequencing technologies have led to unprecedented insight into nuclear organization and its changes with time and/or in response to cell-intrinsic or -extrinsic perturbations (Rowley and Corces, 2018; Kempfer and Pombo, 2020). Not surprisingly, there is an intimate link between genome organization, DNA accessibility and the functional regulation of DNA transactions, such as transcription, replication and DNA repair (Dekker and Mirny, 2016; Misteli, 2020). A first and by now well-characterized barrier to DNA access is the chromatin fiber, in which DNA is wrapped around positively charged nucleosomes consisting of a histone octamer, which typically comprises two copies of the core histones H2A, H2B, H3 and H4. Depending on the cellular context, core histones can be replaced with specialized histone variants. The impact of nucleosome composition and remodeling as well as static and dynamic histone modifications on DSB repair has been extensively investigated and we refer the reader to several excellent reviews summarizing this work (Price and D'Andrea, 2013; Lebeaupin et al., 2015; Ferrand et al., 2021; Hauer and Gasser, 2017). How the three-dimensional (3D) organization of the chromatin fiber in nuclear space can affect DSB repair, and conversely, be affected by the latter, is significantly less well understood.

Using a combination of high-throughput sequencing-based conformation capture approaches and fluorescent *in situ* hybridization (FISH)-based imaging, higher order chromatin organization can be interrogated at the kilobase (kb) or nm-scale, revealing complex chromatin looping that is often tied to cell cycle phases or DNA transactions such as transcription or replication (Bickmore, 2013; Rao et al., 2014; Rowley and Corces, 2018; Misteli, 2020). Chromatin loops are both architectural and functional in nature, providing a platform for 3D genome compaction as well as regulatory interactions. The size of chromatin loops can range from tens of kb to several 100 kb, often containing loops within loops. Loops that are characterized by unique chromatin features are referred to as topologically associated domains (TADs). Recent advances have provided significant insight into the processes that promote and maintain TAD formation, which involves active loop extrusion supported by architectural proteins such as CTCF and the structural maintenance of chromosomes (SMC) cohesin complex (Fudenberg et al., 2017; Rao et al., 2017; Davidson and Peters, 2021). Single cell analyses demonstrate that TAD formation is highly dynamic and often only detectable in a small subset of cells at any given time (Finn et al., 2019). For detailed reviews of recent advances in our understanding of TAD formation and function we refer the reader to (Hansen et al., 2018; Szabo et al., 2019). Of note, individual TADs can segregate into larger chromatin domains, which differ in loop density as well as nucleosome composition and mobility (Janssen et al., 2018; Szabo et al., 2019). While the formation of such chromatin domains is associated with TAD-specific histone modifications, recent findings demonstrate that liquid phase separation may help organize the heterochromatin compartment and perhaps TAD organization more generally (Larson and Narlikar, 2018; Gibson et al., 2019; Sanulli et al., 2019).

In this review, we will describe recent insight into the orchestration of DSB repair in the context of nuclear topology. Specifically, we will discuss how DSB repair processes have adjusted to and in many cases adopted the organizing principles of the nucleus to ensure accurate lesion repair. Finally, we will briefly address the consequences of failed 3D genome maintenance for genome instability and disease.

LOCATION, LOCATION LOCATION – CHROMATIN CONTEXT AFFECTS REPAIR OUTCOME

Large-scale interdisciplinary efforts such as the Encyclopedia of DNA elements (ENCODE) or the 4D Nucleome Projects have helped to map the composition of mammalian chromatin with remarkable resolution (Consortium, 2004; Dekker et al., 2017; Consortium et al., 2020). As we continue to obtain more refined insight into how DNA is organized into functionally and phenotypically distinct chromatin domains, it is time to revisit how these domains affect genome integrity.

Impact of Chromatin Context on DNA Double-Strand Break Repair Pathway Choice

Our understanding of the many chromatin modifications that interface with DSB repair processes is growing continuously and has been extensively reviewed elsewhere (Dabin et al., 2016; Hauer and Gasser, 2017; Ferrand et al., 2021). Nevertheless, a systematic assessment of DSB repair outcome across the various distinct chromatin states that coexist in a single cell has been missing to date. The advent of CRISPR/Cas9 as an effective means to target DSB induction to any given genomic location provides an opportunity to address this knowledge gap. Recent work by van Steensel and colleagues pioneered this effort by combining a multiplexed genome editing approach with a reporter that can distinguish between the two major error-prone DSB repair pathways, MMEJ and NHEJ (Schep et al., 2021). By overlaying a highly quantitative, sequencing-based "DNA repair scar"-counting readout with existing epigenome data, comprehensive MMEJ and NHEJ repair maps were generated for chromatin contexts across >1,000 genomic locations. This work complements and extends previous genome-wide assessments of HR versus NHEJ usage using the AsiSI endonuclease, which cuts the human genome efficiently at <100 sites and revealed a preference for HR in transcribed genomic regions (Aymard et al., 2014). Notably, MMEJ, which like HR relies on the resection of broken DNA ends to expose patches of homology for break alignment and repair, was found to be more frequent in specialized heterochromatic chromatin environments marked by H3 trimethylated at K27 (H3K27me3) (Schep et al., 2021). Together, these findings suggest that despite a common initial end processing step, HR and MMEJ are differentially

controlled by chromatin context, perhaps by regulating the shift from short-range resection to long-range resection generally associated with HR (Symington and Gautier, 2011; Scully et al., 2019).

Supporting a functional role for H3K27me3 in modulating DSB repair outcome, inactivation of EZH2, the histone methyltransferase responsible for most of its deposition, caused a shift in DNA repair away from MMEJ towards NHEJ (Schep et al., 2021). Moreover, EZH2 inhibition was recently shown to shift repair from HR to NHEJ in some ovarian cancer cell lines (Karakashev et al., 2020). Rather than being a lesion-specific effect of H3K27me3, the reduction in HR efficiency upon EZH2 inhibition was due to transcriptional de-repression of MAD2L2, a component of the Shieldin complex that counteracts DNA end resection (Karakashev et al., 2020). Consistent with the defect in HR, EZH2 inhibition selectively sensitized ovarian cancer cell lines with sufficiently high MAD2L2 levels to PARP inhibitors in both orthotopic and patient-derived xenografts. Seemingly in contrast to these findings, EZH2 inhibition has recently been associated with replication fork stabilization and PARPi resistance in BRCA2-deficient and hence HR-defective breast cancer cells (Rondinelli et al., 2017). EZH2-mediated destabilization of replication forks involved H3K27me3 mediated recruitment of the MUS81 endonuclease, thus coupling histone modification to replication fork protection. Together, these observations emphasize that a widely distributed mark of facultative heterochromatin such as H3K27me3 can have a complex impact on genome maintenance, which likely depends on genomic context as well as the type of DNA lesion. Consequently, manipulation of EZH2 resulted in cell line-specific, yet predictable outcomes in response to genotoxic therapy.

The macro-histone variant macroH2A1, which frequently colocalizes with H3K27me3 domains across the genome (Chen et al., 2014), has recently emerged as another modulator of DNA repair pathway choice (Ruiz et al., 2019; Sebastian et al., 2020). Specifically, macroH2A1 controls DSB repair via balanced expression of its two alternative splice variants, macroH2A1.1 and macroH2A1.2. MacroH2A1.1, which unlike macroH2A1.2 can bind poly (ADP-ribose) (PAR), interacts with the MMEJ effectors PARP1 and Ligase 3 in a PAR-dependent manner to facilitate MMEJ, whereas macroH2A1.2 promotes HR by facilitating BRCA1 recruitment to sites of DNA damage (Khurana et al., 2014; Kim et al., 2018). Deletion of macroH2A1.2 shifts repair towards MMEJ resulting in genome instability that is particularly pronounced at the macroH2A1-and H3K27me3-rich inactive X chromosome in female mouse fibroblasts (Sebastian et al., 2020). If and how macroH2A1 and EZH2 functions are related during DSB repair and/or replication stress remains to be determined. However, deregulation of H3K27me3 levels or macroH2A1 variant expression, and the associated HR defects, have both been linked to PARP inhibitor resistance, chromosomal abnormalities, and [...] PARP inhibitor resistance and chromosomal instability in cancer cells in cancer cells (Khurana et al., 2014; Karakashev et al., 2020).

Together, these recent advances exemplify the impact of improved integrative analyses of chromatin composition on our understanding of genome maintenance. They further emphasize the need to i) consider functionally distinct proteoforms, often as the result of alternative splicing, and ii) distinguish lesion-specific from global effects of chromatin perturbation such as the epigenetic deregulation of repair factors.

Chromatin Domains Guide DNA Replication

Recent work suggests that, much like distinct chromatin domains differentially affect DSB repair factor recruitment, they can modulate the initiation and progression of DNA replication, as well as the repair of stalled replication forks (Alabert et al., 2017; Aladjem and Redon, 2017; Bellush and Whitehouse, 2017). While the impact of chromatin on replication timing and DNA polymerase processivity is well described (Marchal et al., 2019; Klein et al., 2021), it was perhaps unexpected that chromatin composition can determine the choice of DNA replisome subunits. Comparative analysis of two replisome-associated proteins involved in the cellular response to replication stress, the translocase FANCM and the poorly characterized DONSON protein (Reynolds et al., 2017), uncovered the existence of distinct replisome complexes. While both proteins facilitate the repair of DNA interstrand crosslinks (ICLs) via a lesion traverse mechanism, FANCM-associated replisomes are most prevalent in late stages of S phase, generally induced by replication stress, and colocalize with a chromatin environment characteristic of late replicating and fragile DNA (Zhang et al., 2020). DONSON, on the other hand, appears to form a distinct replisome complex that is primarily responsible for ICL traverse in early S phase. Notably, FANCM and DONSON show the same bias in replication timing- and chromatin domain-association in cells without ICLs. How distinct chromatin environments regulate replisome composition remains to be determined, as does the functional relevance and potential clinical implications of having different replisomes act throughout S phase. Of note, defects in DONSON or FANCM manifest in microcephalic dwarfism and breast cancer susceptibility, respectively (Reynolds et al., 2017; Catucci et al., 2018). It will be interesting to investigate whether or not these distinct pathological outcomes relate to the observed differences in replication stress responses.

Altogether, we anticipate that continued, refined and comprehensive mapping of functionally distinct chromatin components, DNA repair outcome and genetic dependencies will provide a wealth of clinically actionable insight into repair mechanisms.

DNA DOUBLE-STRAND BREAK REPAIR DOMAINS – NEW INSIGHTS INTO FORMATION AND FUNCTION

Beyond its role in modulating and regulating DNA transactions, chromatin shapes and defines the formation of functionally distinct, specialized nuclear environments. Recently, it has become apparent that DNA repair takes advantage of these features to form contained and often pathway-specific micro-

environments, sequestering DNA lesions for reasons that remain to be fully investigated, but may help prevent illegitimate and potentially harmful repair events. Several novel concepts highlight and extend the impact of nuclear organization to genome maintenance and are discussed below.

Chromatin Loop Extrusion: The DNA Repair Focus Revisited

Microscopically visible DNA damage response (DDR) foci are a striking feature of DSB repair (Rogakou et al., 1999). These foci generally reflect a single DNA lesion and its association with a plethora of often repair pathway-specific damage sensors and repair factors that can cover several hundred kilobases (kb) of lesion-surrounding DNA. At the heart of most DSB-associated chromatin changes is the phosphorylation of S139 on the histone H2A variant H2AX (referred to as γH2AX), orchestrated by early DNA damage signaling events involving one or more of three PI3K family kinases - ATM, ATR and DNA-PKcs (Rogakou et al., 1998; Smeenk and van Attikum, 2013). γH2AX facilitates the recruitment of key downstream repair effector proteins via the γH2AX-binding MDC1 scaffold protein (Smeenk and van Attikum, 2013). Although DSB-surrounding γH2AX chromatin domains have been mapped across the genome in response to numerous DNA damaging agents or endonucleases, the molecular basis that underlies the formation of up to megabase (Mb) size regions of γH2AX has long puzzled the field (Iacovoni et al., 2010). Of note, γH2AX domain boundaries were found to coincide with topologically associated domain (TAD) boundaries (Caron et al., 2012), and super-resolution light microscopy revealed that CTCF, a TAD boundary marker, is juxtaposed to γH2AX foci (Natale et al., 2017). Similar observations were reported for the 53BP1 repair factor, the recruitment of which depends on the RNF8/RNF168 E3 ubiquitin ligases, which in turn bind MDC1 (Hustedt and Durocher, 2016; Ochs et al., 2019). Together, these observations suggest that DNA repair domain formation is governed by high-order chromatin organization.

The organization of the genome into TADs involves ATP-dependent, active extrusion of DNA loops through a cohesin ring (Rao et al., 2017; Ganji et al., 2018; Davidson et al., 2019; Davidson and Peters, 2021). Cohesin consists of the SMC1-SMC3 heterodimeric adenosine triphosphatase (ATPase), the SMC protein partner RAD21 and either of the helical repeat proteins STAG1 or STAG2, and was originally identified as a mediator of sister chromatid cohesion. TAD loops are anchored by inverted CTCF sites, which terminate cohesion-mediated loop extrusion when encountered on opposing strands through yet to be determined mechanisms. For a detailed overview of cohesin function in TAD formation, we refer the reader to a number of excellent reviews (Fudenberg et al., 2017; Rowley and Corces, 2018; Yatskevich et al., 2019).

Loop extrusion does not only facilitate the organization of TADs and chromatin domains, it can also facilitate the ligation of otherwise distal DNA ends. This was first described for the processes of VDJ and class switch recombination, which mediate the rearrangement and assembly of immunoglobulin (Ig) gene elements that are up to several 100 kb apart (Zhang et al., 2019a; Zhang et al., 2019b). Like TAD formation, VDJ recombination and class switching depend on CTCF-associated cohesin rings to allow for accurate ligation of matching gene elements (Ba et al., 2020; Dai et al., 2021). Notably, cohesin was found to accumulate at DSB sites other than the Ig locus, and its depletion resulted in genome instability (Ström et al., 2004; Ünal et al., 2004; Potts et al., 2006; Covo et al., 2010; Meisenberg et al., 2019). Recruitment of cohesin to DSBs was observed throughout the cell cycle, suggesting a function independent of sister chromatin adhesion or HR (Ström et al., 2004; Potts et al., 2006; Caron et al., 2012; Meisenberg et al., 2019). Together with the finding that γH2AX domains overlap with TADs (Caron et al., 2012; Natale et al., 2017), these observations point to a role for loop extrusion in the formation of DSB repair domains. Experimental support for the latter came recently from an elegant set of analyses combining chromosome conformation capture mapping, chromatin immunoprecipitation (ChIP) and AsiSI-mediated DSB induction (Arnould et al., 2021). A marked discrepancy was observed between the distribution of γH2AX and ATM, the kinase primarily responsible for DSB-induced H2AX phosphorylation (Arnould et al., 2021). In contrast to the TAD-sized γH2AX domains, ATM accumulation was restricted to the immediate vicinity of the DSB, suggesting that H2AX phosphorylation is not mediated by the linear spreading of the kinase across TADs. Using tightly controlled DSB synchronization and release combined with ATM inhibition and/or deletion of several cohesion subunits, the authors provide compelling evidence for a model in which H2AX-containing nucleosomes are rapidly phosphorylated as they actively pass by DSB-anchored cohesin. TADs thus delineate the boundaries of γH2AX chromatin domains in a manner that involves one-sided loop extrusion on either side of the break. Importantly, this process was conserved in yeast (Arnould et al., 2021), and the observed kinetics are consistent with previously reported rates of γH2AX foci assembly (Ochs et al., 2019). Moreover, DNA damage was shown to result in the ATM-dependent strengthening of existing TAD structures, perhaps as a mechanism to protect 3D genome integrity during DNA repair (Sanders et al., 2020). Together, these findings highlight how chromosome conformation and TAD-associated loop extrusion have been adopted by the DDR to ensure repair domain formation and genome maintenance (**Figure 1**).

Phase Separation of Double-Strand Break Repair Domains?

In addition to structured chromatin organization, nuclear subdomains can be organized by physicochemical forces (Boeynaems et al., 2018). Examples of such domains include PML bodies, Cajal Bodies, nuclear speckles, and the nucleolus, which were proposed to behave as semifluid spheres suspended in semifluid nucleoplasm almost 2 decades ago (Handwerger et al., 2005). Experimental evidence for the physical nature of such assemblies was provided in 2009, when P granules (RNA and protein-containing bodies) were shown to display liquid-like

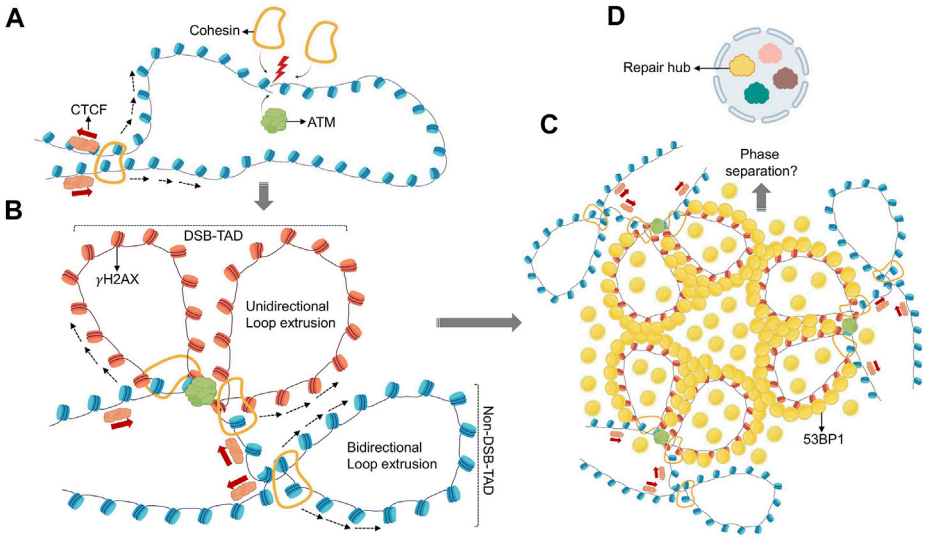

FIGURE 1 | Role of loop extrusion in establishing repair domains. **(A)** DSBs initiate recruitment of ATM kinase and the cohesin complex. **(B)** DSB-associated cohesin anchors initiate unidirectional loop extrusion at both DSB ends, towards TAD anchors. ATM phosphorylates H2AX while nucleosomes are extruded (γH2AX nucleosomes are shown in red). Loop extrusion stops when existing TAD boundaires are encountered. This process generates a TAD-overlapping γH2AX domain. **(C)** γH2AX domains recruit 53BP1 repair factors creating similar, TAD-overlapping 53BP1 profiles. **(D)** 53BP1 mediated phase separation at TAD-associated 53BP1 domains may promote higher-order assembly of multiple 53BP1-TADs, and possibly multiple DSBs, to create spatially segregated repair hubs (yellow). Distinct nuclear subcompartments are symbolized in different colors.

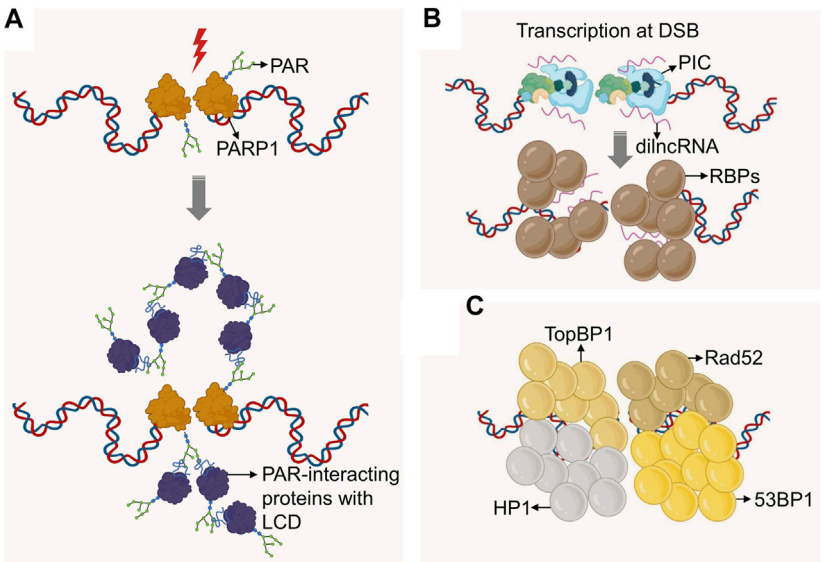

FIGURE 2 | Formation of DNA repair domains via phase separation. **(A)** DSBs recruit PARP1, which mediates DSB-proximal PARylation, attracting PAR-binding proteins, many of which contain Low Complexity Domains (LCD). The latter promote molecular crowding and concomitant phase separation. **(B)** DSBs recruit RNA Polymerase II, which initiates transcription at the DSB site to generate dilncRNAs. These RNAs are bound by IDR-containing RNA binding proteins (RBPs) which can drive phase separation. **(C)** Protein modifications at DSBs recruit proteins such as 53BP1, TopBP1, Rad52 and HP1, all of which were shown to form higher order condensates via phase separation. If these domains are distinct or can be fused remains to be determined. 53BP1 may further promote phase separation via its binding to dilncRNA.

properties and form by phase separation in *C. elegans* (Brangwynne et al., 2009). By definition, phase separation in biological systems occurs when a homogenous mixture of macromolecules such as proteins or nucleic acids in a solution spontaneously separate into a phases of distinct densities. In the context of chromatin, condensates can form either via bridging of

nucleosome-binding proteins (polymer-polymer phase separation) or via multivalent interactions among soluble, chromatin-associated proteins (liquid-liquid phase separation) (Erdel and Rippe, 2018; Miné-Hattab and Taddei, 2019). In the case of liquid-liquid phase separation, which is the focus of this section, the dense phase has liquid-like properties, no fixed stochiometry and accumulates certain macromolecules. Since the non-dense phase is depleted of said macromolecules, it allows the dense phase to attain a compartment-like status. Phase-separated liquid condensates can eventually form more solid-like states exhibiting different material properties, such as dynamic liquid-like droplets or less dynamic gels and solid amyloids (Banani et al., 2017; Miné-Hattab and Taddei, 2019).

Studies looking at the protein composition of phase-separated biological condensates suggest multivalency of adhesive domains and linear motifs as defining features of proteins that drive phase separation. Prominent examples are intrinsically disordered regions (IDRs) and Low Complexity Domains (LCDs). Of note, recent work from the Narlikar and Karpen labs demonstrated that phase separation is also an organizing principle for chromatin domains, particularly heterochromatin (Larson et al., 2017; Strom et al., 2017; Larson and Narlikar, 2018). It may thus be not surprising that phase separation was found to contribute to the formation of DNA repair micro-environments. Underlying mechanistic insight and possible consequences for genome maintenance are discussed below, separated by the phase-separating properties of primary responders or effectors of the DNA damage response (**Figure 2**).

Phase Separation by Poly (ADP-Ribose)

PARP1 is an abundant nuclear protein that attaches a negatively charged (PAR) polymer to itself and to multiple target proteins. This modification is one of the earliest events in the DNA damage response against a wide variety of DNA lesions (Kraus, 2020). Consistent with this, PARylation has been implicated in the repair of single-strand breaks (SSBs), DSBs, the stabilization of DNA replication forks and the modification of the DNA damage-associated chromatin (Ray Chaudhuri and Nussenzweig, 2017). While PARP1 itself has no IDRs, its activation at sites of DNA damage was found to promote transient phase separation via the formation of PAR chains. PAR chains act as a molecular scaffold for the assembly of proteins with disordered or low complexity domains, thereby initiating demixing of distinct liquid phases to achieve dynamic intracellular compartmentalization. Two types of LCDs participate in this process: positively charged arginine–glycine–glycine (RGG) repeats, which act as a PAR sensor, and prion-like protein domains, which amplify PAR-seeded liquid demixing (Altmeyer et al., 2015). This process appears to reflect a general mechanism to dynamically reorganize the soluble nuclear space in response to DNA lesions (**Figure 2A**). Recent work has implicated the highly disordered RGG containing Fused in Sarcoma (FUS/TLS) protein in PAR-seeded liquid demixing (Singatulina et al., 2019). FUS, together with EWS and TAF15, is a member of the FET family and one of the most abundant and highly PARylated nuclear RNA-binding proteins (Britton et al., 2014; Singh et al., 2015; Zhen et al., 2017). FUS condensates have liquid-like properties, the dynamics and structure of which are affected by pathogenic mutations as well as LCD phosphorylation (Patel et al., 2015; Monahan et al., 2017; Murray et al., 2017). Upon DNA damage, the C-terminal RGG repeats of FUS form repair domains in response to PARP activity in a transient and reversible manner (Singatulina et al., 2019). PAR-seeded liquid demixing may thus facilitate the compartmentalization of damaged DNA, and its functional relevance for the DDR is a subject of intense investigation.

Phase Separation by RNA

Analogous to PAR chains, nucleic acids were shown to seed phase-separated structures by recruiting IDR-containing RNA binding proteins (RBPs). Non-coding RNAs (ncRNAs) form molecular scaffolds that connect multiple RBPs into a dynamic network of phase separated droplets (Lin et al., 2015; Aumiller et al., 2016; Pessina et al., 2019; Guo et al., 2021). Messenger RNA (mRNA) was also found to form phase-separated droplets. However, in this case the seed involved specific 3D structures through complementary RNA base pairing (Langdon et al., 2018). Of note, growing evidence points to DNA damage-induced transcription of non-coding RNA at DNA break sites (Sebastian and Oberdoerffer, 2017; Zong et al., 2020). These DNA damage induced long non-coding RNAs (dilncRNA) were found to be necessary for DNA damage response (DDR) focus formation (Francia et al., 2012), form transient RNA:DNA hybrids (Wahba et al., 2013; Ohle et al., 2016), and regulate the extent of end resection and consequently HR. Recently, it was shown that the induction of DSBs resulted in the assembly of functional promoters that include a complete RNA polymerase II preinitiation complex, MED1 and CDK9 (Pessina et al., 2019). Mediator and RNA polymerase II clusters are known to associate in transcription-dependent condensates (Cho et al., 2018), and depletion or inactivation of these factors caused a reduction in DDR foci. Moreover, dilncRNAs drove molecular crowding of DDR proteins, such as 53BP1, into foci that behave like phase-separated condensates (Pessina et al., 2019). Given that phase separation has been proposed as a mechanism for transcription control (Hnisz et al., 2017), a similar role in the DDR may provide an intriguing rationale for DSB-associated transcripts (**Figure 2B**).

Phase Separation by DNA Repair Factors

Phase separation can also be mediated by DNA repair proteins. The NHEJ effector and chromatin binding protein 53BP1 was recently shown to condensate into repair domains that are dynamic and show droplet-like behavior (**Figure 2C**). Repair domain formation by 53BP1 undergoes frequent fusion and fission events, is highly sensitive to changes in osmotic pressure, temperature, salt concentration and the disruption of hydrophobic interactions, consistent with liquid demixing (Kilic et al., 2019). Light-induced optoDroplet formation experiments (Taslimi et al., 2014; Shin et al., 2017) combined with 53BP1 mutagenesis suggest that a C-terminal multivalent domain as well as the C-terminal BRCT domain are sufficient for 53BP1 phase separation properties. The implication of the BRCT domain is intriguing as other BRCT-containing protein such as BRCA1 did not appear to phase-separate, suggesting sequence specificity and/or more complex organizing principles. Providing functional

insight into possible roles of DNA damage-induced phase separation, the tumor suppressor protein p53 was found enriched within 53BP1 repair domains, and conditions that perturb 53BP1 phase separation negatively affected 53BP1-dependent activation of p53 (Kilic et al., 2019). However, a direct role for 53BP1-mediated droplet formation in the repair of DSBs has not been identified to date.

53BP1 foci have regulatory functions beyond the immediate repair of DSBs. Cells that carry replication stress-associated DNA damage, such as lesions resulting from under-replicated DNA, into the next cell cycle form so called 53BP1 nuclear bodies (Harrigan et al., 2011; Lukas et al., 2011). Like DSB-induced 53BP1 foci, 53BP1 nuclear bodies were sensitive to osmotic stress, indicative of phase separation properties (Kilic et al., 2019). Nuclear body formation appears to inhibit repair in G1 to facilitate templated, RAD52-mediated repair of the lesion in the next S phase (Lezaja and Altmeyer, 2018; Spies et al., 2019). Notably, RAD52 was shown to form liquid droplets in *Saccharomyces cerevisiae* (Oshidari et al., 2020). RAD52 droplets cooperate with DNA damage-inducible intranuclear microtubule filaments to promote the clustering of DNA damage sites and facilitate HR (Oshidari et al., 2020). Recent studies suggest that RAD52 may be a "client" rather than a "scaffold" for liquid droplets, pointing to additional factors involved in their formation (Miné-Hattab et al., 2021). It will be interesting to determine if a dynamic transition exists between 53BP1 nuclear bodies and RAD52 droplet formation, which may help regulate repair activity at 53BP1 nuclear bodies in a cell cycle-dependent manner.

A number of other DSB repair-associated proteins have been reported to exhibit phase separation properties (**Figure 2C**). A notable example is heterochromatin protein 1 (HP1), which can associate with sites of DNA damage to aid the recruitment of 53BP1 and RAD51 (Alagoz et al., 2015). HP1 has been implicated in the evolutionarily conserved, liquid-liquid phase separation of heterochromatin domains (Larson et al., 2017; Strom et al., 2017; Sanulli et al., 2019; Wang et al., 2019). However, both the precise nature of HP1 subcompartment formation and its potential role at DSBs remain to be determined (Chiolo et al., 2011; McSwiggen et al., 2019; Erdel et al., 2020). More recently, the ATR activator TopBP1 was shown to self-assemble into micron-sized condensates. Single amino acid substitutions of key residues in the ATR-activation domain of TopBP1, which also contains IDRs, disrupt TopBP1 condensation and, consequently, ATR/Chk1 signaling and replication fork stalling (Frattini et al., 2021). Of note, DSB-dependent formation of early DDR events such as γH2AX and MDC1 foci did not exhibit liquid-like properties (Kilic et al., 2019), pointing to distinct and likely dynamic modes of DSB micro-environment organization. The latter is further consistent with the seemingly independent and/or complementary initiation of phase separation initiation at DNA lesions via either PAR, RNA or DNA repair factors.

Coordination Between Topologically Associated Domains and Phase Separation in DNA Repair

Recent evidence suggests that the formation of phase-separated repair environments is tightly linked to TAD-associated repair micro-domains. Using super-resolution microscopy, Lukas and colleagues were able to provide unprecedented insight into repair domain formation by 53BP1 (Ochs et al., 2019). Specifically, 53BP1 and its interacting factor RIF1 were found to form an autonomous functional module that stabilizes 3D chromatin topology at sites of DNA breakage. This process involves the sequential accumulation of 53BP1 at TAD-associated, compact chromatin, followed by RIF1 accumulation at the boundaries between these domains. The alternating distribution of 53BP1 and RIF1 was found to stabilize neighboring TADs into a higher-order arrangement surrounding a single DSB. Depletion of 53BP1 or RIF1 led to the de-condensation of DSB-surrounding chromatin and aberrant spreading of DNA repair factors. Of possible functional relevance, depletion of either protein also resulted in hyper-resection of DNA ends (Ochs et al., 2019).

Interestingly, topological distortions of 53BP1 domains could also be observed upon depletion of cohesin (Ochs et al., 2019). Together with recent insight into TAD-dependent DDR focus formation, these findings suggest a staged model wherein TAD structure dictates γH2AX foci formation, which in turn promotes their DNA repair-independent, 53BP1-mediated higher-order assembly, perhaps in a process that involves phase separation (**Figure 1**). More work is needed to understand the implications of this 3D re-organization of the DSB-proximal chromatin micro-environment for DNA repair, but the observed changes in DNA resection point to a role in the regulation of DSB repair pathway choice.

DNA LESIONS ON THE MOVE - AGGREGATION OF DOUBLE-STRAND BREAK IN THREE DIMENSIONS

Both TAD- and phase separation-associated repair domain formation can be observed at a single DNA lesion. However, the process of 53BP1-dependent clustering of multiple TAD domains, as well as the inherent biophysical properties of liquid demixing raise the possibility of a higher-order organization of multiple DNA lesions into a single repair "super-focus." Moreover, recent advances in high-resolution live cell microscopy and targeted genome manipulation have uncovered compelling evidence for directed movement of DNA lesions to form aggregates. Although the phenomenon of DSB clustering has been observed in yeast and mammalian cells almost 2 decades ago (Lisby et al., 2003; Aten et al., 2004), until very recently little was known about the underlying mechanistic forces and possible functional relevance. In the following we will discuss novel insight into DSB mobility and one of the pre-eminent nuclear environments they congregate at, the nuclear pore (**Figure 3**).

Movement and Clustering of DNA Lesions

Multiple lines of evidence suggest that DSB mobility can be an active process. Almost 10 years ago, homology search of a single DSB in yeast was shown to involve DNA end resection and RAD51-depedent DSB movement (Dion et al., 2012; Miné-

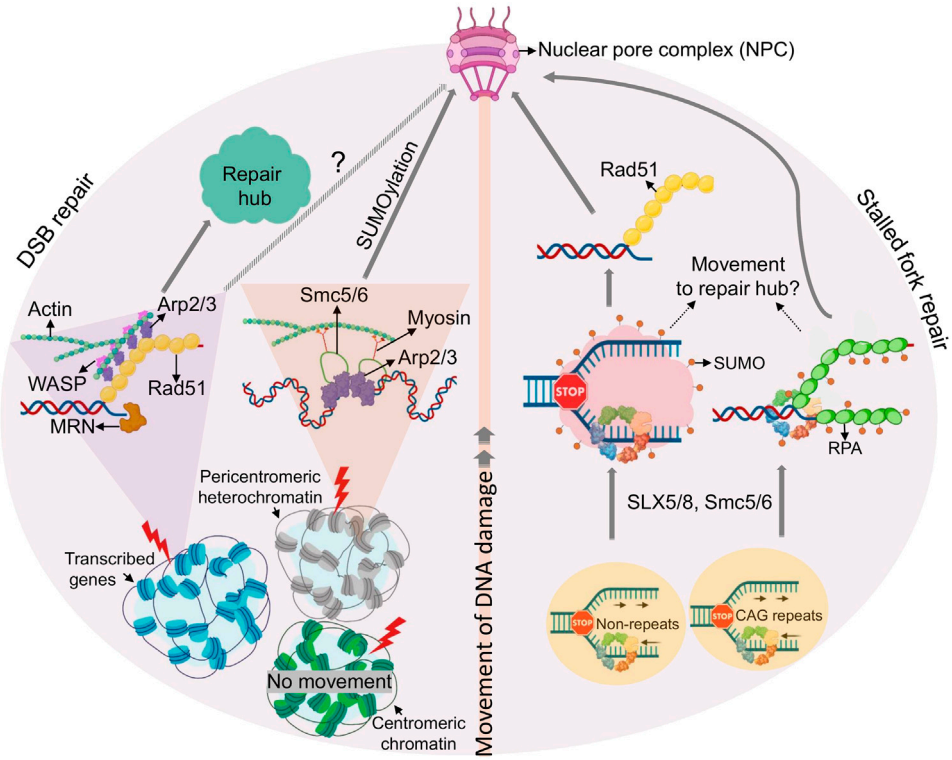

FIGURE 3 | Movement of DSBs and damaged replication forks. Emerging principles of DSB (left) or replication fork mobility (right) are shown. Clockwise from the bottom: Mechanisms of movement are different for DSBs induced in different chromatin environments (see text for details). In brief, DSBs in transcriptionally active chromatin load Rad51 after end resection and move to repair hubs facilitated by actin polymerization. DSBs in pericentromeric heterochromatin initiate resection, but relocate away from hetercochromatin via an SMC5/6 dependent process to facilitate RAD51 loading. Pericentromeric DSBs, and perhaps DSBs within transcribed DSBs regions, are then targeted to the NPC in a manner that involves SUMOylation, myosin, and actin. Counterclockwise from the botton: Stalled replication forks relocate to the NPC in a process involving SUMOylation by Smc5/6 and/or SLX5/8 (shown as red dots). Forks in repetitive sequences are targeted to the NPC via SUMO-RPA, prior to Rad51 loading, whereas arrested forks at non-repetitive genomic loci are targeted to NPC after RAD51 loading.

Hattab and Rothstein, 2012). RAD51-coated DNA can explore a larger nuclear volume than undamaged DNA, which is thought to facilitate homologous pairing and repair. Mean square displacement (MSD) analyses, which plot the average of the squared distances that a particle has travelled against increasing time intervals, suggested that increased DSB mobility was due to an increase in the radius of confinement, rather than a change in the diffusion coefficient of the damaged locus, pointing to a role for chromatin reorganization in this process (Miné-Hattab and Rothstein, 2012; Hauer and Gasser, 2017). DSB mobility has since then emerged as a complex phenomenon that depends on various factors, including cell cycle phase and DSB location (Kalousi and Soutoglou, 2016; Smith and Rothstein, 2017). Telomeric DSBs, for example, are more mobile than the undamaged chromatin (Dimitrova et al., 2008), whereas UV laser microirradiation or endonuclease-mediated induction of DSBs outside of telomeres show limited mobility (Kruhlak et al., 2006; Soutoglou et al., 2007; Roukos et al., 2013). Such discrepancies have sparked intensive efforts to better understand the molecular mechanisms that drive DSB mobility, both in yeast and higher organisms. Much of the initial progress came from studies of broken telomeres, which are relatively easy to monitor in living cells. Telomeric DSBs can result from telomere deprotection and are subject to repair by NHEJ (de Lange, 2018). In mammalian cells, the NHEJ effector 53BP1 was found to promote not only repair but also mobility of broken telomere ends, together with the linker of nucleoskeleton and cytoskeleton (LINC) complex and dynamic microtubules (Lottersberger et al., 2015). 53BP1/LINC-dependent DSB mobility was not limited to telomeres, but was also observed upon irradiation-induced DNA damage. Given the role for 53BP1 in DSB-associated phase separation described in *Phase Separation by DNA Repair Factors*, it will be interesting to determine if the latter contributes to or complements the mechanic movement forces provided by 53BP1, the LINC complex and microtubules. Precedent for a coordination between phase separation and active movement comes from the observation that RAD52 droplets can cooperate with microtubule filaments to promote DSB clustering and repair (Oshidari et al., 2020). While the functional relevance of 53BP1-mediated DSB clustering remains to be established, this process may help restore proximity of DNA ends that have lost their proper interaction and thereby counteract ectopic repair. However, with increasing DNA damage, aberrant end pairing can

have fatal outcomes, as evidenced by aberrant telomere end fusions that result in dicentric chromosome formation (Lottersberger et al., 2015).

A distinct type of telomeric break movement has been described in the context of alternative lengthening of telomeres (ALT), an HR-dependent process to maintain telomeres in the absence of telomerase, which is active in ~15% of cancer types. ALT-associated homologous chromosome synapsis was found to depend on long-range DSB mobility and aggregation into multi-telomere clusters (Cho et al., 2014). Much like HR-prone DSBs in yeast, this process required RAD51, although it further involved the protein dimer Hop1/Mnd1, which also mediates homologous chromosome synapsis during meiosis. Moreover, ALT telomeric DSBs show evidence for directed motion based on MSD analysis (Cho et al., 2014), while DSB movement in yeast was found to be consistent with confined Brownian motion (Dion et al., 2012; Miné-Hattab and Rothstein, 2012). Together, these findings underline the context dependence of telomeric DSB movement.

Telomeres present a unique chromatin environment and the implications of break mobility outside of telomeric regions have only recently been uncovered in vertebrates. In a mass spectrometry approach in *Xenopus* extracts, Gautier and colleagues identified nuclear actin, the actin-nucleating complex ARP2/3, β-actin and the ARP2/3 activator WASP as novel, chromatin-associated DSB repair effectors (Schrank et al., 2018). While DNA damage-induced actin polymerization was reported previously (Belin et al., 2015), little was known about its role in DSB repair and/or at broken DNA. Using the mammalian AsiSI endonuclease system described earlier (Aymard et al., 2014), ARP2/3 and WASP were found to preferentially accumulate at HR-prone DSB sites. Consistent with the latter, HR but not NHEJ efficiency was impaired upon inhibition of actin nucleation, or the depletion of WASP or the nucleation factors FORMIN-2 and SPIRE-1/SPIRE-2. Moreover, nuclear actin polymerization was found to be required for G2-restricted migration of a subset of DSBs and their aggregation into sub-nuclear clusters. Like mobilty in yeast and at ALT telomeres, AsiSI-induced DSB movement was initiated by DNA end resection (Dion et al., 2012; Miné-Hattab and Rothstein, 2012; Cho et al., 2014; Schrank et al., 2018). Interestingly, ARP2/3 loading was found to enhance end resection and RAD51 loading at AsiSI-induced DSBs in a positive feedback loop (Schrank et al., 2018). DSB movement at HR-prone AsiSI DSBs was further found to be consistent with confined Brownian motion, similar to yeast (Schrank et al., 2018). A role for actin nucleation in the movement of yeast or ALT DSBs remains to be demonstrated.

It should be noted that there is some debate as to when during the cell cycle DSBs cluster. Seemingly in contrast to the findings by the Gautier lab, enhanced DSB clustering was first identified in G1 cells (Aten et al., 2004). Preferential aggregation in G1 was confirmed more recently using Hi-C chromosome conformation capture of AsiSI-dependent DSBs (Aymard et al., 2017), although clustering was similarly restricted to HR-prone break sites. DSB clustering in G1 coincided with delayed DSB repair and was dependent on the MRN complex, FORMIN-2 and the LINC complex, consistent with resection-mediated active movement. Given the identical DSB source (AsiSI), discrepancies in the timing of DSB clustering may reflect distinct experimental readouts, such as the resolution of Hi-C versus live cell imaging assays, which is likely to detect significantly smaller aggregates in the case of Hi-C. It will be interesting to determine if distinct "micro" and "macro" aggregate sub-types exist, and how they may differentially contribute to the DDR. One intriguing hypothesis is that transitions in aggregate sub-type may help control repair kinetics during the cell cycle, ensuring HR in S/G2, but preventing HR in G1.

Much like HR, actin-mediated DSB mobility is not restricted to transcribed genes. HR in highly repetitive DNA, such as pericentromeric heterochromatin relies on specialized mechanisms to prevent aberrant recombination events. In *Drosophila melanogaster*, this is achieved by relocalization of DSBs to the nuclear periphery (Chiolo et al., 2011). While proteins responsible for the initial steps of end resection are rapidly recruited within heterochromatin, RAD51 remains excluded, thus preventing homology search and completion of HR. RAD51 loading instead requires resected heterochromatic DSBs to move to the nuclear periphery in a process that involves the SMC5/6 SUMO E3 ligases (Chiolo et al., 2011; Ryu et al., 2015). A similar process has been observed in yeast and at the repetitive rDNA, and has been extensively reviewed elsewhere (Jalal et al., 2017). More recently, Chiolo and colleagues demonstrated that, much like mammalian AsiSI-induced, HR-prone DSBs, the movement of heterochromatic breaks to the nuclear periphery in *Drosophila* requires actin filament formation and the Arp2/3 complex (Caridi et al., 2018). However, while ARP2/3 mediated actin nucleation appears to be sufficient for mobility and clustering of non-heterochromatic DSBs in mammalian cells (Schrank et al., 2018), DSBs within *Drosophila* heterochromatin further require nuclear myosin, which associates with Smc5/6 proteins to initiate movement (Caridi et al., 2018). Notably, two phases of motion have been described for heterochromatic lesions in Drosophila: confined Brownian motion within the heterochromatin domain, and directed motion towards the NPC, outside of heterochromatin (see **Figure 3**) (Caridi et al., 2018; Miné-Hattab and Chiolo, 2020). Given that Arp2/3 promotes non-directed motion of mammalian HR-prone DSBs, actin appears to be able to support both types of motion, implicating additional mobility modulators or species-specific differences. Together, these findings suggest that HR-prone DSBs can initiate movement irrespective of genomic context, but mobility may require additional accessory factors, depending on where the DSBs occur. Why HR-prone breaks move preferentially compared to non-HR prone lesions remains an open question, but further points to a critical role for end resection in this process.

Notably, not all DSBs within compacted chromatin initiate movement, even if they are destined for HR. While DSBs relocalize in the context of pericentromeric heterochromatin as described above, the same does not appear to be true for DSBs in centromeric chromatin, which carries distinct epigenetic marks and occupies distinct nuclear subdomains. The precise nature of this discrepancy remains to be investigated (Tsouroula et al.,

2016). Consistent with the findings by the Gautier lab (Schrank et al., 2018), DSBs in mammalian pericentromeric heterochromatin were found to be positionally stable in G1, where they recruit NHEJ factors, while their resection in S/G2 promoted relocalization away from heterochromatin to allow for RAD51 binding and HR. Centromeric chromatin, on the other hand, was accessible to HR and NHEJ factors throughout the cell cycle and did not require DSB movement for their repair (Tsouroula et al., 2016).

Altogether, these findings add significant new insight into the complexity of the forces that drive DSB mobility, and often DSB clustering, across species, and further place this process at a central position in the control of repair outcome and genome maintenance.

On the Move – But Where to? The Nuclear Pore as a Repair Hub

Once movement of a DNA lesion is initiated, a common theme across species is its relocalization to the nuclear periphery, and specifically the nuclear pore complex (NPC). Movement of DSBs to the NPC has been extensively reviewed elsewhere (Freudenreich and Su, 2016; Schrank and Gautier, 2019). In the following, we will focus on new insight describing the nuclear pore as a specialized repair microenvironment for aberrant replication forks.

Various studies in yeast have shown that collapsed replication forks localize to the NPC in a process that is reminiscent of the movement of DSBs described in *Drosophila* (see *Movement and Clustering of DNA Lesions*, (Chiolo et al., 2011; Ryu et al., 2015)) and similarly depends on SUMO E3 ligases (e.g., SLX5/SLX8, SMC5/6) (Nagai et al., 2008; Freudenreich and Su, 2016; Whalen et al., 2020). Relocation of poly-SUMO-modified arrested forks impedes fork repair by HR until anchorage at the NPC allows for SUMO removal by the SENP SUMO protease Ulp1 and the proteasome, which in turn promotes resumption of DNA synthesis by HR via a process known as Recombination-Dependent Replication (RDR) (Kramarz et al., 2020). Regions undergoing RDR-associated DNA synthesis are prone to chromosomal rearrangements (Lambert et al., 2010; Mizuno et al., 2013), providing a rationale for the spatial segregation of arrested forks within nuclear space.

Relocation of arrested replication forks was observed both at replication obstacles within a unique genomic context and at inherently difficult to replicate repetitive loci. However, the underlying mechanisms appear to be distinct. The relocation of forks collapsed at expanded CAG repeats requires nuclease activities to engage SUMO-RPA onto ssDNA, which prevents Rad51 loading (Whalen et al., 2020). Their anchorage to the NPC is required for RPA removal and efficient Rad51 loading, providing a means to constrain recombination at stalled or collapsed forks until it is required for fork restart. In contrast, relocation of a unique fork block to the NPC was found to occur *after* RAD51 loading, which may be tolerated due to the less recombinogenic nature of a non-repetitive DNA (Kramarz et al., 2020). While both relocation events require SLX5/8-mediated SUMOylation, SUMO-RPA accumulation appears to be specific for lesions in repetitive DNA (Whalen et al., 2020). If and how chromatin composition in these distinct genomic contexts determines whether or not RPA is SUMOylated remains to be determined. Altogether, SUMO-based NPC anchorage mechanisms spatially segregate HR events at broken forks at various steps in the repair process, but with the common goal to constrain recombination until it can be safely executed to allow fork restart.

Extending the parallels between the movement of DSBs and stalled or broken forks, recent work by the Cesare lab identified a role for nuclear actin in fork movement to the nuclear periphery (Lamm et al., 2020). Using live and super-resolution imaging, nuclear F-actin was shown to polymerize in response to replication stress in an ATR kinase-dependent manner that further involved WASP and ARP2/3. Much like at heterochromatic DSBs in *Drosophila*, F-actin and myosin promoted the mobility of stressed replication foci. Actin was further required to resolve replication stress and suppress chromosome and mitotic abnormalities. Finally, nuclear F-actin was detected in human tumor xenografts upon replication stress, indicating disease relevance (Lamm et al., 2020; Lamm et al., 2021). Beyond the response to replication stress, actin dynamics were recently shown to facilitate replication initiation in unperturbed cells by promoting the loading of cyclin-dependent kinase (CDK) and proliferating cell nuclear antigen (PCNA) onto chromatin (Parisis et al., 2017). If the latter contributes to replication re-initiation at stalled forks upon their F-actin-dependent relocalization remains to be investigated.

Not surprisingly, difficult-to-replicate ALT telomeric DNA also localized to the nuclear periphery in an actin-polymerization-dependent manner in response to replication stress (Lamm et al., 2020). Similarly, in yeast, subtelomeric DSBs were found to move to the nuclear pore for repair via break-induced replication, a means to repair single-ended DSBs often associated with the ALT pathways (Chung et al., 2015; Dilley et al., 2016; Oshidari et al., 2018). Movement of subtelomeric breaks require kinesin motor proteins and microtubule polymerization, extending the repertoire of motor proteins at stalled forks beyond F-actin (Chung et al., 2015; Oshidari et al., 2018). Of note, unlike stalled replication forks, endonuclease-mediated DSBs at subtelomeric regions in ALT cells were shown to aggregate predominantly in ALT-associated PML nuclear bodies, which may promote clustering and recombination of telomere ends (Cho et al., 2014) (see also *Movement and Clustering of DNA Lesions*). It will be interesting to determine what accounts for the differential targeting of these distinct, ALT telomere-associated DNA lesions.

Altogether, these recent advances highlight the importance of the nuclear pore as a repair-permissive microenvironment that supports the resolution of both DSBs and replication stress. Future work will need to uncover why the NPC presents a preferential "meeting point" for DNA lesions, and why this environment appears to be selectively associated with HR.

3D GENOME ORGANIZATION-RELATED GENOME INSTABILITY IN CANCER AND DISEASE

Having reviewed the importance of 3D nuclear organization in the context of genome maintenance and accurate DNA

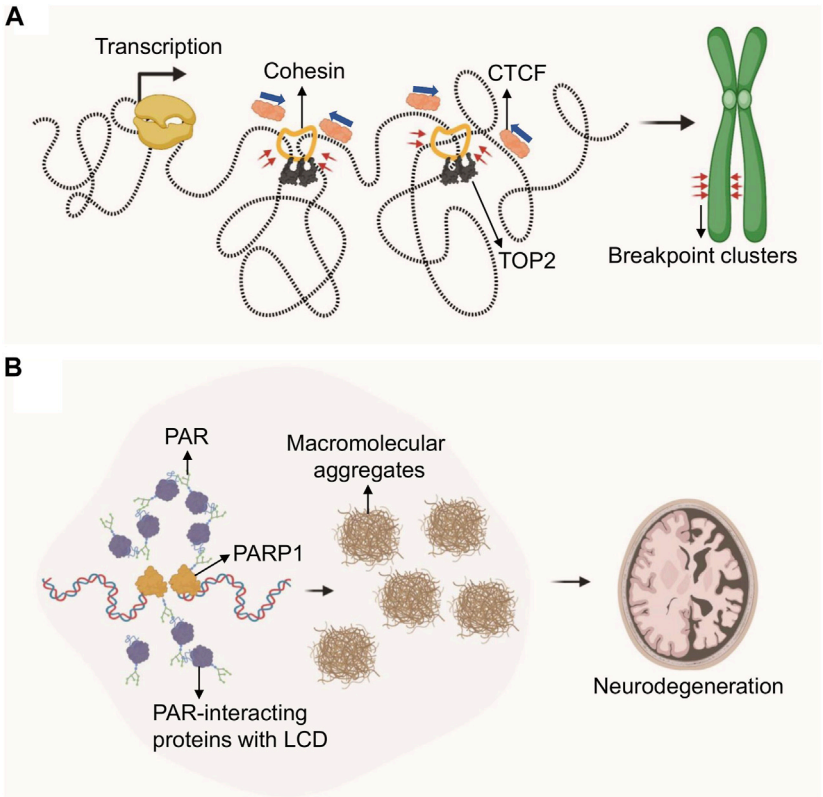

FIGURE 4 | Role of genome architecture in genome instability and disease. **(A)** Loop extrusion by cohesin creates tortional stress on DNA, which is relieved by topoisomerases. TOP2 inhibition via etoposide covalently traps TOP2 at TAD boundaries which generates DSBs in the presence of transcription, ultimately resulting in chromosomal translocations. Etoposide–treatment in cancer is frequently associated with recurrent chromosomal translocations at TAD-boundary-associated breakpoint clusters. **(B)** DNA damage-driven, protein-rich biomolecular condensates are linked to neurodegeneration in A-T or ATLD patients. DDR defects in these patients cause PARP1 hyperactivation and PAR chain accumulation. PAR-dependent protein aggregates are found in A-T patient cerebellum.

repair, it must be noted that the spatial arrangement of chromatin has a direct effect on genome instability, often dictating the outcome of translocations and other aberrant repair events (Hakim et al., 2012; Roukos et al., 2013; Roukos and Misteli, 2014). In the following, we will discuss how nuclear topology can affect mutagenesis and the development of disease.

Topologically Associated Domains, DNA Double-Strand Breaks and Genome Instability

First experimental evidence that TADs, and particularly the anchor regions of these chromosome loops, may pose a threat to genome integrity came from the Nussenzweig lab in 2017 (Canela et al., 2017). Loop anchor regions, as defined by both Hi-C contact maps and ChIP for the anchoring factors CTCF and cohesin (RAD21), were found to be a hot spot for Topoisomerase 2 (TOP2)-mediated DSB breakage. Consistent with this, TOP2B, one of two TOP2 isoforms in mammalian cells, has been shown to physically interact with CTCF and cohesin (Witcher and Emerson, 2009; Uusküla-Reimand et al., 2016) and is enriched in CTCF/cohesin-bound genomic regions (Madabhushi et al., 2015; Uusküla-Reimand et al., 2016). Of note, CTCF/TOP2-associated DSBs at TAD boundaries frequently involve breakpoint clusters that are commonly translocated in cancer, and were shown to drive cell-type- and tumor-specific chromosomal translocations (Canela et al., 2019; Gothe et al., 2019). Thus, loop anchors appear to be genomic fragile sites that can generate DSBs and chromosomal rearrangements. Moreover, these regions are particularly sensitive to treatment with the TOP2 poison and chemotherapeutic agent etoposide, which stabilizes the TOP2 cleavage complex and thus enhances DSB formation. As a result, etoposide treatment is frequently associated with recurrent chromosome translocations involving TAD boundaries in therapy-related myeloid leukemias (t-AML) (Wright and Vaughan, 2014).

While TOP2 chromatin localization and trapping at CTCF/cohesion anchors was shown to be independent of transcription (Canela et al., 2017), the conversion of trapped TOP2 cleavage complexes into DSBs correlates with transcriptional output and directionality (Canela et al., 2019; Gothe et al., 2019). Consistent with the latter, genes that recurrently translocate to drive leukemias are highly transcribed and are enriched at loop

anchors (Gothe et al., 2019). Transcription and 3D chromosome folding thus pose a joint topological threat to genomic stability and are key contributors to the occurrence of genome rearrangements that drive cancer (Canela et al., 2019; Gothe et al., 2019) (**Figure 4A**).

In addition to topoisomerase poisons, mild replication stress was also able to trigger DNA fragility at TAD boundaries, often mapping to difficult to replicate genomic regions known as common fragile sites (CFSs) (Sarni et al., 2020). This effect was particularly pronounced at transcribed large genes that span TAD boundaries and coincided with a delay in replication timing (Sarni et al., 2020). Of note, replication domain boundaries overlap TAD boundaries, suggesting that TADs are regulatory units of replication timing (Pope et al., 2014; Marchal et al., 2019), and providing a rationale for the unique sensitivity of TAD boundaries to replication delays. It will be interesting to determine if this process further involves TOP2-mediated DSB induction.

Phase Separation in Neurodegenerative Disease

While DNA lesion-associated phase separation is emerging as an integral and dynamic aspect of the DNA damage response, recent work by Paull and colleagues suggests that PAR-seeded liquid demixing may ultimately result in insoluble protein-rich biomolecular condensates observed in the cerebellar neurodegenerative disorder associated with ataxia-telangiectasia (A-T) (Lee et al., 2021) (**Figure 4B**). A-T is caused by the loss of ATM kinase, and hypomorphic mutations in the MRE11 repair factor can cause the related A-T-like disorder (ATLD), implicating a prominent role for DNA damage in disease progression (Taylor et al., 2004; Regal et al., 2013). While malignancy and immunodeficiency of A-T and ATLD patients is readily explained by DNA repair defects, the source of neurotoxicity in these patients remains poorly understood (Shiloh, 2020). Genetic ATM separation-of-function mutations previously demonstrated that ATM mutations associated with a loss of its activation by oxidative damage resulted in widespread protein aggregation (Guo et al., 2010; Lee et al., 2018). In seeking to understand the molecular basis for ATM function in protein homeostasis, the Paull lab identified a central role for PARP-dependent nuclear condensates arising from intrinsically disordered proteins associating with PARylated genomic sites (Lee et al., 2021). PARP activation in ATM deficient cells was shown to depend on increased oxidative stress, which in turn caused transcription-associated damage, RNA:DNA hybrid formation and ssDNA lesions. Of note, PARP activation was found to occur independently of oxidative lesions in ATLD patients, suggesting that alternative mechanisms exist to initiate PAR-dependent protein aggregation in ATLD. Of relevance for neurodegenerative disease, PAR-related protein-rich condensates were found to be wide-spread in A-T patient cerebellum (Lee et al., 2021). These findings point to an inherent danger of DNA damage-associated phase separation, particularly in the presence of excessive DNA damage or repair defects that may prevent its dynamic formation and resolution. It will be interesting to determine if other repair-defect- and/or RNA:DNA hybrid-associated neurodegenerative diseases exhibit similar pathology (Moreira et al., 2004; Loomis et al., 2014; Perego et al., 2019).

PERSPECTIVE

Three-dimensional nuclear organization is central to both accurate and aberrant genome maintenance. Continued efforts to map dynamic 3D changes in nuclear space in response to perturbations such as DNA damage, transcription or replication, are critical to advance our understanding of the pathways and factors that control genome maintenance. Recent advances in characterizing the mammalian nucleus in space and time, such as the NIH 4D Nucleome or the ENCODE projects (Consortium, 2004; Dekker et al., 2017), are providing relevant, high-resolution technologies and insight to inform future research in DNA repair. Some of the emerging issues in the field have been indicated throughout this review, but we would like to highlight a few key aspects we consider integral moving forward.

First, how does active movement of DSBs relate to the biophysical separation of repair environments via liquid demixing? Are these events part of the same process, or is there a choice between one and the other, and what would that choice depend on? Can we exploit aggregation and mobility mechanisms to manipulate repair processes, outcome and overall genome maintenance? The latter is supported by intriguing findings that inhibition of actin nucleation can sensitize cancer cells to both PARP inhibition and the DNA polymerase inhibitor aphidicolin (Schrank et al., 2018). Conversely, damage-induced liquid demixing appears to contribute to protein condensates associated with neurodegenerative disorders (Lee et al., 2021), while a potentially beneficial impact of phase separation on DNA repair reactions and concomitant genome maintenance remains to be identified.

Second, the aggregation of multiple DSBs within nuclear space, or at specialized micro-environments such as the NPC, begs the question of why distinct DSBs need to be brought together during repair. At first glance, this seems to be a dangerous proposition, as it may facilitate illegitimate repair events. Indeed, telomere fusions and dicentric chromosome formation are thought to be a result of this process (Lottersberger et al., 2015). What, then, are the benefits of specialized repair micro-environments? Or are DSB clusters merely a natural consequence of a condensation process that evolved to locally increase the concentration of repair factors? And what is the composition of phase separated condensates, HR-associated F-actin-dependent DSB aggregates and DNA lesion-associated NPCs? A better molecular and structural understanding of these specialized repair environments will no doubt help us determine their role in the repair process.

Third, although we may finally understand what leads to often Mb-sized DSB repair foci (Arnould et al., 2021), it remains a mystery as to why DSB marks such as γH2AX and its downstream effector proteins need to cover the extent of DNA they do. Placing these findings in the context of TADs will likely provide additional insight, but more work is needed to understand this most basic feature of DSB repair. Despite

these remaining challenges, however, our understanding of DSB repair has advanced significantly with the consideration of a third nuclear dimension, as well as the complex arrangements of DNA lesions within nuclear space. We look forward to seeing this insight translated into actionable means to manipulate DNA repair to prevent or treat diseases associated with genome maintenance defects.

AUTHOR CONTRIBUTIONS

PO and RS wrote the manuscript with help from MIA. RS designed the figures using BioRender.com with guidance from PO.

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RAD52: Paradigm of Synthetic Lethality and New Developments

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DNA double-strand breaks and inter-strand cross-links are the most harmful types of DNA damage that cause genomic instability that lead to cancer development. The highest fidelity pathway for repairing damaged double-stranded DNA is termed Homologous recombination (HR). Rad52 is one of the key HR proteins in eukaryotes. Although it is critical for most DNA repair and recombination events in yeast, knockouts of mammalian RAD52 lack any discernable phenotypes. As a consequence, mammalian RAD52 has been long overlooked. That is changing now, as recent work has shown RAD52 to be critical for backup DNA repair pathways in HR-deficient cancer cells. Novel findings have shed light on RAD52's biochemical activities. RAD52 promotes DNA pairing (D-loop formation), single-strand DNA and DNA:RNA annealing, and inverse strand exchange. These activities contribute to its multiple roles in DNA damage repair including HR, single-strand annealing, break-induced replication, and RNA-mediated repair of DNA. The contributions of RAD52 that are essential to the viability of HR-deficient cancer cells are currently under investigation. These new findings make RAD52 an attractive target for the development of anti-cancer therapies against BRCA-deficient cancers.

Keywords: Rad52, homologous recombination, single strand annealing, break induced replication, synthetic lethality

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INTRODUCTION

Rad52 was first identified along with a large group of homologous recombination (HR) proteins in a screen for DNA-repair deficient *S. cerevisiae* mutants following ionizing radiation (Game and Mortimer 1974). These proteins (which include Rad52, Rad50, Rad51, Rad54, Rad55, Rad57, Rad59, Rdh54, Mre11, and Xrs2) were collectively called the RAD52 epistasis group genes because of all these genes, *Δrad52* displayed the most severe defect in double-strand break (DSB) repair. Furthermore, RAD52 appeared to be critically important for most, if not all, recombination events in yeast including meiotic recombination, homologous DNA integration, and mating-type switching (Malone et al., 1980; Symington 2002). In contrast, the role of mammalian RAD52 has been largely unexplored due to the lack of a DNA repair or recombination phenotype in RAD52-deficient cells. However, recent discoveries point to multiple novel and intriguing functions of RAD52 in mammalian cells. Recent works have shown that because of RAD52's important role in various aspects of the DNA damage response (DDR), RAD52 mutations can cause synthetic lethality in cells deficient in BRCA1, BRCA2, PALB2, or RAD51C genes. Deficiencies in these genes are responsible for nearly half of hereditary breast and ovarian cancers, and ovarian cancers and for a significant fraction of prostate and pancreatic cancers (Feng et al., 2011; Nogueira et al., 2019; Gottifredi and Wiesmuller 2020). Therefore, Rad52 has potential as a therapeutic target in the treatment of these and some other cancers. Here, we will focus on the recent advancements in

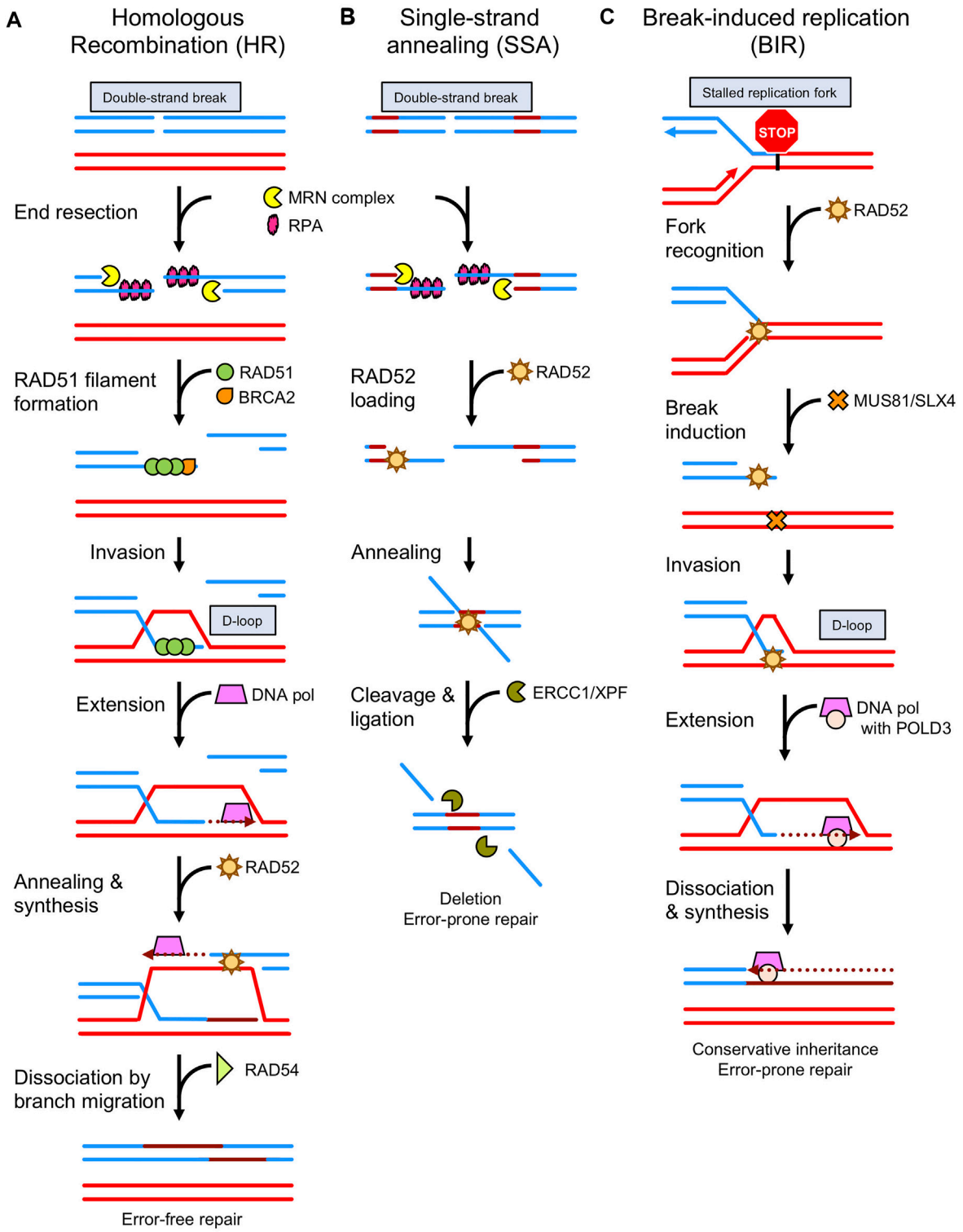

FIGURE 1 | Comparison of RAD51 and RAD52-mediated DNA repair pathways. **(A)** During homologous recombination (HR), the ends of the double strand break (DSB) are resected by nucleases (ex. MRN complex) [see (Zhao et al., 2020) for mechanism of action], exposing single-strand DNA (ssDNA) that becomes bound by RPA. Then the mediator protein, BRCA2 initiates loading of RAD51 on ssDNA helping to displace RPA. RAD51 oligomerizes, forming a nucleoprotein filament, and then searches for the homologous DNA sequence on the intact chromosome. The RAD51 filament invades the intact dsDNA to form a D-loop structure. Further processing by DNA polymerases, chromatin remodelers (ex. RAD54), nucleases, and ligases restore the intact DNA sequence through error-free repair. **(B)** Alternative to HR, single strand annealing (SSA) begins after resection with the binding of RAD52 to ssDNA. RAD52 promotes the annealing of exposed homologous ssDNA regions

(*Continued*)

FIGURE 1 | on either side of the DSB. Processing of the annealed DNA by nucleases (ex. ERCC1/XPF) results in error-prone repair as the sequences between homologous regions are lost. **(C)** RAD52 also recognizes and repairs stalled replication forks *via* break-induced replication (BIR). The structure is cleaved by the endonuclease complex MUS81 and processed by EEPD1 (Kim et al., 2017; Sharma et al., 2020). Bound to the one-ended DNA break, RAD52 invades the dsDNA to form a D-loop. The DNA polymerase contains a non-enzymatic subunit, POLD3, that appears to be specific to this type of repair.

understanding RAD52's role in various DNA repair pathways, and on the work that is underway to develop RAD52 inhibitors that can serve as cancer therapeutics.

Overview of RAD52 Functions in DNA Repair

Genomic DNA is under constant attack by endogenous metabolic byproducts, exogenous chemicals, and environmental stress such as ultraviolet radiation. In response, cells have developed numerous DNA protective and repair mechanisms to maintain genome stability. Mis-repaired DNA damage can be mutagenic and lead to cancer (Hoeijmakers 2009). One of the most harmful types of DNA damage is the DSB, and the most accurate way to repair DSBs is through the HR pathway. The salient step of HR is performed by the recombinase protein RAD51 in conjugation with auxiliary proteins. Following DNA replication and the formation of a sister chromatid, RAD51 will bind the resected, single-stranded DNA (ssDNA) end of a DSB, form a nucleoprotein filament, and search for the homologous DNA sequence on the intact sister chromatid (**Figure 1A**). To gain access to the resected end, RAD51 must compete with the ssDNA-binding protein replication protein A (RPA) that is ubiquitous in all eukaryotes. To successfully compete with RPA's high affinity for ssDNA, RAD51 requires a mediator protein (Sung 1997; Kowalczykowski 2015). The major mediator in budding yeast is Rad52, which promotes the displacement of RPA by Rad51 (Sung and Klein 2006). In mammals, that major RAD51 mediator role is filled by BRCA2 (Esashi et al., 2007; Zelensky et al., 2014; Scully et al., 2019). Nonetheless, mammalian RAD52 retains an ability to physically interact with RAD51 and RPA, but the role of these interactions is a matter of investigation.

The RAD51 filament searches for a homologous DNA on the sister chromatid and performs strand exchange to produce a joint molecule also known as a D-loop. From the D-loop, DNA polymerase then uses the homologous DNA strand as a template and the 3′-end of the broken DNA strand as a primer to commence DNA repair synthesis. The second end of the DSB is captured by RAD52 and annealed to the displaced strand of the D-loop to provide the template for the second strand synthesis. Upon completion of DNA synthesis, the D-loops can be dissociated by RAD54, an ATP-dependent motor protein that interacts with RAD51 and promotes branch migration, or by helicases like BLM (van Brabant et al., 2000; Bugreev et al., 2006; Bugreev et al., 2007). DNA is extended by DNA polymerase and then annealed to the ssDNA portion of the second broken DNA end; followed by gap filling, flap removal, and nick sealing by DNA polymerases. Flap nucleases and ligases then restore the original DNA sequence (Kawale and Sung 2020).

From an accuracy standpoint, it is preferable to repair all DNA damage through HR. However, a preference for sister chromatid limits most HR activity to the late S/G_2 phase of the cell cycle. Additionally, the HR mechanism is time consuming. Even when operating at full capacity, RAD51-dependent HR can only handle ~5 DSBs in a cell at once (Mladenov et al., 2020). In order to repair the ~50 DSBs a normal cell suffers through one cell cycle (Hoeijmakers 2009), the cell relies on another pathway termed classical non-homologous DNA end joining (c-NHEJ) (Vilenchik and Knudson 2003). Here, the Ku70/80 heterodimer, DNA-PKcs and DNA Ligase IV with several auxiliary proteins promote re-joining of DNA ends by ligation (Chang et al., 2017; Ghosh and Raghavan 2021). c-NHEJ is rapid and efficient; requires no homologous sequences and repairs the break with minimal loss of DNA sequence. Currently, there is no known role of RAD52 in c-NHEJ.

At times of high DSB stress (such as during DNA replication), repair of DSBs may also be processed through the alternative DNA end joining (a-EJ) and single-strand annealing (SSA) pathways. In a-EJ, the MRN-CtIP nuclease complex generates short (<20 bps) resected ends at the DSB (Bhargava et al., 2016; Chang et al., 2017). Poly-ADP ribose polymerase-1 (PARP1) and DNA polymerase θ anneal microhomologies (~10 bp) between DNA ends, followed by XRCC1- and DNA ligase III-mediated end processing (Srinivasan et al., 2019) that generate an intact DNA molecule. a-EJ was reported to be partially dependent on RAD52, likely through RAD52's annealing activity (Kan et al., 2017; Hendrickson 2020), which prevents premature usage of a-EJ until the cell enters mitosis (Llorens-Agost et al., 2021). RAD52 also inhibits PARP-mediated single-strand break repair by interfering with colocalization of XRCC1 and DNA ligase III (Wang et al., 2021).

RAD52 plays a major role during SSA. Similar to the main HR mechanism, DSB ends in SSA are resected by helicases (BLM and WRN) and nucleases (DNA2, CtIP, and EXO1) to generate long segments of ssDNA (Ceccaldi et al., 2016). Then RAD52 protein binds to the resected DNA ends (Hanamshet et al., 2016) and promotes the annealing of ssDNA regions of homology (>30 bps) (**Figure 1B**). Following annealing, the ERCC1-XPF complex binds the N-terminal domain of RAD52 to attenuate the SSA activity of RAD52, while enhancing its own endonuclease activity (Motycka et al., 2004). The RAD52-ERCC1-XPF complex localizes to the repair-intermediate and cleaves the 3' ssDNA tails that resulted from RAD52 annealing the homologous sequences together. Gaps are filled by unidentified polymerases and the DNA ends are joined by DNA ligase I (Bhargava et al., 2016). During processing in a-EJ and SSA, one of the two original homologous regions, along with the intervening DNA, are deleted. Thus, in contrast to HR, these alternative pathways are error-prone/mutagenic. It

was shown that SSA could cause interchromosomal translocations between two DSBs occurring simultaneous between two different sets of repeat elements. In this case, SSA resulted in the loss of one repeat on each chromosome (Elliott et al., 2005).

New focus was directed toward the a-EJ and SSA repair pathways once it was shown that PARP inhibitors were effective in treating BRCA-deficient cancers. BRCA-deficient cancer cells are defective in HR. As a consequence, they become dependent on other DNA repair pathways for their survival. PARP1 inhibitors are a clinically approved treatment for certain types of BRCA-deficient cancers (Myers et al., 2020). Recent works have shown that because of its important role in various aspects of the DDR, RAD52 also has potential as a therapeutic target in the treatment of hereditary breast, ovarian, and some other cancers (Feng et al., 2011; Nogueira et al., 2019; Gottifredi and Wiesmuller 2020).

The Biochemical Activities of RAD52

Human RAD52 is a 418 amino acid (46 kDa) protein with two domains. The N-terminal domain contains two DNA binding domains and is highly conserved among eukaryotes (42% identity between *H. sapiens* and S. *cerevisiae* homologs) (Hanamshet et al., 2016). The crystal structure of this highly stable domain showed that the RAD52 N-terminal domain oligomerizes to form an undecameric ring structure (Kagawa et al., 2002; Singleton et al., 2002). The base of this ring forms a large, positively charged channel that accommodates ~40 nt of ssDNA per ring. RAD52 promotes ssDNA annealing (Mortensen et al., 1996; Kagawa et al., 2001; Khade and Sugiyama 2016; Saotome et al., 2018). RAD52-mediated ssDNA annealing persists in the presence of RPA (Sugiyama et al., 1998), and is essential to RAD52's ability to perform SSA repair. A secondary DNA binding site runs parallel to the primary ssDNA binding site at the outer portion of the ring structure. This site accommodates double-stranded DNA (dsDNA) or ssDNA, plays a role during ssDNA annealing, and allows RAD52 to perform DNA strand exchange (Kagawa et al., 2008). Like RAD51, RAD52 can promote the formation of a D-loop between ssDNA and plasmid DNA (Kagawa et al., 2001). Through its two DNA binding sites, RAD52 binds the one-ended DSB and performs strand exchange to produce a D-loop structure in a mechanism termed break-induced replication (BIR) (**Figure 1C**) (Kagawa et al., 2001; Llorente et al., 2008). This activity is abrogated when either DNA binding site is inactivated through mutation (Hanamshet and Mazin 2020). The break is then repaired by POLD3-dependent DNA synthesis (Lemacon et al., 2017). Unlike RAD51, RAD52 does not form long filamentous structures on ssDNA and does not hydrolyze ATP; instead RAD52 forms large co-aggregated stacked ring structures through its C-terminal domain (Ranatunga et al., 2001) that facilitate ssDNA annealing (Kagawa et al., 2008; Saotome et al., 2018).

The C-terminal domain of RAD52 also contains regions that bind to RPA (Shinohara et al., 1998) and RAD51 (Shen et al., 1996). Although human RAD52 binds directly to RPA, this interaction is not essential for the major functions of RAD52 in DNA repair, as the RAD52 N-terminal domain alone was sufficient to maintain viability of BRCA-deficient cells (Hanamshet and Mazin 2020). In yeast Rad52, the binding to RPA is involved in the mediator function of Rad52. Yeast Rad52 binds both RPA and Rad51, which results in the displacement of RPA from resected ssDNA ends and the promotion of Rad51 nucleoprotein filament formation (Sung 1997; New et al., 1998; Shinohara et al., 1998; Gibb et al., 2014; Ma et al., 2017). The role of human RAD52 interactions with RAD51 and RPA remains to be fully understood. We showed that RPA-RAD52 interaction is required for stimulation of RAD52's inverse RNA strand exchange activity by RPA (Mazina et al., 2017).

RAD52 During DDR Pathway Choice

Understanding the rules governing the competition and cooperation between c-NHEJ, HR, SSA, and a-EJ to repair DSBs remains an open research topic. Extensively resected DNA ends act as a signal to promote RAD51-directed repair and suppress c-NHEJ. By default, p53 binding protein 1 (53BP1) suppresses the end resection activity of the MRE11-RAD50-NBS1 complex (MRN) to limit HR during G_1 phase. But once the cell enters S phase, ataxia-telangiectasia mutated (ATM) kinase is recruited to the damage site through an interaction with MRN and activated through autophosphorylation at Ser 1981 (Shiloh and Ziv 2013). ATM then phosphorylates other target proteins such as histone H2AX on Ser139 (γ-H2AX). This phosphorylation event stimulates the recruitment of BRCA1 (Delia and Mizutani 2017). BRCA1 interacts with MRN and CtIP to promote extensive end resection by the exonucleolytic complex EXO1-DNA2 and expose 3' ssDNA ends (Reginato and Cejka 2020).

Following resection, mediator proteins including RAD52, PARP1, and BRCA2 compete with each other and the DNA-damage sensing proteins previously recruited to the site of damage. This competition is partially modulated through cellular signals transduced through posttranslational modifications. The histone acetyltransferase p300/CBP plays a role in regulation of DNA transcription, replication, and repair (Dutto et al., 2018). For instance, it acetylates histones to relax chromatin and increase DNA accessibility to other proteins. RAD52 is also acetylated by p300/CBP at DSB sites, and deacetylated by SIRT2/SIRT3 (Yasuda et al., 2018). The acetylated form of RAD52 persisted at sites of DNA damage longer compared to an acetylation-deficient RAD52 mutant containing ten arginine substitutions. This RAD52 mutant also decreased the ability of RAD51 foci to be retained at DSB sites. A RAD52 acetylation-mimic mutant containing ten glutamines had a higher affinity for RAD51 and RPA in a yeast two-hybrid system. It was speculated that competition between RAD52 and BRCA2 allowed RAD51 nucleoprotein filament expansion following initiation by BRCA2 (Yasuda et al., 2018). In this scenario, RAD52 acetylation would act as a signal to promote homology-directed repair pathways.

DSS1 (Sem1 in yeast) is a small, highly acidic protein that binds BRCA2 and stimulates RAD51 filament formation (Liu et al., 2010). More recently, it was also discovered to bind RAD52 and stimulate its ssDNA annealing and D-loop formation activities (Stefanovie et al., 2020). DSS1 does not appear to

bind DNA on its own; instead it enhances the ssDNA binding activities of BRCA2 and RAD52 to facilitate the initial steps of DSB repair (Zhao et al., 2015).

RAD52 activities are also modulated by several phosphorylation events. Cyclin-dependent kinase 1 (CDK1) regulates the transition through the cell cycle by associating with phase-specific cyclins. In yeast, the homolog of CDK1, Cdc28, coupled with Clb2 or Clb3 cyclins phosphorylates Rad51 at S125 and S375 to increase binding affinity for ssDNA; and Rad52 at Thr412 to promote RAD52 oligomerization (Lim et al., 2020). These residues are conserved from yeast to human (Hanamshet et al., 2016; Kelso et al., 2017), but it remains to be seen if these modifications occur in higher eukaryotes. In humans, RAD52 is phosphorylated at Y104 by the ATM-activated c-ABL kinase. This modification enhances RAD52's ssDNA annealing activity by inhibiting DNA binding at the secondary site (Honda et al., 2011).

The mechanism through which signaling specifies a repair pathway is not understood, but one determining factor appears to be the level of DNA damage. During G_2 phase, RAD51-dependent HR can only function efficiently at the low DSB load that is typical under normal cell growth. RAD51 binding to chromatin saturates at ~20% total RAD51 even at high levels of ionizing radiation (Mladenov et al., 2020). It was shown *in vivo* that efficient RAD51 foci formation at DSBs depended on the prerequisite binding of 53BP1 (Ochs et al., 2016). Exhaustion of 53BP1 (as occurs at high DSBs) limited the RAD51's ability to form stable foci. As a consequence, HR does not significantly contribute to repair when the cell is overloaded with DSBs. In cells experiencing high DSB (~50 simultaneous DSBs), HR handles about 5 repairs at once (10%). While RAD51-dependent repair is suppressed at high DSB, end resection is not. To back up the saturated RAD51 HR pathway, the RAD52-dependent SSA pathway becomes activated. This activation can be achieved through competition between 53BP1 and the E3 ubiquitin ligase protein RNF169. Overexpression of RNF169 or knockout of 53BP1 or BRCA2 in reporter cell lines results in hyperactive SSA repair (Tutt et al., 2001; An et al., 2018). The SSA pathway is activated at IR doses up to 4-fold higher than the saturation level for HR. Above that point RAD52 is also suppressed, leaving only c-NHEJ to repair DSBs (Mladenov et al., 2020).

RAD52 in Protection and Processing of Stalled Replication Forks

During DNA replication, the replisome encounters many roadblocks. The cell has developed several complimentary and competing pathways to recover from the DNA lesions that stall replication forks (Kondratick et al., 2021). Restart of stalled replication forks is complex and fraught with pitfalls that contribute to genomic instability and disease progression in humans (Neelsen and Lopes 2015). A wide range of proteins are recruited to stalled replication forks including ssDNA binding proteins and recombinases (RPA, BRCA2, RAD51, RAD52, RADX), translocases (SMARCAL1, ZRANB3, HLTF, SHPRH, WRN, RECQ1, ATAD5), and endo/exonucleases (MRE11, EXO1, DNA2, MUS81) (Kondratick et al., 2021; Nickoloff et al., 2021). An active area of research is aimed at understanding the interplay between these factors. Depending on the type of block, stalled replication forks can be repaired through several mechanisms (**Figure 2A**). Damaged DNA bases are bypassed *via* translesion synthesis, in which specialized DNA polymerases are recruited to the fork by the ubiquitylation of PCNA. These polymerases have low fidelity for base pairing, allowing them to bypass DNA lesions at the expense of potential mutagenesis. In yeast, Rad52 recruits the E2/E3 ligases Rad6/Rad18 to ubiquitylate PCNA (Cano-Linares et al., 2021).

DNA replication stress often leads to the uncoupling of leading and lagging strand synthesis and the accumulation of ssDNA gaps (Zellweger et al., 2015). These types of stalled replication forks are repaired by BIR, in which the ATAD5-RLC removes PCNA and recruits RAD51 (Park et al., 2019). RAD51 filament protects the fork through a mechanism that does not require its ATPase activity (Mason et al., 2019), and presumably recruits translocases, such as RAD54 (Bugreev et al., 2011), SMARCAL1, and/or ZRANB3 (Kondratick et al., 2021) that reverse replication forks and create a "chicken-foot" structure that is cleaved by MUS81 to generate a one-ended DSB.

First described in recombinant dependent replication of bacteriophage T4 (Luder and Mosig 1982), and later in yeast (Morrow et al., 1997), BIR's role in mammalian systems is only now beginning to be appreciated (Costantino et al., 2014). The molecular mechanism of BIR has been extensively studied in yeast systems (Malkova and Ira 2013). At the one-ended DSB, the end is resected and Rad52 initiates formation of a Rad51 nucleoprotein filament on ssDNA. It invades the homologous region of the intact sister strand to form a D-loop. Then a replisome assembles containing a non-essential subunit of DNA polymerase δ called Pol32 (Lydeard et al., 2007). Unique to BIR, the D-loop then moves with the replication fork during leading strand synthesis (Smith et al., 2007). Lagging strand synthesis is delayed until the sister chromatin separates, resulting in conservative DNA replication (as opposed to traditional semi-conservative) (Wilson et al., 2013). The recently discovered RADX protein binds ssDNA and directly interacts with the RAD51 to destabilize the nucleofilament and ensure that the resumed DNA replication proceeds at the proper rate (Adolph et al., 2021).

When the DNA damage load overwhelms RAD51's capabilities, then collapsed replication forks are restarted by the RAD52-dependent BIR pathway. This pathway has been studied in BRCA2-deficient cells where the RAD51 pathway is no longer viable. In this environment, fork reversal is deregulated and leads to excessive degradation by MRE11 (Mijic et al., 2017; Taglialatela et al., 2017). The exonuclease activities of MRE11 and EXO1 generate extensive ssDNA that increases chromosome breaks and genome instability. These partially resected forks are cleaved by MUS81 to create one-ended DSBs. In CHK1-deficient cells where the G_2/M cell cycle checkpoint is lost, cell survival is dependent on RAD52 and MUS81 to relieve replication stress by creating DSBs as the cell tries to complete the cell cycle (Murfuni et al., 2013).

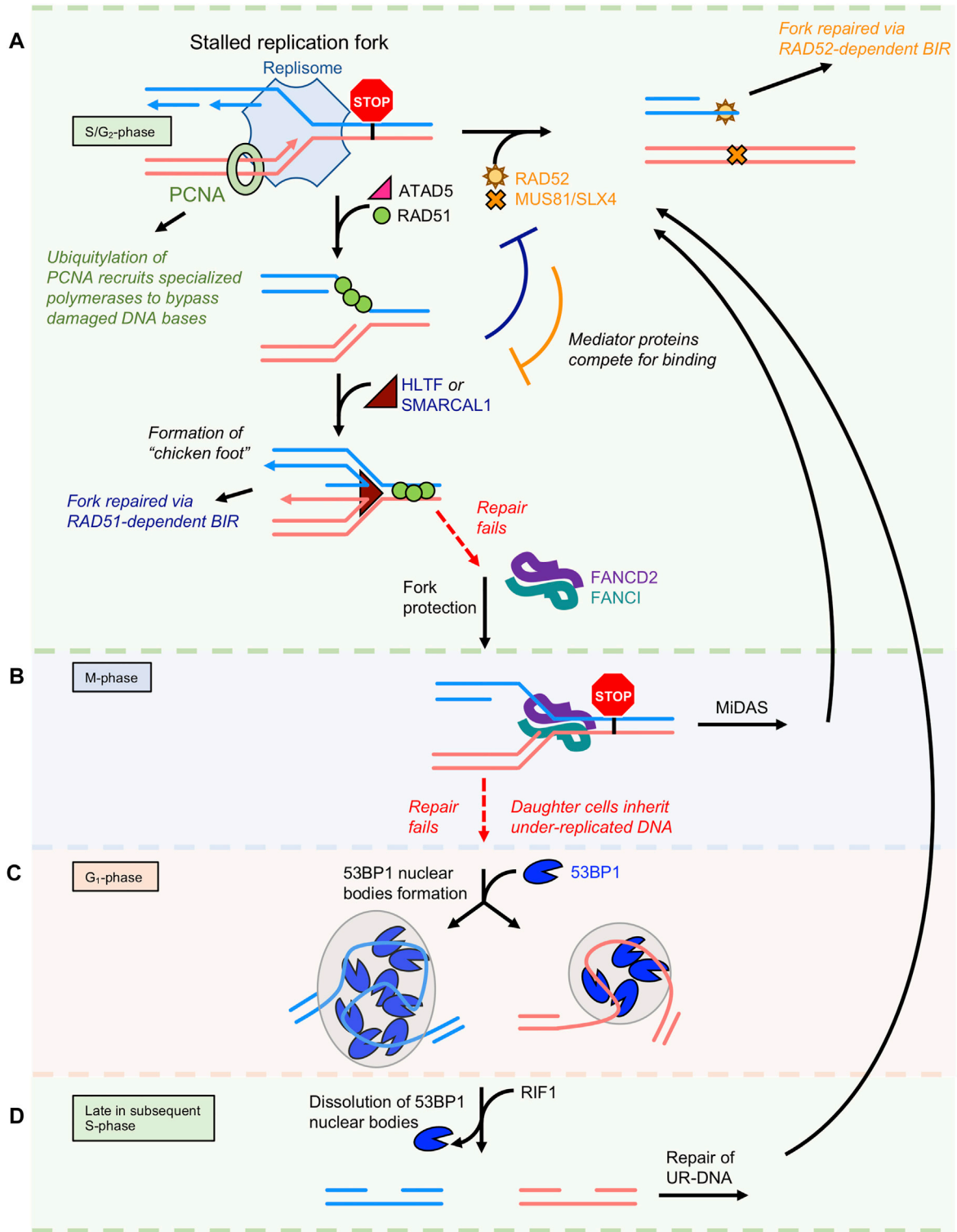

FIGURE 2 | Repair of stalled replication forks *via* BIR. **(A)** Multiple DNA repair pathways compete to repair stalled replication forks during S/G_2 phase of the cell cycle. **(B)** Once the cell enters M-phase, unrepaired forks become bound by the FANCD2/FANCI complex. It will attempt to repair the lesion again by a RAD52-dependent BIR-like pathway termed mitotic DNA synthesis (MiDAS). **(C)** If still unsuccessful, the cell with complete mitosis with each daughter cell inheriting under-replicated ssDNA that is protected by the 53BP1 protein during G_1. **(D)** In the subsequent S-phase, the cell has one final attempt to repair under-replicated DNA *via* BIR. After this point, the cells must undergo apoptosis or pass on an incomplete genome.

In Rad52-dependent BIR (Malkova et al., 1996), ssDNA annealing by Rad52 and Rad59 are responsible for pairing homologous sequences. It is also possible the DNA pairing (D-loop formation) activity of Rad52 plays a role in BIR initiation. In yeast, Rad59 removes the inhibitory effect of Rad51 on Rad52's ability to anneal ssDNA and promote single-strand template repair (Gallagher et al., 2020). Rad52-dependent BIR also requires the translocase protein Rdh54 and the exonuclease/resolvase complex MRX (Signon et al., 2001) to complete the process. Rad52-mediated BIR in yeast is highly mutagenic due to high level of template switching during replicative repair (Kockler et al., 2021). Recent studies suggest that RAD52-driven BIR may promote genome instability in human cancers. The Halazonetis group used the overexpression of oncogenic cyclin E in U2OS cells to induce DNA replication stress and identify POLD3 or POLD4 (homologs of yeast Pol32), MUS81 and SLX4 (endonuclease complex), and RAD52 as required for BIR (Costantino et al., 2014; Sotiriou et al., 2016).

There exist difficult-to-replicate regions of the genome termed common fragile sites. They tend to be at AT-rich sequences in long coding regions where transcribing RNA polymerases inevitably collide with replicating DNA polymerases (Helmrich et al., 2011). An under-replicated DNA (one copy instead of two) event probabilistically occurs at least once a cell cycle (Al Mamun et al., 2016). At colliding polymerases, the forks stall and become bound by the FANCD2/FANCI complex that tether sister chromatids to each other (**Figure 2B**). The cell attempts to repair these DNA lesions *via* mitotic DNA synthesis (MiDAS). The mechanism of MiDAS appears equivalent to BIR, as it produces conservative DNA replication and requires MUS81-EME1, SLX4, POLD3, and RAD52 (Al Mamun et al., 2016).

When MiDAS fails to repair the damage before cell division, then the daughter cells inherit under-replicated DNA marked as lesions sequestered during G_1 phase by 53BP1 nuclear bodies (Lukas et al., 2011) (**Figure 2C**). Late in the subsequent S-phase, 53BP1 nuclear bodies dissolve by the RIF1-mediated activation of late replication origins. This triggers recruitment of RAD52 and gives the cell a second chance to repair the damage (Spies et al., 2019) through a BIR-equivalent pathway (**Figure 2D**). Many questions remain regarding the signaling and molecular mechanisms that govern the repair of known fragile sites (Bertolin et al., 2020). If these events are as common as the literature suggests, how can their repair rely on error-prone RAD52-dependent BIR mechanisms? How would these genes survive multiple generations if they are prone to break, and repair results in DNA sequence loss?

RAD52 in RNA-Dependent DNA Repair

HR is known to use homologous DNA sequences as a template to carry out high-fidelity repair of DSB and other lethal lesions. However, recent data shows that HR can also use a homologous RNA transcript to repair DSB damage (Keskin et al., 2014; Mazina et al., 2017; Michelini et al., 2018). This defies the central dogma, in which genetic information flows from DNA to RNA. Strong support for the use of an RNA template in HR came from experiments in *Saccharomyces cerevisiae*. Keskin et al. developed a DSB-inducible system to monitor repair by a homologous RNA transcript (Keskin et al., 2014). They showed that RNA can be directly used as a template for DSB repair in the absence of reverse transcriptases. Further, the efficiency of this process increased dramatically in the absence of RNase H. It was proposed that upon DSB formation at an actively transcribed locus, the homologous RNA transcript forms a DNA:RNA heteroduplex intermediate that bridges the two DNA ends together and serves as a template for gap filling synthesis (Keskin et al., 2014; Mazina et al., 2017; Michelini et al., 2018) (**Figure 3A**).

In addition to this bridging-template mechanism, RNA transcripts were also implicated in DNA replication-restart. RNA is known to form R-loops with homologous DNA, the three-stranded structures consisting of an RNA-DNA hybrid and the displaced ssDNA strand. Thus, up to 5% of human and 8% of yeast genome is susceptible to DNA:RNA hybrid or R-loop formation (Chedin 2016; Wahba et al., 2016). It was proposed by Kogoma that R-loops may prime a restart of DNA replication forks stalled at damaged DNA in *E. coli* (Kogoma 1997) (**Figure 3B**). While DNA repair by canonical HR requires sister chromatids as a source of homologous DNA template sequences and therefore is limited to S/G_2 phase, RNA-dependent DNA repair may occur in non-dividing cells, like terminally differentiated neurons (Welty et al., 2018).

Rad52 was implicated in RNA-dependent DSB repair by genetic data from *S. cerevisiae* (Keskin et al., 2014; Mazina et al., 2017). Rad52 knockouts in yeast reduced the level of RNA-dependent DNA repair. The role of RAD52 in RNA-dependent DSB repair is also supported by data from human cells (Wei et al., 2015; Yasuhara et al., 2018). Currently, the function of RAD52 in RNA-dependent DSB repair is under intense investigation. We recently reported an unconventional type of strand exchange, known as inverse strand exchange, that yeast and human Rad52 promote between RNA and homologous dsDNA (Mazina et al., 2017) (**Figure 3A**). This activity is different from the conventional (forward) strand exchange activity of major recombinases of the RAD51 family. In case of RAD51, the active species in DNA strand exchange is a nucleoprotein filament that RAD51 forms with ssDNA. The filament binds dsDNA to promote the search for homology and strand exchange. In contrast, RAD52 forms the active nucleoprotein complex with dsDNA which promotes strand exchange with free RNA or ssDNA. The bacterial DNA repair protein, RecA, was first discovered to have this type of DNA strand exchange (Zaitsev and Kowalczykowski 2000). In eukaryotes, this activity is unique to Rad52, neither the major recombinase Rad51 nor the yeast Rad52 paralog Rad59 perform inverse RNA strand exchange. These biochemical results are consistent with genetic data in *S. cerevisiae*, which show that RNA-templated DSB repair is dependent on Rad52 but not on Rad1, Rad9, or on end resection factors Sae2, Exo1, and Mre11 (Mazina et al., 2017; Meers et al., 2020). Moreover, the RAD52 R55A mutant defective in inverse RNA strand exchange fails to promote RNA-dependent DNA repair in budding yeast. Recently, it was found that RNA-templated DNA repair occurs in yeast cells through two mechanisms: DSB-dependent

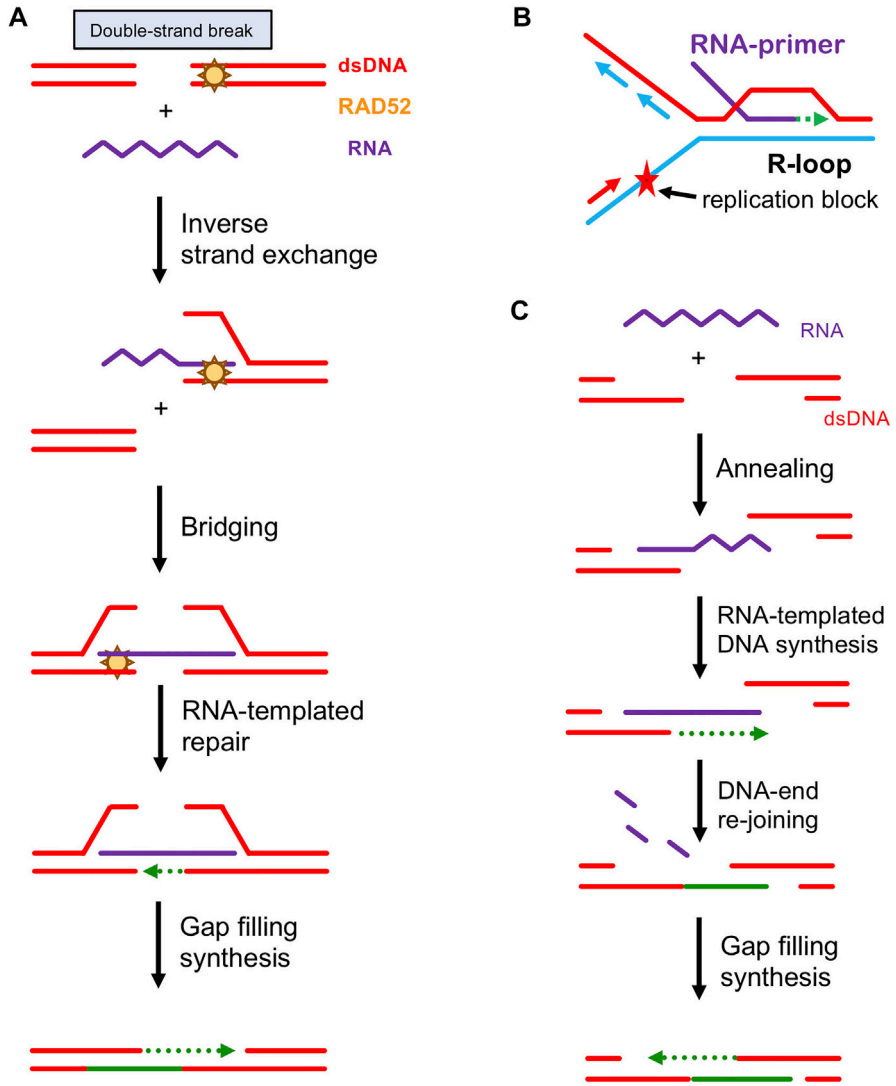

FIGURE 3 | Proposed Mechanisms of RNA-Dependent DSB Repair. **(A)** Repair of DSBs *via* inverse RNA strand exchange. Rad52 forms a complex with DSB ends either blunt ended or minimally processed by exonucleases/helicases. Then, RAD52 promotes inverse RNA strand exchange with a homologous RNA transcript. The RNA transcript in the resultant DNA:RNA hybrid provides a template for DNA repair synthesis. The single-stranded tails are removed by flap nucleases, the gaps are filled in, and any remaining nicks are sealed by DNA ligases, restoring the original DNA sequence in an error-free manner. **(B)** Restart of DNA synthesis stalled at DNA damaged site primed by an R-loop. **(C)** A tentative role of RAD52 annealing activity in DSB repair. RAD52 promotes annealing between the ssDNA ends of an exonucleolytically processed DSB and homologous RNA transcript. The RNA transcript provides a template for DNA repair synthesis that extends the ssDNA end ensuring an overlap with the ssDNA of another DSB end. This is followed by re-joining of the DSB ends *via* ssDNA annealing, removal of DNA:RNA heteroduplex by RNase H, filling the gaps by DNA polymerases and sealing the nicks by DNA ligases.

and DSB-independent (Meers et al., 2020). Only the DSB-dependent mechanism requires RAD52, which is consistent with the RNA inverse strand exchange activity of RAD52 that occurs in the proximity of DNA ends. Overall, genetic data in *S. cerevisiae* support the biological role of inverse RNA strand exchange *in vivo*.

In addition to inverse RNA and DNA strand exchange, Rad52 is known to promote annealing between complementary ssDNA molecules (Mortensen et al., 1996). More recently it was found that RAD52 can also promote annealing between ssDNA and complementary RNA (Keskin et al., 2014; McDevitt et al., 2018). It was suggested that this RNA/DNA annealing activity may also contribute to DSB repair by bridging the exonucleolytically processed DNA ends (**Figure 3C**).

RNA transcripts can be transcribed by reverse transcriptases encoded by retrotransposons or retroviruses. Genetic data in *S. cerevisiae* show that the resultant cDNA may be used efficiently for DSB repair *via* the conventional RAD51-dependent HR mechanisms (Keskin et al., 2014). In the absence of reverse transcriptases, short DNA synthesis on RNA templates can be carried out by DNA polymerases, which have limited reverse transcriptase activity. It was shown that several polymerases

including yeast replicative polymerases (δ and α) possess minimal reverse transcriptase activity *in vitro* (Storici et al., 2007). Human Pol η and Pol θ are capable of utilizing an RNA template (Su et al., 2019; Chandramouly et al., 2021). Recently, it was shown that yeast Pol ζ is required for RNA-dependent DNA repair (Meers et al., 2020). In yeast, it was proposed that as DNA Pol δ encounters a DSB at an actively transcribed locus, Rad52 generates an DNA:RNA heteroduplex (R-loop) at the proximity of the DSB. Then, polymerase switching occurs and the RNA in this heteroduplex is used as a template for repair by Pol ζ (Meers et al., 2020).

Several recent reports linked the function of RAD52 in human cells to a specific type of HR occurring within transcriptionally active genome regions. This type of HR was named transcription-coupled homologous recombination (TC-HR) (Welty et al., 2018) or transcription-associated homologous recombination repair (TA-HRR) (Yasuhara et al., 2018). It was found that several HR proteins including RAD52, RAD51, RAD51C and RPA form a larger number of nuclear foci in response to DNA damage in active transcription regions (Wei et al., 2015). In contrast, several other HR proteins like NBS1, BRCA1, and BRCA2; or NHEJ proteins Ku70 and DNA ligase IV did not show such preference for foci formation in active transcription regions. Unlike canonical HR that occurs in S/G_2 cell cycle phase, TC-HR can also operate in G_0/G_1 phase (Welty et al., 2018).

It was found that RAD52 recruitment to DNA damage sites occurs in a DNA:RNA hybrid-dependent manner during TC-HR (Wei et al., 2015; Yasuhara et al., 2018). Inhibition of transcription at the site of DNA damage or overexpression of RNase H reduced RAD52 recruitment. Also, it was suggested that RAD52 can be recruited through direct binding to DNA:RNA hybrids or R-loops (Yasuhara et al., 2018). While RAD52 can indeed bind to these structures, its preferential substrate is ssDNA, not DNA:RNA hybrids (Mazina et al., 2017; Welty et al., 2018). On the other hand, the preferential binding of RAD52 to the ssDNA strand displaced in R-loops, does not seem strong enough to support that as a mechanism of RAD52 recruitment. Recently, it was shown that RAD52 displays an increased affinity for DNA: RNA hybrids containing m5C-modified RNA *in vitro*; m5C(s) are generated in mRNA by the RNA methyltransferase TRDMT1 that is recruited to DNA damage sites (Chen et al., 2020). Additional quantitative characterization of this binding may further clarify the role of m5C RNA modification in RAD52 recruitment to DNA damage sites.

It is also possible that intermediate factors are involved in RAD52 recruitment to DNA:RNA hybrids. It was reported that RAD52 recruitment requires Cockayne syndrome B protein (CSB), a key protein of transcription-coupled nucleotide-excision repair (Wei et al., 2015; Teng et al., 2018). These authors suggest that CSB recognizes DNA:RNA hybrids and then recruits RAD52 and RAD51C to DNA damage sites. However, the universality of this mechanism requires further investigation; reactive oxygen species used in this study as a source of DNA damage are known to generate multiple types of DNA damage including those that are specifically repaired by nucleotide excision repair (NER), which may not be common for other types of DNA damaging agents. Indeed, a CSB-independent mechanism of RAD52 recruitment has been reported (Tan et al., 2020). RAD52 is known to physically interact with other proteins involved in DNA repair, including RPA that stimulates the inverse RNA strand exchange activity of RAD52 (Mazina et al., 2017). RPA is a ubiquitous ssDNA binding protein, that was also found to bind ssRNA and to promote R-loop formation *in vitro* (Mazina et al., 2020). *In vivo*, RPA association with R-loops is well documented (Wei et al., 2015; Nguyen et al., 2017). It is possible that RPA is involved in RAD52 recruitment to DNA: RNA hybrids. Overall, the mechanism of RAD52 recruitment to transcriptionally active sites remains to be fully understood.

Upon its recruitment, RAD52 plays a pivotal role in the initiation of RNA-dependent DNA repair. RAD52 knockout in immortalized RPE-hTERT cells significantly reduced RPA and RAD51 foci formation after ionizing radiation and the rate of sister chromatid exchanges (Yasuhara et al., 2018). Importantly, recruitment of RAD51 to the sites of DNA damage was dependent on RAD52 specifically in transcriptionally active loci. Recent data indicate that RAD52 may also contribute to recruitment of POLD3, a subunit of DNA polymerase δ that is critical for BIR (Tan et al., 2020). Knockout of RAD52 in U2OS cells led to activation of NHEJ and increased chromosome aberrations indicating an important role of RAD52-mediated transcription-dependent DNA repair in the maintenance of genome stability.

Furthermore, RAD52 may play an important role in the resolution of DNA:RNA hybrids (or R-loops) by recruiting XPG nuclease, a member of the NER pathway (Yasuhara et al., 2018). These data together with the data by Lan's group on interaction between RAD52 and CSB (Wei et al., 2015; Teng et al., 2018) indicate an intriguing crosstalk between the NER and HR pathways during DNA repair at active transcription sites. Moreover, in both of these studies, RAD52 plays a central role in linking HR and NER pathways during transcription-dependent DNA repair.

The relationship between the function of RAD52 in TA-HRR/ TC-HR and its inverse RNA strand exchange activity raises an interesting question. Yasuhara et al., reported that the formation of DNA:RNA hybrids was not affected in a RAD52 knockout, arguing against the role of inverse RNA strand exchange activity of RAD52 in formation of these hybrids (Yasuhara et al., 2018). However, this study tracked formation of DNA:RNA hybrids during the initial 2 min response following DSB induction, whereas DSB repair *via* RAD52-mediated inverse RNA strand exchange likely requires an extended period of time comparable with a few hours as required for DSB repair *via* canonical HR. Therefore, it seems that RAD52 may play different roles at different stages of transcription-dependent DNA repair. In a rapid response, it may act by recruiting other DNA repair factors to the site of DNA damage at transcriptionally active sites, which parallels the mediator function of RAD52 in yeast where it promotes loading of RAD51 on RPA-covered ssDNA at the site of DNA damage (Sung 1997). While at later stages of DSB repair, RAD52 may promote formation of DNA:RNA hybrids in which RNA can be used as a template for DSB repair. The studies are currently under way to better understand the mechanisms of

RNA-dependent DNA repair and the specific role(s) that RAD52 plays in this process.

RAD52's Role in Cancer Development

Cancer cells exhibit a high degree of DNA damage and genomic instability. It is known that BRCA1 and BRCA2 play important roles in HR-dependent repair of DSBs. However, BRCA-deficient tumors show increased dependence on alternative pathways such as SSA and a-EJ to overcome their "BRCAness" phenotype, characterized by reduced DSB repair, impaired replication fork protection, and hypersensitivity to DNA damaging agents (Stok et al., 2021). Through its strand annealing and DNA pairing activities, RAD52 is central to the SSA and BIR pathways (Gottifredi and Wiesmuller 2020). These alternative pathways are highly mutagenic and provide a conducive environment for chromosomal translocations to occur through non-specific, error-prone joining of two heterologous chromosomes (Malkova and Ira 2013; Blasiak 2021). For example, the hyper-resection of DSB ends in the absence of DNA damage sensor proteins such as 53BP1, DNA-PKcs, and EXOSC10 in S/G_2 phase promotes mutagenic SSA activity (Domingo-Prim et al., 2019; Mladenov et al., 2019; Toma et al., 2019; Morales et al., 2021).

The hyper-mutagenic activity of BIR is primarily attributed to the significantly increased frequency of frameshift mutations, which may occur at a rate 2,800-fold higher than that of spontaneous mutations. These mutations are likely generated by the intermittent dissociation of Pol δ-synthesized DNA from its template during bubble migration (Sakofsky and Malkova 2017). This increases the propensity to incorporate mismatched nucleotides into the newly synthesized DNA which is normally repaired by mismatch repair (MMR) (Deem et al., 2011). However, the efficiency of MMR during BIR is significantly lower than that during S-phase replication. Another BIR-like mechanism, namely, alternative lengthening of telomeres (ALT) is implicated as a RAD52-dependent process involved in the development of human cancers (Sakofsky and Malkova 2017). One of the hallmarks of rapidly dividing cancer cells is their ability to efficiently maintain telomere length. While most cancer cells utilize telomerase to perform this activity, ~15% of human cancers employ ALT. ALT-associated PML bodies contain telomeres, telomere-binding proteins, and the scaffold protein PML (Grobelny et al., 2000). RAD52 is required to promote ALT, and *in vitro* RAD52 can promote D-loop formation with telomeric ssDNA (Zhang et al., 2019). However, a RAD52-independent ALT pathway that relies on the endonuclease cofactor SLX4 has also been identified (Verma et al., 2019). Cells lacking both RAD52 and SLX4 are synthetically lethal due to the accumulation of genomic abnormalities, and thus are potential therapeutic targets in cancers that are telomerase deficient.

Several studies demonstrated that RAD52 is important for enhanced viability of cancer cells. The correlation between RAD52 overexpression and accelerated hepatocarcinogenesis in TGF-α/c-myc transgenic mice was the first significant evidence that highlighted the importance of RAD52 in tumor development (Hironaka et al., 2003). Deletion of RAD52 in an ATM-deficient background was shown to decrease T-cell lymphoma incidence and increase the life span of double-mutant mice (Treuner et al., 2004). ATM kinase activates cell cycle arrest, DNA repair, or apoptosis to restore proliferation of normal cells and maintain genomic stability, or eliminate heavily damaged cells. The loss of ATM kinase causes ataxia-telangiectasia, a syndrome associated with increased chromosomal abnormalities and high predisposition to breast cancer, brain cancer, lymphoma, and leukemia (Treuner et al., 2004; Estiar and Mehdipour 2018). Lieberman et al. showed that RAD52 deletion in Squamous Cell Lung Carcinoma increased the death of cells undergoing carcinogen-induced transformation *in vivo*. They also observed an increased antitumor activity in $RAD52^{-/-}$ cells through an enhanced capacity of cytotoxic T lymphocytes and natural killer cells to directly kill tumor cells (Lieberman et al., 2017; Nogueira et al., 2019).

Several studies reported an association between high RAD52 expression level in tumor samples with poor patient prognosis and disease prognosis (Jewell et al., 2010; Lieberman and You 2017; Ho et al., 2020). In a study of cancer cells containing an inactivated RECQL4 gene and upregulated RAD52, inhibition of RAD52 sensitized the cancer cells to ionizing radiation (Kohzaki et al., 2020). Chronic expression of the CDK1 inhibitor p21 in pre-cancerous p53-deficient cells enables a subpopulation to develop with increased proliferation through deregulation of origin licensing during DNA replication (Galanos et al., 2016). It was shown that in these hyperproliferative cells, the p21-induced replication stress caused increased RAD52 expression and reliance on RAD52-dependent DNA repair pathways (Galanos et al., 2018). Further studies are needed to understand the contexts under which RAD52 expression can serve as a factor in determining the proper treatment to increase the success of patient outcomes.

Synthetic Lethality and RAD52 as a Therapeutic Target

In normal cells, genome stability is maintained by a network of DDR pathways. Inactivation of DDR pathways due to intrinsic genome instability coerces tumor cells to rely on the remaining alternative DNA repair/signaling pathways. Not surprisingly, the pro-oncogenic role of RAD52 is especially pronounced in cancer cells that are deficient in DDR pathways, like ATM-deficient cancers (Treuner et al., 2004). But the most remarkable pro-cancer RAD52 phenotype is seen in cancer cells deficient in any of the following DNA repair proteins: BRCA1, BRCA2, PALB2, XAB2 or RAD51 paralogs: RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 (Feng et al., 2011; Chun et al., 2013; Lok et al., 2013; Sharma et al., 2021). Powell's group showed that cells in which one of these proteins were mutated or depleted became dependent on RAD52 for viability; thus, mutations in RAD52 are synthetically lethal with mutations/depletion in these proteins. The term synthetic lethality refers to scenarios in which the simultaneous disruption of two biological pathways results in cell death, but disruption of either one in isolation does not (Dobzhansky 1946). It was proposed that RAD52 operates in a DSB repair sub-pathway that is distinct from the major BRCA-dependent HR pathway (Jalan et al., 2019). Recent data indicate

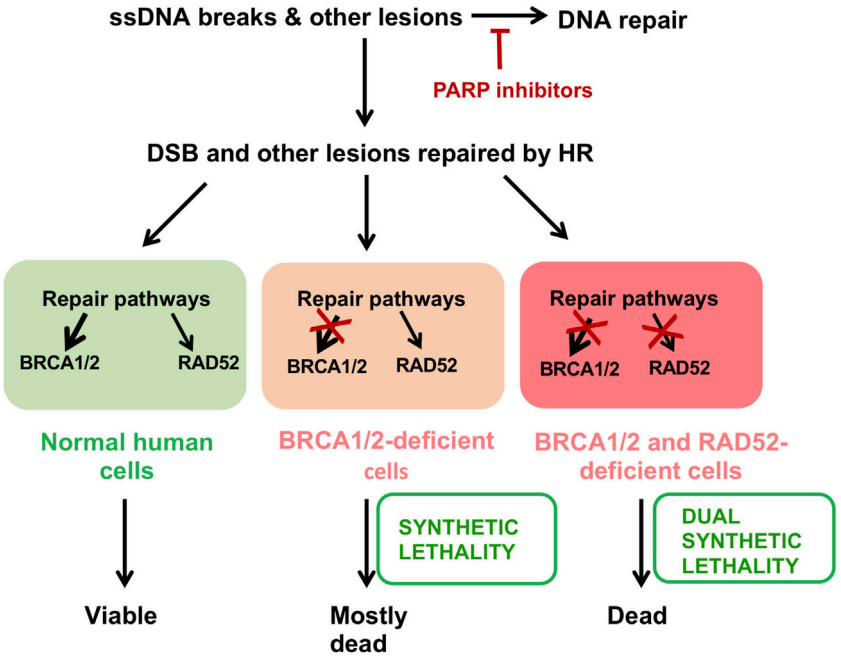

FIGURE 4 | Targeting cancer cells *via* synthetic lethality. PARP inhibitors trap PARP on DNA lesions and suppress repair of ssDNA breaks. This leads to generation of DSBs and other lesions that can only be repaired by HR. Normal cells are capable of repairing these lesions. Dysfunction of BRCA1/2 and related genes cause synthetic lethality with PARP inhibitors so that most of these cells die. Selective pressure forces the cancer cells to become more dependent on alternative RAD52-dependent DNA repair pathways. A combinational treatment of PARP and RAD52 inhibitors enhances the efficacy of each individual treatment *via* dual synthetic lethality and may cause a delay in the development of cancer drug resistance.

that RAD52 "catalytic" activities encoded by the N-terminal domain which include DNA pairing (D-loop formation), ssDNA and RNA annealing, inverse RNA and DNA strand exchange; are responsible for the viability of BRCA-deficient cells (Hanamshet and Mazin 2020). Which of these specific activities of RAD52 that are critical for the viability remains to be identified. The role of the C-terminal domain and its potential mediator function (similar to yeast Rad52) with RAD51 and RPA, remains to be investigated as well.

Hromas with co-workers showed RAD52/BRCA synthetic lethality depends on EEPD1, a structure-specific endonuclease that cleaves stalled replication forks (Hromas et al., 2017). Depletion of EEPD1 suppressed the synthetic lethality of RAD52-depleted BRCA1$^-$ cells, as DNA breaks are shunted toward (or processed by) the a-EJ pathway. Thus, the synthetic lethal relationship between BRCA and RAD52 is dependent on the generation of dead-end DNA intermediates that no remaining DNA repair pathway can handle in BRCA- and RAD52-deficient cells.

The synthetically lethal relationship between RAD52 and BRCA-related genes has important practical implications because mutations in BRCA1/2 and several related genes are responsible for nearly half of familial breast and ovarian cancers. Adamson et al. recently showed in populational studies that a RAD52 S346X polymorphic variant significantly reduces breast cancer risk among germline BRCA2 mutation carriers. This variant encodes a truncated RAD52 lacking the last 8 amino acids composing a nuclear localization signal. Cytoplasmic retention renders this RAD52 variant nonfunctional leading apparently to attrition of BRCA2-deficient breast cancer cells (Adamson et al., 2020; Biswas and Sharan 2020).

Targeting DNA repair proteins in synthetically lethal relationships has emerged as a prime strategy of novel cancer therapeutics (Huang et al., 2020; Myers et al., 2020). Thus, inhibitors of the DNA repair protein PARP represent the newest generation of cancer therapeutics (Lord and Ashworth 2017; D'Andrea 2018). However, the majority of cancer patients treated with PARP inhibitors (PARPi) eventually develop resistance to these agents, which stresses the need for new therapeutics (Lord and Ashworth 2013). Because in humans, RAD52 mutations cause no discernible HR phenotype, the synthetically lethal BRCA/RAD52 relationship makes RAD52 an attractive therapeutic target.

The synthetically lethal relationship between RAD52 and BRCA was first exploited using an oligopeptide aptamer to inhibit RAD52 in BRCA-downregulated acute myeloid leukemia cells. As expected, these cells arrested in G_2 and showed increased apoptosis (Cramer-Morales et al., 2013; Xu et al., 2020). Later, our and several other groups developed small molecule RAD52 inhibitors to specifically suppress the growth of BRCA-deficient cancer cells (Chandramouly et al., 2015; Hengel et al., 2016; Huang et al., 2016; Sullivan et al., 2016; Hengel et al., 2017; Sullivan-Reed et al., 2018). One of these compounds, D-I03, showed anti-proliferative activity against BRCA1-deficient breast cancer cells both *in vitro* and *in vivo* (Sullivan-Reed et al., 2018). However, the highest anti-proliferating activity of D-I03 was

observed in combination with the PARP inhibitor Talazoparib. This is consistent with the different mechanisms of action of PARP and RAD52 inhibitors. While PARP inhibitors increase the DNA damage load for the HR pathway and inhibit alternative a-EJ pathway, RAD52 inhibitors block the escape route for BRCA-deficient cancer cells through RAD52-dependent mechanism(s) of DNA repair (**Figure 4**). Combination treatment may also help to attenuate formation of drug resistance in cancer, the main nemesis of anti-cancer therapies. More work is needed for development of truly drug-like RAD52 inhibitors that can be used in clinic.

CONCLUSIONS AND FUTURE PERSPECTIVES

In yeast, Rad52 is a key protein of HR. The biochemical studies show that it may play a mediator function by assisting Rad51 recombinase loading on ssDNA occupied by RPA. But these studies may not tell the whole story, as genetic data indicate a stronger Rad52 phenotype in DSB repair and HR than that of Rad51 recombinase. In contrast to yeast, mammalian RAD52 knockouts show a mild phenotype in DNA repair and recombination in otherwise normal cells. However, RAD52 function became essential for viability of BRCA-deficient cancer cells. RAD52 is a multifunctional protein with several important activities including DNA pairing (D-loop formation) and ssDNA annealing. Recent studies uncovered an important role of RAD52 in RNA-dependent DNA repair and in R-loop resolution. RAD52 can promote DNA:RNA annealing and inverse strand exchange between RNA and homologous dsDNA at the proximity DSBs. Determining which of these activities play a critical role for viability of BRCA-deficient cancer cells remains a subject of investigation. Better understanding of RAD52 function will clarify the mechanisms of DNA repair in eukaryotes, and in humans particularly. Importantly, the synthetically lethal RAD52/BRCA relationship provides an opportunity to develop new anti-cancer drugs targeting BRCA-deficient cancers. Use of these inhibitors in combination with PARP inhibitors or other targeted therapies is a promising approach to increase the efficacy of the treatment and attenuate formation of drug resistance in cancer.

AUTHOR CONTRIBUTIONS

All authors conceived the paper and developed the plan; MR wrote the first draft; and all authors corrected and completed the paper.

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Phasor Histone FLIM-FRET Microscopy Maps Nuclear-Wide Nanoscale Chromatin Architecture With Respect to Genetically Induced DNA Double-Strand Breaks

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A DNA double-strand break (DSB) takes place in the context of chromatin, and there is increasing evidence for chromatin structure to play a functional role in DSB signaling and repair. Thus, there is an emerging need for quantitative microscopy methods that can directly measure chromatin network architecture and detect changes in this structural framework upon DSB induction within an intact nucleus. To address this demand, here we present the phasor approach to fluorescence lifetime imaging microscopy (FLIM) of Förster resonance energy transfer (FRET) between fluorescently labeled histones in the DSB inducible via AsiSI cell system (DIvA), which has sufficient spatial resolution to map nuclear-wide chromatin compaction at the level of nucleosome proximity with respect to multiple site-specific DSBs. We also demonstrate that when phasor histone FLIM-FRET is coupled with immunofluorescence, this technology has the unique advantage of enabling exploration of any heterogeneity that exists in chromatin structure at the spatially distinct and genetically induced DSBs.

Keywords: DNA repair, chromatin, histones, fluorescence lifetime imaging microscopy (FLIM), Förster resonance energy transfer (FRET)

INTRODUCTION

Inside the nucleus of a living cell, DNA is folded around histone proteins into nucleosomes and compacted into a multi-layered three-dimensional (3D) structure called chromatin (Luger et al., 2012; Bickmore, 2013; Bonev and Cavalli, 2016). At any moment in time, a DNA double-strand break (DSB) can occur anywhere within this dynamic structural framework, and somehow, a cellular surveillance system termed the "DNA damage response" (DDR) (Jackson and Bartek, 2009) has the capacity to instantaneously detect DSB induction and recruit repair machinery to this type of genetic damage (Kalousi and Soutoglou, 2016; Hauer and Gasser, 2017; Marnef and Legube, 2017). Initially, chromatin was viewed as an obstacle to DSB repair that the DDR must first "open" and then restore upon DSB resolution. More recently, however, it has become apparent that the chromatin compaction status of a DSB plays a more active role in DNA damage signaling and DSB repair pathway choice (Soria et al., 2012; Lemaître et al., 2014; Clouaire and Legube, 2015; Polo and Almouzni, 2015). Local reorganization in chromatin network architecture has been shown to

spatiotemporally modulate the arrival and retention of different DNA repair factors at DSB sites (Hinde et al., 2014; Smith et al., 2019). Thus, in order to understand how genome integrity is maintained at a cellular level, there is an emerging need to study DSB repair within the context of chromatin and the 3D nuclear landscape of a living cell.

The chromatin "opening" and "compacting" events that follow DSB induction (Klement et al., 2014; Kalousi et al., 2015; Thorslund et al., 2015; Luijsterburg et al., 2016) are underpinned by nanoscale changes in the spacing between nucleosomes (Hauer and Gasser, 2017), and these dynamics occur on a spatial scale that is well below the diffraction limit of optical microscopy (Luger and Hansen, 2005; Ou et al., 2017; Ohno et al., 2018; Ochs et al., 2019; Whelan and Rothenberg, 2021). Thus, with the aim of rendering any DDR-induced changes to a local chromatin structure visible in a living cell, we recently demonstrated that Förster resonance energy transfer (FRET) between fluorescently labeled histones is a sensitive real-time readout of nucleosome proximity during DSB repair (Lou et al., 2019) that can be spatially mapped throughout the nucleoplasm by the phasor approach to fluorescence lifetime imaging microscopy (FLIM) (Liang et al., 2020). From coupling FLIM detection of FRET between histone H2B tagged to eGFP (H2B-eGFP) and mCherry (H2B-mCh) with DSB induction via near-infrared (NIR) laser micro-irradiation, we quantified a rapid chromatin decompaction event central to a DNA repair locus that was surrounded by a border of compact chromatin foci and found this chromatin structure to be critical for the timely accumulation of DNA repair factors at a DSB site (Lou et al., 2019). Thus, this phasor histone FLIM-FRET assay has the potential to be an invaluable tool for biologists studying DSB repair, since it has sufficient spatiotemporal resolution to reveal what is normally an invisible layer of regulation to a cellular DDR.

Here in this study, we demonstrate the capacity of the phasor histone FLIM-FRET assay to spatially map chromatin architecture with respect to DNA damage in the DSB inducible via AsiSI cell system (DIvA) (Iacovoni et al., 2010). DIvA cells harbor a 4-hydroxytamoxifen (4OHT)-inducible AsiSI restriction enzyme that allows for induction of approximately 100 site-specific DSBs throughout the genome upon 4OHT treatment (Iacovoni et al., 2010; Massip et al., 2010; Aymard et al., 2014). Thus, by multiplexing phasor histone FLIM-FRET with immunofluorescence (IF) against phosphorylated serine 139 of histone variant 2AX (γH2AX), we are able to spatially map nuclear-wide chromatin compaction at the level of nucleosome proximity with respect to DIvA DSB locations. From image analysis of this three-color experiment across multiple DIvA nuclei, we find in agreement with our previous study employing NIR laser micro-irradiation (Lou et al., 2019) that DSB induction induces a global chromatin compaction event that surrounds sites of DNA damage, which statistically represent nuclear locations that are in a more "open" chromatin state. While a benefit of NIR laser micro-irradiation as a method for DSB induction was temporal resolution, an important advantage of the DIvA cell system is having access to the spatial heterogeneity that underlies this quantified chromatin response. Thus in a final experiment, to demonstrate this utility, we perform a four-color experiment that enables the chromatin structure reported by histone FRET to be studied as a function of the DSB repair pathway. We anticipate that this unique capacity of the phasor histone FLIM-FRET assay in DIvA alongside IF has the potential to facilitate discovery into how exactly chromatin structure regulates a DSB DNA damage response.

RESULTS

Phasor histone FLIM-FRET microscopy coupled with IF maps nuclear-wide changes in chromatin compaction with respect to DSB induction in the DIvA cell system. To quantify the local versus global chromatin compaction status of nucleus architecture with respect to multiple site-specific DSBs, here we combine phasor histone FLIM-FRET analysis with IF of γH2AX in the DIvA cell system. FRET is an optical phenomenon that reports fluorescent protein–protein interaction on a scale of 1–10 nm, and in the context of chromatin labeled with donor–acceptor fluorescent histones (Llères et al., 2009), FRET reports nucleosome proximity with nanoscale resolution. Thus, to implement histone FRET in the DIvA cell system, we first transfected DIvA cells with H2B tagged to eGFP (H2B-eGFP) in the absence (donor control) versus presence of mCherry (H2B-mCh) (**Figures 1A,B**). Then in fixed and washed DIvA nuclei expressing the donor control versus donor–acceptor FRET pair, we acquired FLIM data in the H2B-eGFP (donor) channel where quenching of the donor lifetime in the presence of H2B-mCh (acceptor) reports histone FRET (**Figures 1C,D**). Quantification of this donor control versus histone FRET experiment in the DIvA cell system by the phasor approach to lifetime analysis enabled the FRET efficiency of compact chromatin to be characterized as 16% (i.e., donor lifetime shift from 2.5 to 2.1 ns) (**Figure 1C**) and definition of a cursor-based palette to spatially map compact (red pixels) versus open chromatin (teal pixels) throughout DIvA nuclei (**Figure 1D**).

To next employ histone FRET as a readout of chromatin network architecture with respect to sites of DSB induction in the DIvA cell system, we first confirmed via IF for γH2AX Alexa Fluorophore 647 (γH2AX-AF647) in DIvA cells fixed 2 h after 4-hydroxytamoxifen (4OHT) treatment that multiple DSBs do form across the genome (**Figure 1E**). Then from careful design of a multi-colored imaging experiment that aimed to measure histone FRET between H2B-eGFP and H2B-mCh (**Figures 1F,G**) in the presence of γH2AX-AF647 IF (**Figure 1H**), we spatially mapped compact versus open chromatin in the presence versus absence of multiple DSB foci (**Figure 1I**) without artifact from 4OHT addition (**Supplementary Figure S1**). Quantification of this multiplexed imaging experiment via calculation of the fraction of pixels exhibiting histone FRET (our readout of a compact chromatin state) (**Figure 1J**) revealed genetic DSB induction to initiate significant nuclear-wide chromatin compaction when applied across multiple cells (**Figure 1K**). This result alongside a qualitative comparison of γH2AX-AF647 localization with

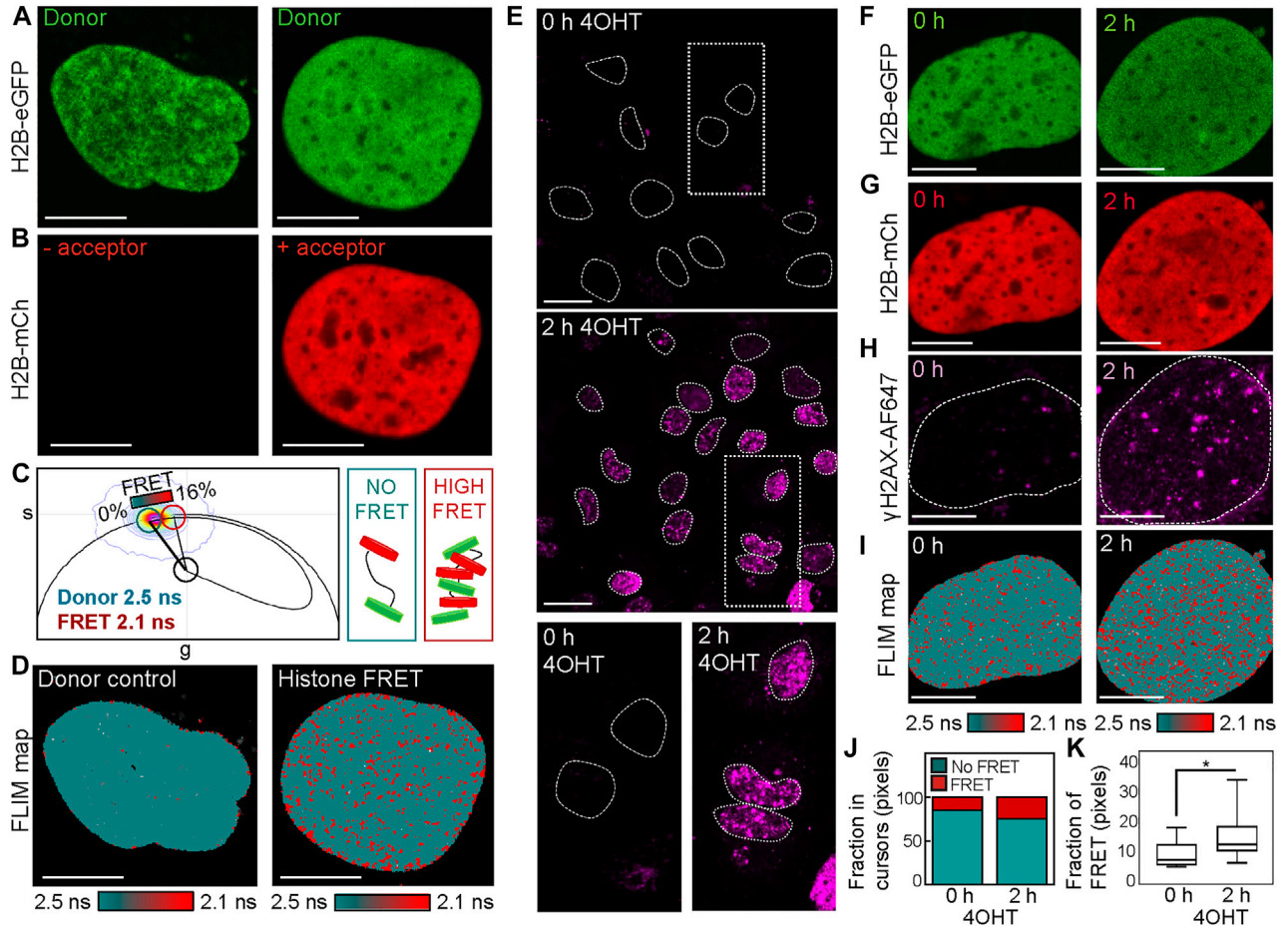

FIGURE 1 | Phasor histone FLIM-FRET coupled with γH2AX IF reveals DSB induction in the DIvA cell system to induce nuclear-wide chromatin compaction. **(A,B)** Fixed DIvA nuclei expressing H2B-eGFP **(A)** in the absence **(left)** versus presence **(right)** of H2B-mCh **(B)** (scale bar 10 μm). **(C)** Phasor distribution of H2B-eGFP in the absence (donor control) versus presence of H2B-mCh (histone FRET experiment) with a theoretical FRET trajectory superimposed (black curve) that extends from the unquenched donor lifetime (teal cursor, 2.5 ns). This FRET trajectory enables characterization of the histone FRET efficiency as 16% (red cursor, 2.1 ns) and definition of a palette to detect open (teal) versus compact (red) chromatin. **(D)** FLIM maps of H2B-eGFP in the absence **(left)** versus presence **(right)** of H2B-mCh pseudo-colored according to the FRET palette defined in the phasor plot of panel **(C)**. **(E)** IF against γH2AX in DIvA before versus after 2 h of treatment with 4OHT (scale bar 20 μm). **(F–H)** Fixed DIvA nuclei co-expressing the histone FRET pair H2B-eGFP **(F)** and H2B-mCh **(G)** with IF against γH2AX **(H)** in the absence **(left)** versus presence **(right)** of 2 h of treatment with 4OHT (scale bar 10 μm). **(I)** FLIM maps of the cells presented in panels **(F–H)** pseudo-colored according to the FRET palette defined in panel **(C)**. **(J)** Quantification of the fraction of pixels in the phasor cursor that reports no FRET (open chromatin) versus histone FRET (compact chromatin) in the cells presented in panel **(I)**. **(K)** Quantification of the fraction of pixels in the phasor cursor that reports histone FRET (compact chromatin) across multiple cells before versus after 2 h of 4OHT treatment (N = 11 and 47 cells, respectively, three biological replicates). The box and whisker plot shows the minimum, maximum, and sample median. $^*p < 0.05$ (unpaired *t*-test).

histone FRET after 4OHT treatment (**Figures 1H,I**, right) suggested DSB sites to occupy the few "open" chromatin regions that exist within the detected nuclear-wide chromatin compaction event. Thus, to further investigate this observation, we next performed a γH2AX-AF647–based mask analysis of the histone FRET maps derived after 4OHT treatment, to enable quantification of the local (inside the DSB site) versus global (outside the DSB site) chromatin response to DSB induction.

To generate a mask that enables histone FRET analysis of chromatin compaction inside versus outside of DSB foci (**Figures 2A–D**), a threshold based on γH2AX-AF647 IF was employed (**Figure 2E**). This binary mask allowed for selection of pixels within the FLIM map that occupy DSB sites versus the surrounding nucleoplasm (**Figure 2F**), and quantitation of the fraction of pixels exhibiting histone FRET in either environment (**Figure 2G**). From application of this analysis to multiple cells after 4OHT treatment (**Figure 2H**), we confirmed DSB sites to statistically be in a more "open" chromatin state than the surrounding chromatin environment, which was compacted upon DSB induction (**Figure 1**). Interestingly, this differentially regulated reorganization in local versus global chromatin structure that was induced by multiple DSBs being genetically cut at distinct nuclear locations is in direct agreement with our previous study, which coupled histone FRET with NIR laser micro-irradiation to cut multiple DSBs at a single nuclear location (Lou et al., 2019). Thus, while NIR laser

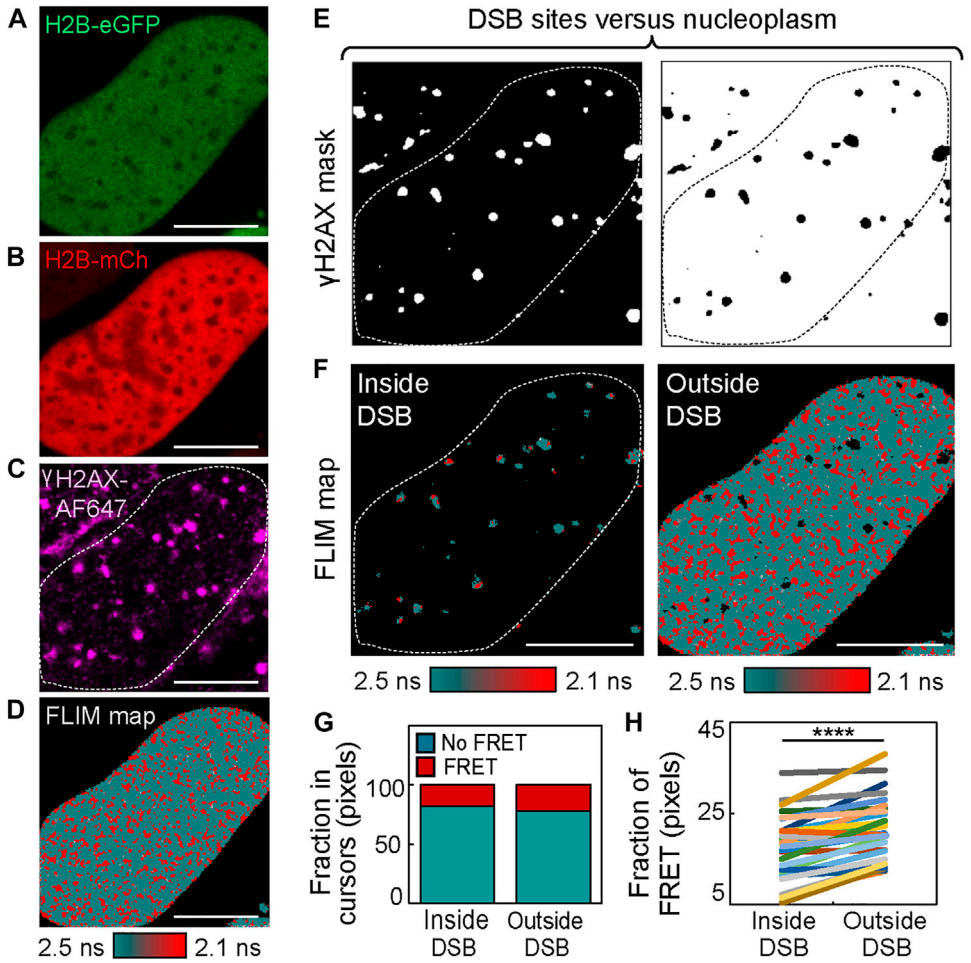

FIGURE 2 | IF-based mask analysis of histone FRET in the DIvA cell system reveals chromatin to be "open" at sites of DSB induction. **(A–C)** DIvA nucleus co-expressing the histone FRET pair H2B-eGFP **(A)** and H2B-mCh **(B)** that has been fixed with IF against γH2AX **(C)** after 2 h of treatment with 4OHT (scale bar 10 μm). **(D)** FLIM map of the cell presented in panels **(A–C)** pseudo-colored to report histone FRET (red pixels) versus non-FRET (teal pixels). **(E)** Masks based on γH2AX IF presented in panel **(C)** that select chromatin inside **(left)** versus outside **(right)** of DIvA DSBs. **(F)** Pseudo-colored histone FRET maps with threshold defined by masks presented in panel **(E)** applied to select inside **(left)** versus outside **(right)** of DSBs. **(G)** Fraction of pixels reporting FRET (compact chromatin) versus no FRET (open chromatin) within masked FLIM maps presented in panel **(F)**. **(H)** Quantitation of the fraction of histone FRET (compact chromatin) inside versus outside of DSBs after 2 h of 4OHT treatment across multiple cells (N = 29 cells, three biological replicates). The box and whisker plot shows the minimum, maximum, and sample median. $^{****}p < 0.0001$ (paired *t*-test).

micro-irradiation was advantageous in terms of temporal resolution and enabling observation of early changes in DSB chromatin structure, a clear advantage of the DIvA cell system for histone FRET assessment of DSB chromatin during repair is the potential for this assay to explore any spatial heterogeneity that underlies this response.

IF-guided image analysis of phasor histone FLIM-FRET microscopy data acquired in DIvA cells quantifies chromatin network organization and enables exploration of DSB foci heterogeneity. To demonstrate the potential of phasor histone FLIM-FRET microscopy and IF in DIvA cells to enable both 1) a quantitative insight into the nuclear-wide spatial organization of compact chromatin with respect to DSBs and 2) exploration of heterogeneity in the local chromatin response at DSBs, here we performed two types of image analysis to acquired FLIM maps of histone FRET. The first type of analysis extracts the nuclear-wide localization of high FRET compact chromatin foci within a FLIM map, treats them as particles, and then quantifies their spatial distribution in terms of particle size. From application of this analysis to DIvA nuclei that were untreated versus treated with 4OHT (**Figures 3A,B**), we find the extracted network of high FRET compact chromatin foci (**Figure 3C**), to undergo a spatial reorganization in response to DSB induction that results in an increase in foci area (**Figures 3D,E**). This result, alongside the finding that DSB induction initiates a nuclear-wide chromatin compaction event at the level of nucleosome proximity (**Figures 1**, **2**), suggests that, in addition to this global but nanoscale reorganization in chromatin structure, which occurs outside of DSB sites, a DSB DNA damage response also initiates sub-micron changes to higher order chromatin network organization (**Figure 3**).

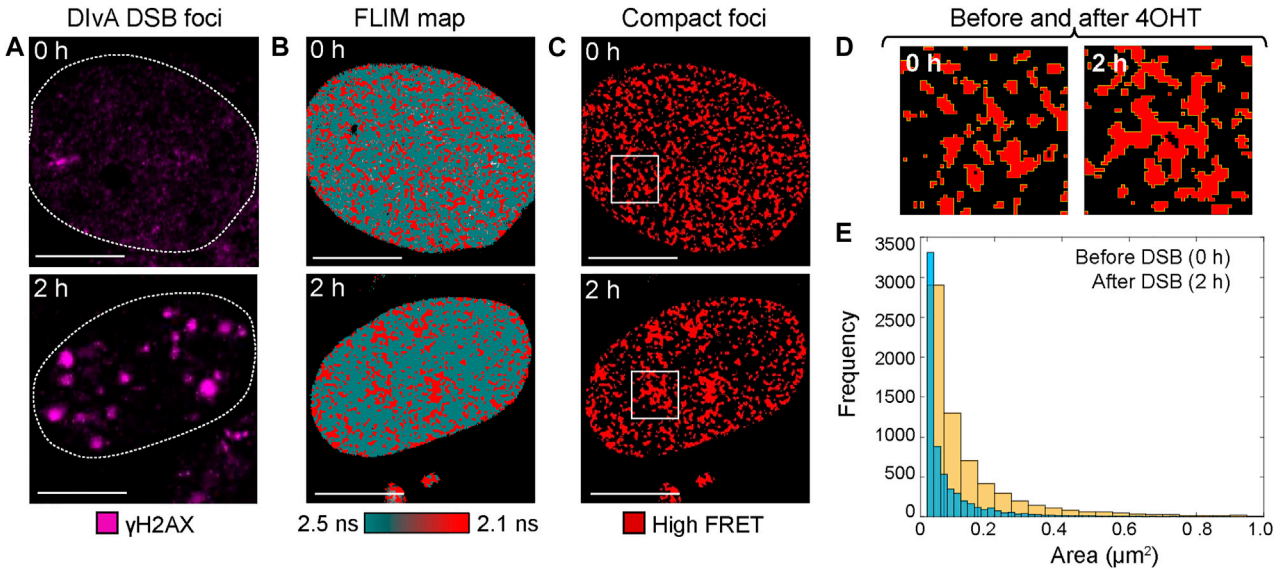

FIGURE 3 | Particle analysis of histone FRET in DIvA cell FLIM maps reveals sub-micron changes in compact chromatin network organization upon DSB induction. **(A)** DIvA nucleus expressing the histone FRET pair (H2B-eGFP and H2B-mCh) that has been fixed with IF against γH2AX before **(top)** versus after **(bottom)** 2 h of treatment with 4OHT (scale bar 10 μm). **(B)** FLIM maps of the cells presented in panel **(A)** pseudo-colored to report histone FRET (red pixels) versus no FRET (teal pixels). **(C)** Localization of compact chromatin foci extracted from the histone FRET maps presented in panel **(B)**. **(D)** Zoom of region of interest (white box) in localization maps presented in panel **(C)**. **(E)** Histogram of the size of compact chromatin foci as detected by histone FRET in DIvA before (blue) versus after 2 h (yellow) of treatment with 4OHT (N = 6 cells, two biological replicates).

To next investigate heterogeneity in the local chromatin response reported by histone FRET at DIvA DSB sites, we performed IF against not only γH2AX, which is expected to highlight the total population of DSBs present, but also different DNA repair proteins that highlight the DSB sub-population set to undergo repair by one of two dominant DSB repair pathways. In particular, we performed an IF-guided mask analysis of histone FRET maps acquired in DIvA nuclei co-expressing H2B-eGFP and H2B-mCherry, which were treated with 4OHT for 2 h (**Figures 4A–C**) and fixed with IF against 1) tumor suppressor p53 binding protein 1 (53BP1-AF405) that highlights DSBs marked for non-homologous end joining (NHEJ) (**Figures 4D–F**) and 2) breast cancer type 1 susceptibility protein (BRCA1-AF647) that highlights DSBs marked for homologous recombination (HR) (**Figures 4G–I**). Collectively, these experiments enabled quantification of chromatin compaction inside versus outside of DSB foci marked for NHEJ (**Figure 4J**) and HR (**Figure 4K**), as well as investigation into whether NHEJ versus HR DSB repair takes place in different chromatin environments (**Figure 4L**).

We find from this analysis that both NHEJ and HR DSB sites are statistically in a more "open" chromatin state than their surrounding undamaged chromatin environment (**Figures 4J, K**), which is in keeping with our γH2AX-guided analysis (**Figure 2H**). Also, intriguingly, if we take into account the baseline chromatin compaction status of each DIvA nucleus analyzed (i.e., normalized with respect to FRET fraction in pixels outside DSB sites), we find that while 53BP1 DSB foci marked for NHEJ are not significantly different from γH2AX DSB foci, BRCA1 DSB foci marked for HR are statistically more "open" than γH2AX DSB foci (**Figure 4L**). The molecular mechanism and physiological function of why HR DSB foci are more "open" needs to be further investigated; however, it is in keeping with previous studies that link BRCA1 with roles in chromatin de-condensation (Bochar et al., 2000; Ye et al., 2001), and it does suggest that heterogeneity in terms of chromatin structure does exist as a function of the DSB repair pathway.

DISCUSSION

In this study, we applied phasor histone FLIM-FRET microscopy to the measurement of nuclear-wide chromatin compaction at the level of nucleosome proximity and demonstrated that this assay can quantify sub-micron changes in the spatial organization of this nanoscale feature upon DSB induction in the DIvA cell system. From coupling this technology with immunofluorescence against histone modifications that highlight DSB sites (e.g., γH2AX) and DNA repair proteins involved in DSB resolution (e.g., 53BP1 and BRCA1), we also highlight the capacity of phasor histone FLIM-FRET to explore spatial heterogeneity in the local DSB chromatin structure as a function of DSB repair pathway choice—NHEJ versus HR. In doing so, we found that DIvA DSBs induce a global chromatin network compaction event that reduces the average spacing between nucleosomes and reorganizes them into larger clusters, in parallel with the local opening of chromatin at DSB sites—especially those marked for repair via HR. Interestingly, this result, which stems from multiple site-specific DSBs being induced at distinct locations throughout the DIvA nucleoplasm, is in strong agreement with our previous study that implemented phasor

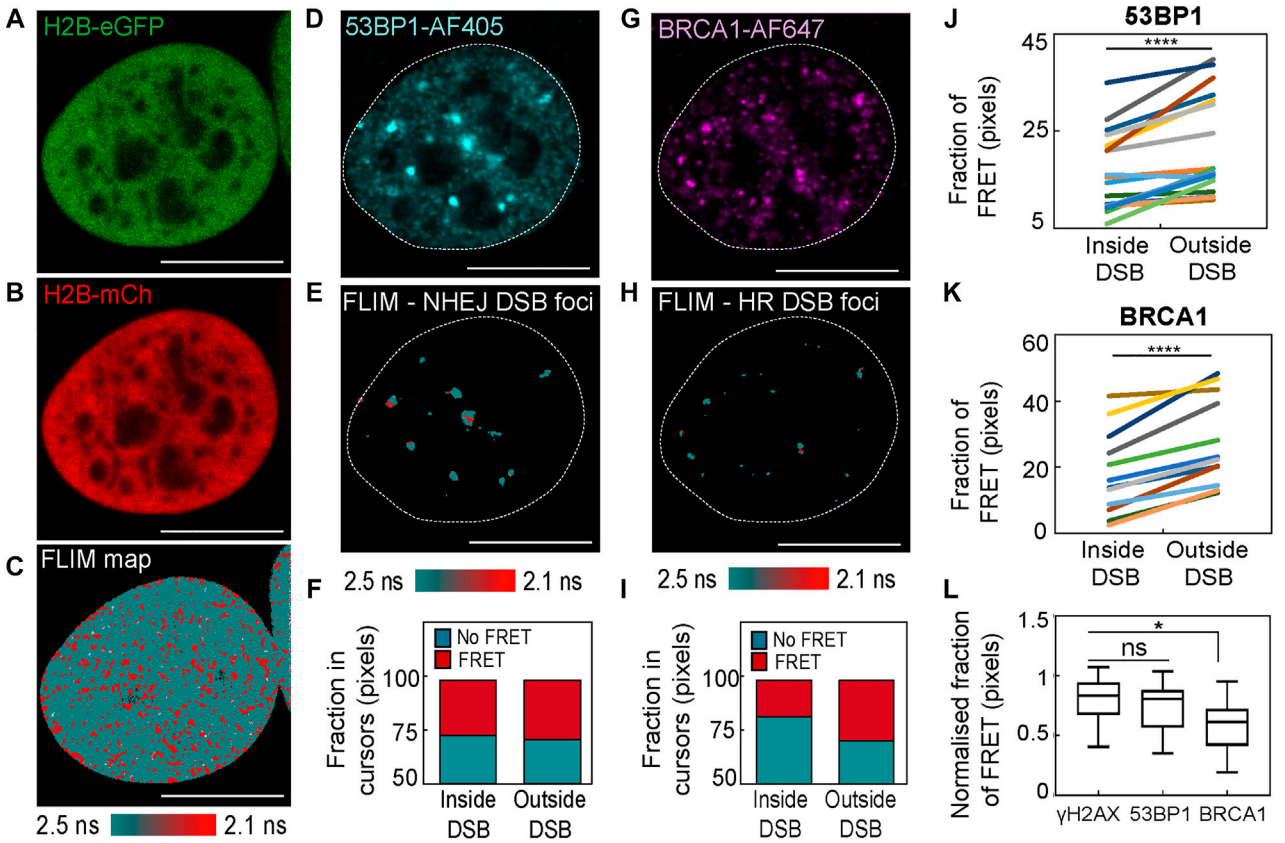

FIGURE 4 | An IF-based mask analysis of DSB histone FRET heterogeneity in the DIvA cell system. **(A,B)** DIvA nucleus co-expressing the histone FRET pair H2B-eGFP **(A)** and H2B-mCh **(B)** that has been treated with 4OHT for 2 h and fixed with IF against different DNA repair proteins. **(C)** FLIM map of the cell presented in panels **(A,B)** pseudo-colored to report histone FRET (red pixels). **(D)** IF against NHEJ DNA repair protein 53BP1 (53BP1-AF405) in the cell presented in panels **(A–C)**. **(E,F)** Pseudo-colored histone FRET map from panel **(C)** with mask based on the 53BP1-AF405 signal **(D)** applied to select NHEJ DSB foci **(E)** and the fraction of pixels reporting histone FRET (red pixels) within versus outside of this mask **(F)**. **(G)** IF against HR DNA repair protein BRCA1 (BRCA1-AF647) in the cell presented in panels **(A–C)**. **(H,I)** Pseudo-colored histone FRET map from panel **(C)** with mask based on the BRCA1-AF647 signal **(G)** applied to select HR DSB foci **(H)** and the fraction of pixels reporting histone FRET (red pixels) within versus outside of this mask **(I)**. **(J)** Quantification of the fraction of histone FRET (compact chromatin) inside versus outside of 53BP1 identified NHEJ DSBs after 2 h of 4OHT treatment across multiple nuclei (N = 18 cells, two biological replicates). **(K)** Quantification of the fraction of histone FRET (compact chromatin) inside versus outside of BRCA1 identified HR DSBs after 2 h of 4OHT treatment across multiple nuclei (N = 12 cells, two biological replicates). **(L)** A quantitative comparison of the fraction of histone FRET (compact chromatin) inside γH2AX labeled foci (all DSBs) versus 53BP1 foci (NHEJ DSBs) and BRCA1 foci (HR DSBs) normalized to the fraction of histone FRET in the surrounding nucleoplasm (N ≥ 12 cells, two or three biological replicates). The box and whisker plot shows the minimum, maximum, and sample median. In **(J,K)**, $^{****}p < 0.0001$ (paired *t*-test). In **(L)**, $^{*}p < 0.05$ and ns > 0.05 (unpaired *t*-test).

histone FLIM-FRET in HeLa cells exposed to NIR laser micro-irradiation, which induces multiple DSBs at a single nuclear location (Lou et al., 2019). Thus, chromatin "opening" at a DSB site alongside chromatin compacting of the surrounding DNA appears to be a universal mechanism for efficient repair of DSBs whether they be induced genetically or by a source of radiation.

The next question is the following: What biological function do these detected changes in chromatin structure serve for DSB resolution? In the context of DNA repair, there is already evidence obtained via super-resolution microscopy that a nanoscale reorganization in the chromatin structure regulates DNA repair protein access and retention at DSB sites (Ochs et al., 2019; Whelan and Rothenberg, 2021). Along this line, in our previous study that employed NIR laser micro-irradiation, we found that the compacted chromatin boundary of a DSB repair locus serves to modulate the mobility and access of the NHEJ repair factor tumor suppressor 53BP1 to the central "opened" region of this type of genomic lesion (Lou et al., 2019). Thus, given the demonstrated potential of the histone FRET assay to explore DSB chromatin structure, here as a function of DNA repair pathway choice when coupled with IF in DIvA, future experiments will be dedicated toward bettering understanding what is the role of DSB chromatin structure in controlling 53BP1 versus BRCA1 access and identifying whether chromatin plays a role in the decision to proceed toward DSB resolution via NHEJ versus HR.

MATERIALS AND METHODS

Cell Culture, Transient Transfection, and IF

DIvA cells (originally provided by Gaëlle Legube, LBCMCP, CNRS, Toulouse, France) were grown in Dulbecco's modified

Eagle's medium (Lonza) supplemented with 10% bovine growth serum (Gibco), 1x Pen-Strep (Lonza), and 1 μg/ml puromycin (Thermo Fisher Scientific) at 37°C in 5% CO_2. DIvA cells were then plated 24 h before transfection onto 35 mm glass bottom dishes and transiently transfected with H2B-eGFP and H2B-mCherry via use of Lipofectamine 3000 according to the manufacturer's protocol. Transiently transfected DIvA cells were then treated (or left untreated) with 300 nM of 4OHT for 2 h and then fixed with 4% paraformaldehyde for 15 min, permeabilized with 1 mg/ml Triton X-100 for 15 min at room temperature, and blocked with 1% bovine serum albumin for 30 min. Three rounds of washing with phosphate-buffered saline (PBS) were performed in between each of these fixation steps. For IF against γH2AX (S139) (Catalog number 9718S, Cell Signaling), 53BP1 (Catalog number 4937S, Cell Signaling), and BRCA1 (Catalog number SAB2702136-100UL, Sigma), the fixed DIvA cells were incubated with primary antibody (1:200) overnight at 4°C and then secondary antibody labeled with Alexa Fluor 405 (AF405) or Alexa Fluor 647 (AF647) for 1 h at room temperature. The three rounds of washing step with PBS were also performed in between each of these IF steps. In general, PBS washing not only was critical for fixation and IF but also counteracted a 4OHT-induced shift in the fluorescence lifetime of H2B-eGFP that was unrelated to histone FRET (**Supplementary Figures S1A–E**).

Confocal Laser Scanning Microscopy and FLIM Data Acquisition

All fixed cell microscopy measurements were performed on an Olympus FV3000 laser scanning microscope coupled to a 488 nm pulsed laser operated at 80 MHz and an ISS A320 FastFLIM box. A ×60 water immersion objective 1.2 NA was used for all experiments, and the cells were imaged at room temperature. Prior to acquisition of FLIM data in the donor channel (H2B-eGFP) for histone FRET analysis, multi-channel intensity images (two-, three-, and four-color) were acquired from each selected DIvA nucleus to verify that the FRET acceptor (H2B-mCh) was present in excess of H2B-eGFP (i.e., acceptor–donor ratio > 1) and to record the localization of DSB breaks labeled with either H2AX (γH2AX-AF647) or 53BP1 (53BP1-AF405) and BRCA1 (BRCA1-AF647). This involved sequential imaging of a two-phase light path in the Olympus FluoView software. The first phase was set up to image H2B-eGFP and H2B-mCh via use of solid-state laser diodes operating at 488 and 561 nm, respectively, with the resulting signal being directed through a 405/488/561/6033 dichroic mirror to two internal GaAsP photomultiplier detectors set to collect 500–540 nm and 600–700 nm. The second phase was set up to image 53BP1-AF405 and BRCA1-AF647 or just γH2AX-AF647 via use of solid-state laser diodes operating at 405 and 633 nm, respectively, with the resulting signal being directed through a 405/488/561/633 dichroic mirror to two internal GaAsP photomultiplier detectors set to collect 420–460 nm and 600–700 nm. Then in each DIvA nucleus selected, a FLIM map of H2B-eGFP was imaged within the same field of view (256 × 256-pixel frame size, 20 μs/pixel, 90 nm/pixel, 20 frame integration) using the ISS VistaVision software. This involved excitation of H2B-eGFP with an external pulsed 488 nm laser (80 MHz) and the resulting signal being directed through a 405/488/561/633 dichroic mirror to an external photomultiplier detector (H7422P-40 of Hamamatsu) that was fitted with a 520/50 nm bandwidth filter. The donor signal in each pixel was then subsequently processed by the ISS A320 FastFLIM box data acquisition card to report the fluorescence lifetime of H2B-eGFP. All FLIM data were pre-calibrated against fluorescein at pH 9 which has a single exponential lifetime of 4.04 ns.

FLIM-FRET Analysis

The fluorescence decay recorded in each pixel of an acquired FLIM image was quantified by the phasor approach to lifetime analysis (Digman et al., 2008; Hinde et al., 2012). As described in previously published papers (Hinde et al., 2012; Liang et al., 2020), this results in each pixel of a FLIM image giving rise to a single point (phasor) in the phasor plot, which when used in the reciprocal mode enables each point in the phasor plot to be mapped to each pixel of the FLIM image. Since phasors follow simple vector algebra, it is possible to determine the fractional contribution of two or more independent molecular species coexisting in the same pixel. For example, in the case of two independent species, all possible weightings give a phasor distribution along a linear trajectory that joins the phasors of the individual species in pure form. While in the case of a FRET experiment, where the lifetime of the donor molecule is changed upon interaction with an acceptor molecule, the realization of all possible phasors quenched with different efficiencies describes a curved FRET trajectory in the phasor plot that follows the classical definition of FRET efficiency.

In the context of the histone FRET experiments presented, the phasor coordinates (g and s) of the unquenched donor (H2B-eGFP) and background (cellular autofluorescence) were first determined independently in fixed DIvA cells transfected versus un-transfected with H2B-eGFP. This enabled definition of a baseline from which a FRET trajectory could be extrapolated and then used to determine the dynamic range of FRET efficiencies that describe chromatin network organization in the DIvA cell system (Lou et al., 2019; Liang et al., 2020). From superimposition of this FRET trajectory with the combined phasor distribution measured for H2B-eGFP in fixed DIvA cells co-transfected with H2B-mCh, we find the DIvA chromatin network to exhibit compaction states that range from 0 to 16% in FRET efficiency. This corresponds to a shift in the H2B-eGFP donor lifetime from approximately 2.5 ns ($g = 0.39 \pm 0.05$, $s = 0.49 \pm 0.05$) to 2.1 ns ($g = 0.47 \pm 0.05$, $s = 0.50 \pm 0.05$). We therefore defined two cursors centered at these phasor coordinates to spatially map where chromatin is open (teal cursor) versus compact (red cursor) throughout a FLIM data acquisition in a fixed DIvA nucleus. Also, to quantify the extent to which DIvA chromatin was compacted before versus after DSB induction across multiple nuclei, we calculated the fraction of pixels counted as compact (i.e., FRET state in red cursor). All FLIM-FRET quantification was performed in the SimFCS software developed at the LFD.

DSB Foci Segmentation and Foci FLIM-FRET Analysis

To quantify the local chromatin structure of DSB foci versus the undamaged nuclear-wide chromatin architecture in DIvA nuclei, we applied an intensity threshold mask based on a DSB protein IF intensity image to FLIM maps pseudo-colored according to histone FRET (compact chromatin) versus no FRET (open chromatin). This involved 1) smoothing each DIvA nucleus' IF image of DSB localization (i.e., γH2AX-AF647, 53BP1-AF405, or BRCA1-AF647) with a 3×3 spatial median filter, 2) transforming this smoothed image into a binary mask based on an intensity threshold that was sufficiently harsh to reject non-specific IF staining but retain DSB foci, 3) applying the IF-guided mask to its associated FLIM map pseudo-colored according to histone FRET, and 4) quantification of the fraction of compact chromatin within (i.e., DSB foci) versus outside (i.e., nucleoplasm) the IF-guided mask.

Compact Chromatin Foci Size Analysis

To quantify the size of compact chromatin foci detected within a FLIM map pseudo-colored according to histone FRET (compact chromatin) versus no FRET (open chromatin), a binary image of compact chromatin foci was exported from the software SimFCS to ImageJ, and then a particle analysis routine was applied that identified particles based on the following criteria: 1) particle size was from 0 to infinity, 2) all adjacent non-zero pixels were considered one particle, and 3) holes inside connected pixels were considered part of the identified particle. The area of identified particles was calculated as the number of pixels times the area of a single pixel.

Statistics and Figure Preparation

Statistical analysis was performed by using GraphPad Prism software. Figures were prepared by using Adobe Illustrator, Microsoft PowerPoint, SimFCS, and ImageJ.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JL and EH conceived the study, designed the experiments, and wrote the manuscript. JL and ZL conducted experimentation. JL and AS analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.770081/full#supplementary-material

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REVIEW
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Chromatin and Nuclear Dynamics in the Maintenance of Replication Fork Integrity

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Replication of the eukaryotic genome is a highly regulated process and stringent control is required to maintain genome integrity. In this review, we will discuss the many aspects of the chromatin and nuclear environment that play key roles in the regulation of both unperturbed and stressed replication. Firstly, the higher order organisation of the genome into A and B compartments, topologically associated domains (TADs) and sub-nuclear compartments has major implications in the control of replication timing. In addition, the local chromatin environment defined by non-canonical histone variants, histone post-translational modifications (PTMs) and enrichment of factors such as heterochromatin protein 1 (HP1) plays multiple roles in normal S phase progression and during the repair of replicative damage. Lastly, we will cover how the spatial organisation of stalled replication forks facilitates the resolution of replication stress.

Keywords: DNA replication, chromatin, DNA repair, nucleus, DNA damage

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1 INTRODUCTION

Faithful replication of the genome is important to allow successful inheritance of genetic material from parental to daughter cells in all organisms. Eukaryotic DNA replication is preceded in G1 phase with the licencing of pre-replicative complexes (pre-RC), containing head-to-head dimers of minichromosome maintenance (MCM) helicase, to replicative origins throughout the genome in an origin recognition complex (ORC)-dependent manner (Parker et al., 2017). After the G1 to S phase transition, these pre-RCs are activated in a process called origin firing, which is highly dependent on Dbf4-dependent kinase (DDK) cyclin-dependent kinase (CDK) activity (Boos and Ferreira, 2019). Origin firing does not happen simultaneously across the genome; instead, different regions are replicated at different stages throughout S phase and this process is coordinated by various factors such as the local chromatin environment and sub-nuclear localisation. Control of origin firing is vital to ensure coordinated replication of the entire genome (Boos and Ferreira, 2019). Upon origin firing, Cdc45, and GINS are recruited to MCM to form the active CMG helicase which unwinds DNA into single stranded DNA (ssDNA) in a bidirectional manner, forming two replication forks (Burgers and Kunkel, 2017). Replication then occurs from these forks and DNA in synthesised in a semi-conservative manner. Due to the unidirectionality of the CMG helicase and replicative polymerases, the leading strand is synthesised continuously by polymerase epsilon (Polε) and the lagging strand is synthesised in discontinuous Okazaki fragments by polymerases alpha (Polα) and delta (Polδ) (Burgers and Kunkel, 2017).

Throughout S phase, replication forks may be challenged by various sources of replication stress that stall DNA synthesis through a variety of mechanisms. Some endogenous sources include challenging secondary DNA structures within repetitive sequences, barriers to replisome movement such as torsional stress or DNA-protein crosslinks (DPCs) and collisions with the transcription machinery (Zeman and

Cimprich, 2014). Importantly, the high levels of endogenous replication stress observed in cancer cells are exploited to develop novel anti-tumour drugs (Ubhi and Brown, 2019). In addition, several exogenous agents can be used experimentally to induce replication stress; for example, hydroxyurea (HU) stalls replication by depleting the cellular supply of deoxyribonucleotides (Bianchi et al., 1986). In the event of replication stress, the cell coordinates a variety of pathways to maintain genome stability and ensure the completion of replication.

These processes occur in a highly organised genome. The genome is spatially arranged in a hierarchical manner from nuclear compartments and chromosome territories (CTs) to topologically associated domains (TADs) and nucleosomes with a range of chromatin states (Gibcus and Dekker, 2013). Genome-wide chromosome conformation capture methods (such as Hi-C) are important tools to delineate how different regions of chromatin interact with each other, allowing the identification of TADs: loops of chromatin with borders that restrict the activity of regulatory elements between different TADs (Dixon et al., 2012; Li et al., 2018). In addition, genomic regions are identified according to their transcriptional status as either A or B compartments. A compartments are transcriptionally active, open and gene-rich whereas B compartments are transcriptionally silent, compact and gene-poor (Gibcus and Dekker, 2013). At the smaller scale, genetic material is packaged into units called nucleosomes comprised of DNA wrapped around a histone octamer of two H2A-H2B dimers and two histone H3-H4 dimers which are connected by H1-bound linker DNA (Luger et al., 2012). The local chromatin environment defined by composition of these nucleosomes, histone post-translational modifications (PTMs) and enrichment of non-histone proteins can affect the levels of transcription and chromatin compaction. In this sense, chromatin is categorised into relaxed, transcriptionally active euchromatin and compact, transcriptionally inactive heterochromatin. For example, histone marks enriched within active chromatin include methylated H3K4 and acetylated H3K9 and inactive marks include trimethylated H3K9 and H3K27 (Kouzarides, 2007). Regions of chromatin can also be localised within specific sub-nuclear compartments; for example, the nuclear/nucleolar periphery, nuclear pores, within nucleoli and in the nuclear interior.

Other than transcriptional regulation, genome organisation has major regulatory roles in the repair of DNA damage and in faithful DNA replication. In this Review, we will outline how the organisation of the genome, ranging from higher order spatial arrangement to the local chromatin environment, impacts the complex process of replication and the response to replication stress.

2 HOW CHROMATIN AFFECTS UNPERTURBED REPLICATION

2.1 Replication Timing

2.1.1 Higher Order Compartments and Nuclear Location

The spatial positioning of specific genomic regions greatly influences their replication timing (RT). This region-specific replication timing program is established during early G1 phase during a period called the timing decision point (TDP), coinciding with the restoration of genome organisation following cell division (Dimitrova and Gilbert, 1999; Dileep et al., 2015). Furthermore, TADs identified through mapping of the entire genome in several cell types correlate with the basic units of replication timing known as replication domains (RD); therefore, the global organisation of the genome contributes to RT at a greater level than the activation of individual replication origins (Pope et al., 2014). Notably, early and late replicating regions correlate with transcriptionally active A and silent B compartments, respectively, and this correlation can be followed even with resolution of nuclear sub-compartments (Ryba et al., 2010; Yaffe et al., 2010; Rao et al., 2014). Histone modifications enriched at specific nuclear compartments involved in transcriptional activation/repression also contribute to this control of replication timing (see below). Higher order reorganisation of the genome during mouse stem cell differentiation, which is coordinated with changes in transcription and cell fate determination, is also associated with changes in the RT program (Hiratani et al., 2008, 2010). Recently it was shown that reorganisation of the TADs is important in modulating origin firing efficiency (Li et al., 2018). During G1 phase, TADs can be rearranged by displacement of CTCF and chromatin decompaction mediated by transcription, which results in the relocation of replication origins to the TAD boundaries. At the spatial boundary of TADs, origin firing is more efficient due to the presence of proliferating cell nuclear antigen (PCNA) clusters that facilitate replication (Li et al., 2021). This describes a mechanism by which transcription plays a role in the regulation of replication initiation, a phenomenon described in multiple studies (Blin et al., 2019; Chen et al., 2019; Li et al., 2021).

In mammalian cells, the progression of S phase follows a distinct pattern, where euchromatin is replicated in early S phase, facultative heterochromatin at the nuclear/nucleolar periphery in mid S phase and constitutive heterochromatin in late S phase (Heinz et al., 2018) (**Figure 1**). Some heterochromatic regions of the genome, known as lamina-associated domains (LADs) are associated with the nuclear periphery and bind to the nuclear envelope via lamin proteins. These regions are generally replicated during mid-S phase, gene-poor and categorised under the transcriptionally repressed B compartment. In support of the nuclear periphery environment playing roles in replication timing, artificial targeting of mouse chromocentres containing constitutive heterochromatin to the nuclear periphery advances their replication timing from late to mid-S phase (Heinz et al., 2018). It has been hypothesised that the nuclear periphery contains the replication factors required for origin firing during mid S phase and therefore provides an environment where artificially tethered chromatin is replicated during this time. This advance in replication timing is not due to *de novo* deposition of facultative heterochromatin marks following artificial tethering, although constitutive histone marks are progressively lost at these regions following subsequent divisions (Heinz et al., 2018).

An important factor that connects genome organisation with the replication timing program is RIF1, a protein mainly

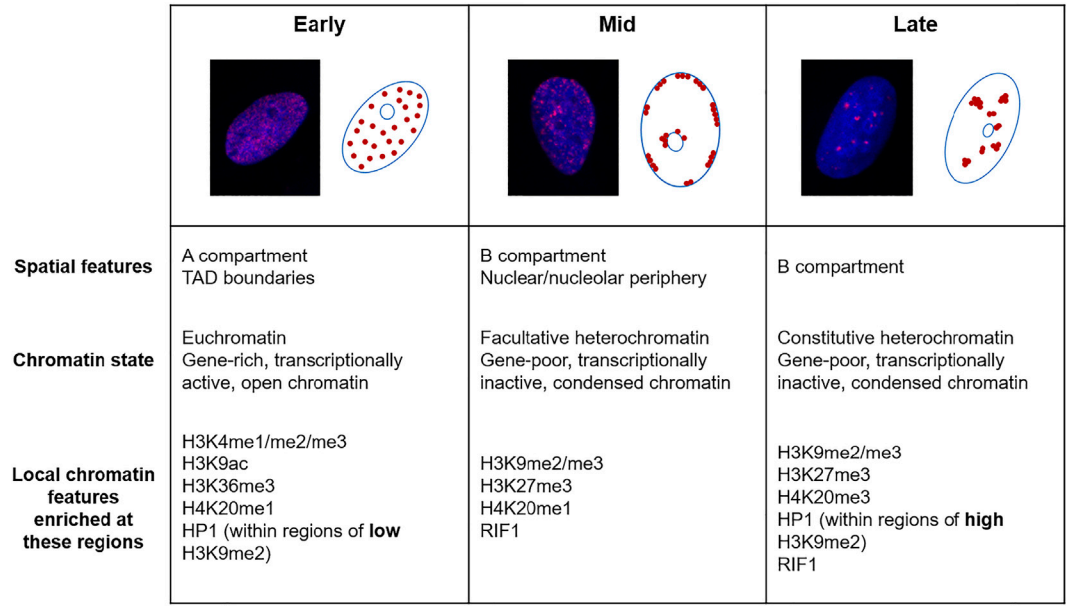

FIGURE 1 | The replication timing of the eukaryotic genome. Fluorescence microscopy images of U2OS cells labelled with 5-ethynyl-2′-deoxyuridine (EdU, red) and DAPI (blue) and schematics showing the three distinguishable sub-phases of replication; early, mid and late. Below are descriptions of the chromatin replicated in each of these sub-phases.

involved in maintaining the late replication status of specific genomic regions (Hayano et al., 2012; Yamazaki et al., 2012; Peace et al., 2014; Foti et al., 2016; Hafner et al., 2018; Gnan et al., 2021). Specifically, RIF1 interacts with the nuclear lamina and plays a key role in regulating the replication timing of the LADs (Roux et al., 2012; Foti et al., 2016). Recruitment of RIF1 to RIF1 associated domains (RADs) leads to chromatin reorganisation of these regions and delays the replication timing through regulating origin firing (Yamazaki et al., 2012; Davé et al., 2014; Hiraga et al., 2014; Hiraga et al., 2017; Foti et al., 2016; Alver et al., 2017). This control of replication origin firing by RIF1 is highly dependent on its conserved interaction with protein phosphatase 1 (PP1) (Davé et al., 2014; Hiraga et al., 2014; Hiraga et al., 2017; Mattarocci et al., 2014; Alver et al., 2017; Gnan et al., 2021). Mechanistically, recruitment of PP1 to replication origins by RIF1 reverses the phosphorylation of the MCM complex by DDK, a modification that is required for initiation of replication, and therefore prevents premature replication at specific genomic loci (Davé et al., 2014; Hiraga et al., 2014).

2.1.2 Link Between Histone Modifications and Replication Timing

In both mouse and human cell types, there is a broad correlation between early replicating regions with "active" chromatin marks such as methylated H3K4, H3K36me3, H4K20me1 and acetylated H3K9 but not "repressive" marks including di- and trimethylated H3K9 and H3K27me3 (Hiratani et al., 2008; Yokochi et al., 2009; Ryba et al., 2010; Picard et al., 2014) (**Figure 1**). In one study in human cells, the strongest correlation between later replication and repressive histone marks was detected with H3K9me2, which is commonly enriched at the nuclear periphery (Ryba et al., 2010).

In mouse embryonic stem cells (mESCs), the degree of DNA methylation affects replication timing through affecting histone modifications particularly at pericentric heterochromatin (Jørgensen et al., 2007a; Takebayashi et al., 2021). Partial loss of DNA methylation (Dnmt1 single knockout), as well as loss of other repressive chromatin modifiers, results in earlier replication of pericentric major satellite repeats (Jørgensen et al., 2007a). Interestingly, complete abolishment (Dnmt1, Dnmt3a, and Dnmt3b triple knockout) results in abnormal enrichment of H3K27me3 at some loci of pericentric heterochromatin, where it instead causes a delay in replication timing (Takebayashi et al., 2021). However, this redistribution of H3K27me3 does not cause RT changes in other regions of the genome and replication timing at these loci appears to be more dependent on transcriptional changes caused by loss of DNA methylation. In another study, it was demonstrated that H3K27me3 enrichment broadly correlates with mid-S phase replicating origins (Picard et al., 2014).

Monomethylation and dimethylation of lysine 20 of histone H4 (H4K20me1 and me2) are found throughout the genome and coincide with chromatin compaction and transcriptional inactivation, whereas trimethylation (H4K20me3) is exclusively enriched at pericentric heterochromatin and imprinting control regions (Schotta et al., 2004; Karachentsev et al., 2005; Delaval et al., 2007). The methyltransferase PR-Set7 deposits H4K20me1 at replication origins and facilitates loading of the pre-RC complex during licencing. This activity is regulated by ubiquitin-mediated degradation of PR-Set7 during S phase to prevent re-replication (Tardat et al., 2010). In contrast, SET8-dependent methylation of H4K20 has been implicated in

repressing replication origin licencing through facilitating chromatin compaction (Shoaib et al., 2018). H4K20me1 is present in approximately half of origins and enriched in early- and mid-replicating regions (Picard et al., 2014). The control of the proportion of nucleosomes with each methylation state of H4K20 is critical to regulate origin selection to ensure no re-replication of DNA, allowing faithful replication of the genome (Schotta et al., 2008; Beck et al., 2012). In addition, H4K20me2 is enriched at replication origins, where it acts as a binding site for ORC1, and H4K20me3 is vital for ensuring the late replication of pericentric heterochromatin (Kuo et al., 2012; Brustel et al., 2017).

2.1.3 Modulation of Replication Timing by Heterochromatin Protein 1

Heterochromatin protein 1 (HP1) is a non-histone protein that is recruited to regions of heterochromatin through binding to methylated H3K9 (Lachner et al., 2001). This factor is an identifier of heterochromatin in several organisms and plays a major role in maintaining the local chromatin environment of heterochromatin. Therefore, the different isoforms of HP1 (HP1α, β and γ in humans) play varying roles in regulating processes including transcription, chromatin compaction (Eissenberg et al., 1990; Kwon and Workman, 2011), cell differentiation (Mattout et al., 2015; Casale et al., 2019) and DNA repair (Dinant and Luijsterburg, 2009; Bártová et al., 2017). In *Drosophila,* HP1 has contrasting roles in replication timing depending on nuclear localisation and H3K9 methylation status. HP1 has been implicated in promoting the early replication timing of some regions with low H3K9me2 enrichment and in mediating later replication of regions with greater H3K9 methylation, such as pericentric heterochromatin (Schwaiger et al., 2010) (**Figure 1**). Additionally, HP1 is sufficient to induce the late replicating characteristic normally seen in heterochromatic regions when artificially tethered to earlier replicating loci (Pokholkova et al., 2015). Interestingly, HP1 interacts with ORC, a complex that promotes origin firing, and this association is important in HP1α localisation and heterochromatin formation (Pak et al., 1997; Prasanth et al., 2010).

However, mouse HP1 was shown to be dispensable for the maintenance of late replication within pericentric heterochromatin, with the methylation status of H3K9 being more important in establishing the RT of these regions (Wu et al., 2006). Therefore, it is still unclear whether the roles of HP1 in controlling the late replication of pericentric heterochromatin is conserved across organisms or whether H3K9 methylation, a repressive mark which recruits HP1, is more important.

2.2 Restoration of Chromatin Following Replication

2.2.1 Formation of Nucleosomes Behind the Fork

During replication, histones must be dissociated from the DNA to allow progression of the replication fork and it is important that they are accurately restored on the parental strand and duplicated onto the daughter strand. Movement of the replisome destabilises chromatin, leading to decondensation and increased mobility of linker histone H1 (Gasser et al., 1996; Contreras et al., 2003; Kuipers et al., 2011). Indeed, mobility of H1 regulated by its phosphorylation is involved in controlling replication timing, presumably by allowing chromatin relaxation (Contreras et al., 2003; Alexandrow and Hamlin, 2005; Katsuno et al., 2009; Thiriet and Hayes, 2009).

Histone deposition is not identical across the leading and lagging strands of replication, and therefore strand-specific mechanisms of chromatin restoration have been identified which are dependent on several components of the replisome. For example, MCM2 and polymerase α directly bind to parental H3-H4 and recycle it onto the lagging strand, and Dpb3 and Dpb4 (accessory subunits of polymerase ε) facilitate the deposition of H3-H4 onto the leading strand (Gan et al., 2018; Petryk et al., 2018; Yu et al., 2018; Li et al., 2020). It is important that these strand-specific mechanisms of histone deposition are highly regulated to allow symmetric inheritance of histones, alongside their post-translational modifications, during duplication of the genome.

As the replication fork progresses, parental histones are recycled onto the nascent DNA and newly synthesised histones are added in any gaps with the help of histone chaperones (Alabert and Groth, 2012; Stewart-Morgan et al., 2020). For example, chromatin assembly factor 1 (CAF-1) is an important chaperone which interacts with the replication factor PCNA to deposit histones as replication occurs and has roles in maintaining heterochromatin and transcriptional regulation (Yu et al., 2015). The anti-silencing function 1 (ASF1) chaperone interacts with Mcm2-7 and deposits parental and new H3-H4 dimers directly behind the replication fork in a process that is disrupted during replication stress (Groth et al., 2007; Jasencakova et al., 2010). Another important histone chaperone is called facilitates chromatin transcription (FACT), which also interacts with the replication machinery and has roles in both the eviction and deposition of histones during replication (Formosa, 2012). In order to maintain the epigenomic features of the parental DNA, specific histone variants must also be deposited onto the nascent DNA using specialised histone chaperones. This restoration of chromatin does not always occur in a replication-dependent manner; for example, centromere protein A (CENP-A, a H3 variant) deposition by Holliday junction recognition protein (HJURP) at centromeres and H3.3 deposition by death-associated protein 6 (DAXX) at pericentric and telomeric heterochromatin occur after replication in the following G1 phase (Ahmad and Henikoff, 2002; Jansen et al., 2007; Foltz et al., 2009; Goldberg et al., 2010; Lewis et al., 2010).

2.2.2 Maintenance of Histone Modifications

Following the deposition of nucleosomes onto nascent DNA, it is important that histone PTMs from the parental chromatin are inherited so that the newly synthesised chromatin maintains the same transcriptional regulation, conformation, and replication timing. Methylation and acetylation marks are maintained on parental histones, but these are only present on half of the chromatin following replication due to the incorporation of new unmodified histones, so further processes are required to

ensure full restoration of PTMs (Scharf et al., 2009; Xu et al., 2012a; Alabert et al., 2015). If a residue is mono-/dimethylated (e.g., H3K9me1/me2, H3K27me1/me2 and H4K20me1/me2), this mark is diluted following replication and newly incorporated histones obtain these PTMs *de novo* within one cell cycle to become identical to the parental histones (Alabert et al., 2015). On the other hand, trimethylation (e.g., H3K9me3 and H3K27me3) is established slowly and continuously, independent of replication, across several cell divisions (Alabert et al., 2015; Reverón-Gómez et al., 2018). Consequently, oscillations in the levels of histone marks can be observed throughout the cell cycle, and these are not equal at all loci and for all types of histone modification. For example, the H3K4me3 mark enriched at active promoters recovers much quicker than repressive marks such as H3K9me3 and H3K27me3 (Alabert et al., 2015; Reverón-Gómez et al., 2018; Stewart-Morgan, Petryk and Groth, 2020).

2.2.3 Roles of Heterochromatin Protein 1 in Maintaining Heterochromatin

In mouse cells, the interaction of HP1 with the histone chaperone CAF-1, particularly the large p150 subunit, is essential for S phase progression (Quivy et al., 2008). Disruption of this interaction impedes replication of pericentric heterochromatin in a manner that is independent of the canonical histone deposition roles for CAF-1. Intriguingly, p150 is essential for mouse embryo viability during the period of development where HP1-enriched domains are formed (the 8–16 cell stage), suggesting major importance for the p150-HP1 interaction in preserving the survival of cells that are rapidly proliferating and therefore undergoing frequent DNA replication (Houlard et al., 2006).

Additionally, the p150-HP1 complex colocalises with the histone methyltransferase SETDB1 to promote monomethylation of non-nucleosomal histone H3.1 (Loyola et al., 2009). This is important to allow formation of H3K9me1 at newly incorporated histones immediately following DNA replication, which then nucleates formation of trimethylated H3K9 catalysed by Suv39H1/H2. Additionally, the HP1-CAF-1-SETDB1 complex has roles in depositing HP1 at sites of pericentric regions already enriched in H3K9me3. Therefore, CAF-1 has numerous roles in preserving repressive H3K9me3 and HP1 enrichment at pericentric heterochromatin following replication (Loyola et al., 2009).

3 ROLE OF THE LOCAL CHROMATIN ENVIRONMENT DURING REPLICATION STRESS

Replication stress (RS) is defined as the slowing or blocking of replication fork progression by a range of endogenous and exogenous sources. Mild RS only results in the slowing of the replication fork velocity and the activation of dormant origins in order to complete replication (Técher et al., 2017). However, as RS gets more severe, the cellular response becomes more intricate. Prolonged RS may lead to uncoupling of the replisome, resulting in production of stretches of replication protein A (RPA)-bound ssDNA which activate the ataxia telangiectasia and Rad3-related (ATR) kinase (Byun et al., 2005). Downstream effectors of ATR such as checkpoint kinase 1 (CHK1) activate the intra-S phase checkpoint which protects the genome from further instability by inhibiting late origin firing and cell cycle progression and promoting DNA repair pathways (Iyer and Rhind, 2017). In a process called fork reversal, the stalled replication fork is remodelled to form a four-way junction to provide protection against excessive degradation (Neelsen and Lopes, 2015). Although this fork reversal, in combination with the recruitment of multiple factors including RAD51 and FANCD2, prevents degradation by nucleases such as MRE11, DNA2 and MUS81, some controlled resection is required to allow rescue of replication (Bryant et al., 2009; Schlacher et al., 2012; Thangavel et al., 2015; Lemaçon et al., 2017). Replication restart following this remodelling of the fork requires the recruitment of several HR proteins, importantly RAD51, which facilitate homology-directed restart (Ait Saada et al., 2018). Therefore, components of homologous recombination, a pathway canonically associated with DSB repair, play various roles in fork remodelling, protection and restart in the event of replication stress. In this section, we will discuss how several aspects of the local chromatin environment are involved in the regulation of these pathways that play central roles in the resolution of replication stress (**Table 1**).

3.1 Histone Variants

In humans, there are several variations of histones H2A, H2B, H3, and H4 which have different sequences to their canonical histone counterparts and may be localised to specific genomic regions to influence the structure and function of the chromatin (Martire and Banaszynski, 2020). Here, we will describe the importance of some of these histone variants during replication and particularly in the event of replication stress.

3.1.1 Histone H2AX

The histone H2A variant H2A.X has major implications in genome stability: specifically, H2A.X phosphorylated at serine-139 rapidly after DSB induction, creating a mark known as γH2AX (Rogakou et al., 1998). γH2AX formation catalysed by the ATR signalling cascade also occurs following replication stalling before the collapse of forks in DSBs, although inhibition of Chk1, a downstream factor of ATR, has been shown to induce γH2AX formation (Ward and Chen, 2001; Sirbu et al., 2011). This association of γH2AX presumably facilitates recruitment of DNA repair proteins and studies in yeast have revealed a role in repairing replicative damage in cells without intra-S phase checkpoint activation (Redon et al., 2003). Interestingly, γH2AX distribution following replication stress is not equal across the genome and greater association is seen in commonly fragile regions containing compact chromatin which are depleted of transcription start sites and CpG islands (Lyu et al., 2019). Therefore, this modification could be primarily important to promote certain pathways involved in the resolution of replicative damage in specific chromatin contexts, or this enrichment could reflect persistent replication stress particularly in fragile regions of the genome that are difficult

to repair. In addition, ATR kinase activity and H2AX phosphorylation also occur during unperturbed S phase to facilitate the correctly timed transition into G2 phase (Saldivar et al., 2018). Consequently, γH2AX not only has direct roles during stressed replication but also is essential to maintain genome integrity by preventing premature entry into mitosis and under-replication of the genome.

3.1.2 Macro H2A

MacroH2A is a subfamily of histone H2A variants comprised of three isoforms called macroH2A1.1, macroH2A1.2 (which are splice variants from the same gene) and macroH2A2 (Rasmussen et al., 1999; Costanzi and Pehrson, 2001). Structurally, these proteins are composed of a H2A domain with an N-terminal non-histone macro domain and are approximately three times the size of other H2A variants (Pehrson and Fried, 1992). Some functions of this histone variant have been linked to X chromosome inactivation (Costanzi and Pehrson, 1998; Mermoud et al., 1999; Rasmussen et al., 2000), transcriptional regulation (Ouararhni et al., 2006; Gamble et al., 2010; Creppe et al., 2012) and nucleosome organisation (Angelov et al., 2003; Abbott et al., 2004; Chakravarthy and Luger, 2006; Muthurajan et al., 2011; Chakravarthy et al., 2012). Additionally, macroH2A is enriched at heterochromatin regions, particularly those marked by H3K9me3, and has roles in higher genome organisation (Douet et al., 2017; Kozlowski et al., 2018).

Alongside functions in homology-directed DSB repair (Xu et al., 2012b; Khurana et al., 2014), macroH2A1.2 also has roles during replication stress, where it is deposited onto chromatin by the FACT histone chaperone (Kim et al., 2018). This activity is particularly important at common fragile sites (CFS), regions that are more prone to replication stress induced damage. MacroH2A deposition is also assisted by the LSH chromatin remodeller and promotes BRCA1 and RAD51 recruitment to stalled forks to promote fork protection and facilitate repair (Kim et al., 2018; Xu et al., 2021). Recently, it was shown that loss of this macroH2A deposition is associated with an increase of H4K20me2 at stalled forks which then favours 53BP1 recruitment to stalled forks rather than BRCA1, causing a detrimental effect on fork protection (Xu et al., 2021). MacroH2A1.1 and 1.2 are enriched on the mammalian female inactive X (Xi) chromosome, a highly condensed genomic region with increased susceptibility to replication stress (Costanzi and Pehrson, 1998; Koren and McCarroll, 2014). The macroH2A1.2 variant is involved in supressing replication stress at the Xi, whereas the splice variant macroH2A1.1 activates the alternative end joining DSB repair pathway, leading to Xi anaphase defects in the absence of H2A1.2 (Sebastian et al., 2020). Overall, these studies suggest an importance for macroH2A in facilitating the proper repair of difficult-to-replicate genomic loci such as CFS's and the inactive X chromosome. The replication stress-dependent recruitment of macroH2A to fragile sites is mainly transient but some remains after resolution of replication stress and protects these difficult to replicate regions from future replicative damage (Kim et al., 2018). Since macroH2A has roles in repressing gene expression, X inactivation and nucleosome organisation, it would be important to see whether this continued enrichment alters transcription and genome organisation at these sites.

3.1.3 H2A.Z

H2A.Z is a variant of histone H2A that is incorporated throughout the cell cycle and has the ability to alter the physical properties of the nucleosome and create specialised chromatin structures (Fan et al., 2002, 2004). This histone variant has conserved functions in the regulation of transcription in euchromatic genes (Zhang et al., 2005; Hardy et al., 2009). Nucleosomes associated with H2A.Z also enhance replication origin firing efficiency through promoting H4K20 dimethylation and ORC1 recruitment (Long et al., 2020). In yeast, incorporation of the H2A.Z homologue Htz1 by Swi2/Snf2-related chromatin remodelling complex (SWR-C) is important in maintaining genome stability following both DSBs (Kalocsay et al., 2009; Horigome et al., 2014) and replication stress (Van et al., 2015; Srivatsan et al., 2018). Specifically, Hitz1 has roles in preventing the misincorporation of nucleotides during replication and in promoting the repair of replicative damage (Van et al., 2015; Srivatsan et al., 2018). Incorporation of Htz1 during replication stress has been hypothesised to prevent collapse of forks into DSBs by two mechanisms: either it stabilises the fork to prevent replisome dissociation, or it is incorporated after replisome dissociation to prevent further degradation of the fork by nucleases (Srivatsan et al., 2018). Furthermore, the balance of H2A.Z on chromatin is vital: as removal by the INO80 chromatin remodeller is also important in maintaining replication fork stability and to allow HR to occur (Papamichos-Chronakis et al., 2011; Lademann et al., 2017).

3.1.4 CENP-A at Centromeres

Despite being located between pericentromeric regions expressing features of heterochromatin, mammalian centromeres display active epigenetic marks (e.g., H3K4me2 and H3K36me2) combined with production of long non-coding RNA transcripts (Sullivan and Karpen, 2004). In human cells, centromeres are composed of tandem alpha satellite repeats and enriched in CENP-A, a histone H3 variant specific for centromeres which has key roles in formation of kinetochores to allow proper chromosome segregation (Barra and Fachinetti, 2018). Because of their involvement in chromosome segregation, the maintenance of centromere integrity during replication is vital to ensure proper cell division.

Unlike many canonical histone variants, CENP-A is not deposited onto newly replicated DNA during S-phase but is instead incorporated in the following G1-phase by its dedicated histone chaperone, HJURP (Jansen et al., 2007). Contrary to this, CENP-A has recently been shown to possess key roles in maintaining centromere integrity during DNA replication (Giunta et al., 2021). CENP-A deposition prevents the formation of centromeric R-loops formed as a consequence of transcription-replication conflicts (TRCs) during late S phase, thereby promoting fork progression. Centromeres depleted of CENP-A display multiple replication-associated defects such as error-prone mitotic DNA synthesis (MiDAS) and chromosomal

translocations, breakages, and fragmentation. These chromosomal aberrations are the result of recombination between alpha satellite repeats in an R-loop dependent manner rather than being due to defects in chromosomal segregation during mitosis (Giunta et al., 2021). The specialised function for CENP-A in removing R-loops is important because some alpha-satellite RNA transcripts remain associated with the centromere and could therefore disrupt the progression of incoming replication forks (McNulty et al., 2017).

3.2 Role of Linker Histone H1

In human cells, eviction of the linker histone H1 by the histone chaperone SET results in sensitivity to DNA damage (Mandemaker et al., 2020). Although depletion of SET does not alter normal S phase progression, it does cause resistance to the replication stress-inducing agent HU; suggesting important roles for chromatin-bound H1 during perturbed replication (Mandemaker et al., 2020). In agreement, overexpression of SET causes slower S phase progression upon treatment of HU (Kalousi et al., 2015). SET also promotes retention of the heterochromatin factors KAP1 and HP1 on chromatin which causes chromatin compaction and inhibition of HR repair of collapsed forks (Kalousi et al., 2015). Potentially, loss of SET and subsequent H1 retention and chromatin decompaction could allow for greater access of DNA repair factors to replicative lesions, thereby promoting proper repair and cell survival.

In addition, the linker histone dH1 in *Drosophila* has been implicated in preventing the accumulation of R-loops, a structure which can induce replication stress (Bayona-Feliu et al., 2017). Upon depletion of dH1, transcriptional repression is relieved within heterochromatin regions, leading to the accumulation of R-loops. Interestingly, R-loop accumulation was not seen upon depletion of HP1a, suggesting that this effect is only seen upon loss of H1 and is not a general effect of transcriptional derepression in heterochromatin (Bayona-Feliu et al., 2017), although loss of repression in repetitive regions has been associated with R-loop prevention in *C. elegans* (Zeller et al., 2016). Notably, in mouse cells depleted of histone H1, fork stalling as a result of transcription-replication conflicts has also been observed (Almeida et al., 2018). H1 loss leads to chromatin decompaction which causes acceleration of both transcription initiation and replication leading to pathogenic accumulation of R-loops and collisions between the transcription and replication machineries (Almeida et al., 2018).

The linker histone H1 has been shown to have roles in both preventing the onset of replication stress and in facilitating the repair of replicative damage. Currently, it is unclear how H1 elicits these effects: whether it is due to its roles in organising nucleosome particles into stable higher order structures and maintaining proper spacing between nucleosomes or in the formation of heterochromatin and maintaining transcriptional repression (Happel and Doenecke, 2009).

3.3 Histone Modifications and Replication Stress

3.3.1 Histone Ubiquitination

Ubiquitination of histone H2AK13 and K15 adjacent to DNA lesions plays a central role in the DNA damage response, where these marks facilitate the recruitment of several DNA repair factors (Smeenk and Mailand, 2016). During unchallenged S phase, H2A ubiquitination by RNF168 and activation of the DNA damage response (DDR) is required for normal replication progression and to prevent fork stalling (Schmid et al., 2018). In addition, RNF168 plays a role in preventing accumulation of reversed forks through restarting stalled replication, particularly at difficult to replicate repetitive regions of the genome (Schmid et al., 2018). Formation of γH2AX by ATR and ATM kinases occurs upstream of H2A ubiquitination and is involved in coordinating this DDR activation during S phase (Schmid et al., 2018; Nakamura et al., 2021). Interestingly, ATM inhibition increases activation of the histone ubiquitin response upon fork breakage by camptothecin. This is possibly because ATM is involved in a negative feedback loop alongside PLK1 where end resection at single-ended DBSs deactivates the histone ubiquitination pathway (Nakamura et al., 2021).

Monoubiquitinated histone H2B is a dynamic histone mark first associated with transcription and repair (Kao, 2004; Fleming et al., 2008) also possessing other roles in controlling chromatin compaction during DSB repair (Moyal et al., 2011; Nakamura et al., 2011). In yeast, the ubiquitin ligase responsible for H2B monoubiquitination, Bre1, is maintained on replicating DNA and is further enriched at stressed replication forks (Trujillo and Osley, 2012; Lin et al., 2014; Hung et al., 2017). During replication, H2B ubiquitination plays a role in regulating nucleosome assembly onto replicating DNA, which facilitates normal fork progression and replisome stability during replication stalling by HU (Trujillo and Osley, 2012). In contrast, H2Bub has also been shown to restrict fork progression upon HU-induced stress through coordinating chromatin assembly and activation of the Rad53-dependent intra-S checkpoint (Lin et al., 2014). Intriguingly, other roles for H2Bub during replication stress are linked to its involvement in regulating DNA damage tolerance (DDT) pathways (Northam and Trujillo, 2016; Hung et al., 2017). This mark promotes polymerase eta (Polη)-dependent translesion synthesis (TLS) and suppresses the more mutagenic polymerase zeta (Polζ)-dependent pathway following fork stalling by HU and UV treatment (Northam and Trujillo, 2016). In another study, monoubiquitinated H2B was shown to promote recombination-dependent lesion bypass following treatment with alkylating agents, specifically by altering the chromatin dynamics and allowing RAD51 recruitment (Hung et al., 2017). This mark then facilitates repair of these bypassed lesions post-replication, possibly by promoting activation of the G2/M checkpoint. Therefore, this histone modification has cell cycle-specific roles for maintaining genome replication following replicative damage checkpoint (Hung et al., 2017). Whilst these studies have elucidated the importance of H2B

monoubiquitination and chromatin dynamics during replication stress, it remains unclear how this modification has seemingly contradictory roles in both stalling of replication through checkpoint activation and coordinating DDT pathways to allow DNA synthesis beyond lesions and subsequent post-replicative repair.

3.3.2 Histone H3 Modifications

Methylation of H3K4 has been implicated to have contrasting roles in the protection of stalled replication forks in mammalian cells depending on which methyltransferase deposits this mark (Ray Chaudhuri et al., 2016; Higgs et al., 2018). In mouse cells, MLL3/4 is responsible for depositing H3K4me1 and H3K4me3 at stressed forks, where it promotes fork degradation by MRE11 in BRCA-deficient mouse cells with existing defects in fork protection (Ray Chaudhuri et al., 2016). Therefore, loss of H3K4 methylation by MLL3/4 rescues fork protection in BRCA-deficient cells, leading to chemoresistance (Ray Chaudhuri et al., 2016). In contrast, monomethylation of H3K4 at stressed forks by human SETD1A promotes RAD51-dependent replication fork protection from degradation by DNA2 and CHD4 (Higgs et al., 2018). This fork protection is mediated through chaperoning of histone H3.1 by the Fanconi anaemia factor FANCD2 (Higgs et al., 2018), a factor involved in interstrand crosslink repair, RAD51-mediated replication fork protection and the replication of fragile sites (Schlacher et al., 2012; Sato et al., 2012; Madireddy et al., 2016; Higgs et al., 2018). It is interesting that methylation of H3K4 by different methyltransferases leads to opposite effects on fork protection and this could mean that these enzymes are either active at different times during the replication stress response, are recruited differentially to certain genomic loci or are responsible for methylation within a specific local chromatin context (e.g., presence of H2BUb) (Wu et al., 2008; Higgs et al., 2018). Additionally, H3K4me is commonly found in active regions, so it would be important to investigate whether these mechanisms are mostly important at these regions or whether the mark is added *de novo* throughout the genome in response to replication stress. Notably, fork protection upon loss of MLL3/4 was observed only in BRCA-deficient cells whereas fork degradation by SETD1A loss was observed in BRCA-WT cells (Ray Chaudhuri et al., 2016; Higgs et al., 2018). Therefore, the promotion of fork degradation by MLL3/4 mediated methylation of H3K4 may specifically occur when HR-mediated fork protection is lost.

In yeast, H3K4 methylation plays a role in slowing down fork progression through highly transcribed regions to prevent transcription-replication conflicts, thereby ensuring faithful replication (Chong et al., 2020). Indeed, transcriptionally active genes are commonly decorated with H3K4me3 (Santos-Rosa et al., 2002; Guenther et al., 2007). Another histone H3 modification with roles in preventing TRCs is methylated H3K9 (Zeller et al., 2016), a mark commonly enriched at heterochromatin that plays a role in HP1 recruitment and transcriptional repression (Bannister et al., 2001; Lachner et al., 2001; Nakayama, 2001). In *C. elegans*, H3K9me2/me3 function to repress genes in repetitive elements, thereby suppressing R-loop formation and preventing replication stress (Zeller et al., 2016).

Trimethylated H327 is also a mark of transcriptionally inactive chromatin and is deposited by enhancer of zeste homologue 2 (EZH2), a component of the polycomb repressive complex 2 (PRC2), during both G1 and S phases (Hansen et al., 2008; Morey and Helin, 2010). Alongside its important roles in transcription and cell identity (Wiles and Selker, 2017), H3K27me3 also plays a role during replication stress (Rondinelli et al., 2017). Methylated H3K27 is involved in the recruitment of MUS81 to stalled forks, an endonuclease which creates DSBs to facilitate HR-mediated fork restart (Hanada et al., 2007; Rondinelli et al., 2017). Whilst activation of this pathway is required for replication restart in BRCA2-deficient cells, excessive fork degradation by MUS81 can be toxic. Therefore, in a similar manner to with the MLL3/4/H3K4me/MRE11 axis (Ray Chaudhuri et al., 2016), loss of EZH2 and subsequent fork protection from MUS81 endonuclease activity leads to chemoresistance in BRCA-deficient cells (Rondinelli et al., 2017). These roles of epigenetic modifications in protecting the replication fork, thereby allowing cancer cells to become resistant to therapies, have major clinical implications. For example, expression levels of EZH2 could be used as a biomarker to predict resistance to drugs such as poly (ADP-ribose) polymerase (PARP) inhibitors and allow more stratified treatment for BRCA-deficient cancers (Rondinelli et al., 2017).

3.3.3 Histone H4 Modifications

In addition to its roles in replication timing, the methyltransferase PR-Set7 responsible for H4K20 methylation has roles in facilitating S phase progression and during replication stress, where it interacts with the key replication factor PCNA (Jørgensen et al., 2007b). In addition, depletion of this factor affects the number and velocity of replication forks and causes activation of p53 and Chk1 dependent checkpoints. Loss of H4K20 methylation in this manner increases the frequency of replicative DNA breaks which then recruit repair factors such as RPA, RAD51 and 53BP1 (Jørgensen et al., 2007b; Tardat et al., 2007). Importantly, unmethylated H4K20 which marks newly incorporated histones plays a role in regulating the post-replicative repair of DNA lesions obtained during S phase (Saredi et al., 2016). H4K20me0 provides a binding site for TONSL-MMS22L in G2/M phases, and this complex then promotes HR repair (Duro et al., 2010; Saredi et al., 2016). Overall, these studies show roles for H4K20 methylation status in the regulation of replication fork progression and, in addition, the repair of replicative lesions after S phase completion.

Histone acetyltransferase 1 (HAT1) catalyses the acetylation of histone H4 at lysine 5 and lysine 12 and this activity is important in the processing of newly deposited histones during replication (Nagarajan et al., 2013). Depletion of HAT1 results in replication stalling and sensitivity of cells to replication stress induced by HU (Nagarajan et al., 2013; Agudelo Garcia et al., 2020). The importance of HAT1 in maintaining genome stability during replication stress is due to its roles in protecting stalled forks from degradation by MRE11 (Agudelo Garcia et al., 2020). In contrast, acetylation of H4K8 by p300/CBP-associated factor (PCAF)

promotes nucleolytic degradation of stalled forks in BRCA-deficient cells, suggesting differential roles for histone acetylation on replication fork protection depending on which residue is modified and by which acetyltransferase (Kim et al., 2020). Acetylation of H4K8 by PCAF provides a binding site for MRE11 and EXO1 nucleases, and this activity is suppressed by ATR-mediated phosphorylation of PCAF to maintain tight control of stalled fork degradation. In BRCA2-deficient tumours where fork protection is compromised, low levels of PCAF activity is associated with resistance to PARP inhibition through restoring fork protection (Kim et al., 2020).

3.4 HP1 During Replication Stress

In a more recent study, HP1β has been shown to have some important roles during normal replication progression and in the presence replication stress in mammalian cells. Knockout of HP1 in mouse embryonic fibroblasts (MEFs) led to reduced cell growth and fork speeds in combination with enhanced formation of DNA damage foci (Charaka et al., 2020). Upon depletion of deoxyribonucleotides, both mouse and human (Hela) cells depleted of HP1 displayed increased fork stalling and defective fork restart. In support for key roles of HP1 during replication stress, depletion also sensitises human and mouse cells to HU and cisplatin, resulting in increased levels of chromosomal aberrations (Charaka et al., 2020). It is currently unknown exactly how HP1 plays a role in the cellular response to replication stress, however it could share some similarities with its roles in DSB repair. For example, HP1 depletion leads to reduced recruitment of BRCA1 to DSBs, so perhaps the importance of HP1 in the resolution of replication stress is dependent on an ability to recruit BRCA1, which then promotes RAD51-dependent fork protection (Schlacher et al., 2012; Lee et al., 2013). On the other hand, HP1 retention on heterochromatin has been implicated in *preventing* homology-directed repair of heterochromatic DSBs and mobilisation of HP1β plays a role in the activation of DDR signalling (Ayoub et al., 2008; Kalousi et al., 2015). Currently, it is unclear which specific pathways HP1 plays a role in to resolve replication stress and whether it is strictly important for RS in heterochromatin or whether it has global roles.

4 MOVEMENT OF STRESSED REPLICATION FORKS

The mobility of DNA lesions, namely DSBs, has been well documented and has been shown to be vital for proper repair in specific circumstances. In mammalian cells for example, whilst heterochromatic DSBs are positionally stable in G1 and are repaired by non-homologous end joining (NHEJ), they are relocated to the periphery of heterochromatin domains during S and G2 phases to allow recruitment of HR factors (Tsouroula et al., 2016). The mobility of DSBs is a conserved response that allows formation of the RAD51 filament and repair of DSBs, especially within heterochromatin, and SUMOylation is an important post translational modification which controls this process (Oza et al., 2009; Ryba et al., 2010). Therefore, certain nuclear compartments provide a protective environment for DNA repair, and this is also important in the event of replication stress. In this section we will discuss how some stressed forks do not remain positionally stable and are relocated to specific nuclear compartments in a similar manner to DSBs.

4.1 The Nuclear Pore Complex and Replication Stress

4.1.1 Importance in Repetitive vs. Non-repetitive Sequences

During S phase in yeast, replication stress prone expanded CAG repeats relocate to the nuclear periphery where they interact with components of the nuclear pore complex (NPC) to prevent chromosomal breakages (Su et al., 2015). For this movement to occur, the repair proteins RPA, Rad59 and Rad52 are SUMOylated by Mms21 SUMO E3 ligase which permits their interaction with the SUMO interacting motif (SIM) of Slx5 (Whalen et al., 2020). SUMOylation of the ssDNA-binding protein RPA inhibits Rad51 binding at the stalled fork and this inhibition is lost when collapsed forks associate with NPCs, possibly by degradation of SUMOylated proteins promoted by the SUMO-targeted ubiquitin ligase (STUbL) Slx5/8 (Su et al., 2015; Whalen et al., 2020). Therefore, homology-directed fork restart is suppressed until movement of these repetitive sequences to the nuclear pore. Intriguingly, in a study where replication stress was induced at a unique sequence in yeast, RAD51 binding and activity were able to occur before anchoring to the NPC (Kramarz et al., 2020). Here, SUMOylation by Pli1 was shown to promote fork mobility, and anchoring of the fork to the NPC caused removal of the SUMO chains and allowed recombination-dependent restart. Alternatively, recombination-dependent restart could still even occur without NPC anchoring when SUMOylation by Pli1 was selectively inhibited (Kramarz et al., 2020).

Overall, these studies suggest that different mechanisms of replication stress resolution occur depending on the nature of the DNA sequence (**Figure 2**). In repetitive sequences (i.e., expanded CAG repeats), RAD51 binding is limited until tethering to the NPC, which is essential to allow replication restart and prevention of DSBs (Su et al., 2015; Whalen et al., 2020). Conversely, within non-repetitive sequences, RAD51 activity occurs at stalled forks before relocation to the NPC, and restart can occur with or without NPC tethering (Kramarz et al., 2020). Therefore, mobility of stalled forks and tethering to the NPC may only be necessary for repetitive sequences, which are likely to undergo detrimental recombination events if in close proximity to other repetitive sequences. The involvement of SUMO is similar in both repetitive and unique sequences, where it promotes mobility of stalled forks but is removed upon tethering to the NPC for replication restart to occur (Su et al., 2015; Kramarz et al., 2020; Whalen et al., 2020).

4.1.2 Anchoring of Stressed Telomeres to the Nuclear Pore Complex

Telomeres in yeast and human cells are relocated to the nuclear pore complex in response to genomic stress (Khadaroo et al., 2009; Churikov et al., 2016; Pinzaru et al., 2020). In budding yeast, this relocalisation of eroded telomeres is SUMO- and STUbL-

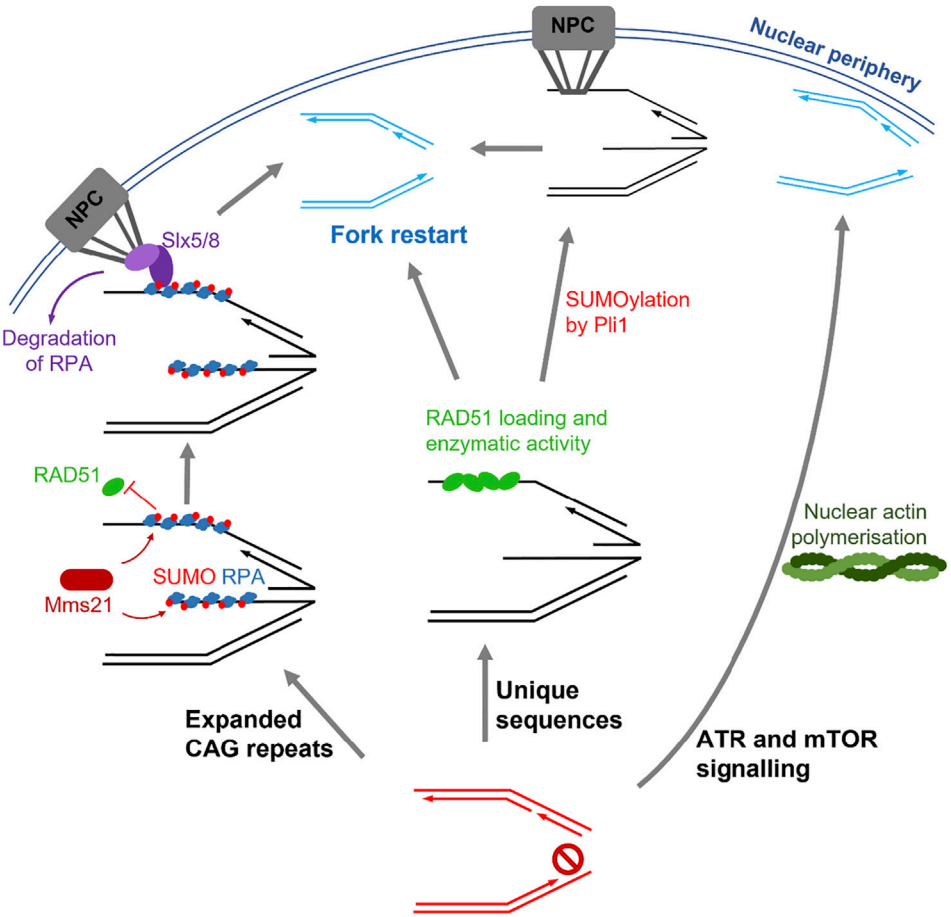

FIGURE 2 | Summary of the pathways involved in stressed fork mobility. Left: fork stalling induced by expanded CAG repeats leads to SUMOylation of repair proteins such as RPA by Mms21, which block RAD51 binding. Tethering of the fork to the nuclear pore complex (NPC) by Slx5/8 binding releases this inhibition and allows fork restart. Centre: Replication fork stalling within unique sequences results in loading of RAD51 in the nucleoplasm followed by SUMOylation events by Pli1, which allow NPC tethering and fork restart. Alternatively, fork restart can also occur in the absence of SUMOylation and NPC tethering. Right: ATR and mTOR signalling during replication stress leads to polymerisation of nuclear actin. Stalled forks then move along the actin filaments to the nuclear periphery, where fork restart can occur.

dependent and promotes type II recombination [the mechanism of alternative lengthening of telomeres (ALT) in yeast] (Churikov et al., 2016). In human cancers, replication stress at telomeres can be arise due to dysfunction of the telomere protection factor POT1 (Pinzaru et al., 2016; Pinzaru et al., 2020). This type of replicative stress causes increased MiDaS at telomeres and relocation of a small fraction of them to nuclear pore. This movement is promoted by polymerisation of nuclear actin and is required to maintain telomere integrity in POT1-deficient cells. Indeed, disruption of the nuclear pore complex in cells harbouring a DNA binding deficient POT1 mutant exacerbates telomere repeat instability and detrimental telomeric recombination events (Pinzaru et al., 2020).

4.1.3 Processing of R-Loops at the Nuclear Pore Complex

Several studies have implicated roles for the NPC in the resolution of transcription-replication conflicts. It has been hypothesised that transcribed genes are transiently localised to the nuclear pores to aid in nuclear export of transcription products in a process called gene gating (Blobel, 1985). In yeast, localisation of transcribed genes to the NPC is important in preventing pathological R-loop formation, suggesting that gene gating and the subsequent nuclear transport of nascent RNA is important in preventing TRCs (García-Benítez et al., 2017). The human NPC component Tpr has also been implicated in the processing of DNA-RNA hybrids (Kosar et al., 2021). Interestingly, activation of the ATR-dependent checkpoint during replication stress releases transcribed genes from the nuclear pore to facilitate fork restart (Bermejo et al., 2011). These results suggest that proximity to the nuclear pore is important in preventing R-loop-dependent replication stress within transcribed genes, but upon exposure to other sources of replication stress, these genes are released from the NPC possibly to *allow* R-loop formation which consequently promotes DDR signalling and

TABLE 1 | The roles of histone variants and modifications in replication stress.

Histone variant/ modification	Chaperone/ enzyme	Functions in replication stress	Reference(s)
H2A.X	—	Phosphorylation at serine-139 (γH2AX) by ATM/ATR is an early marker of damage and involved in repairing replicative lesions in checkpoint-blind yeast γH2AX maintains normal S/G2 phase transition during unperturbed replication	Ward and Chen (2001), Redon et al. (2003), Sirbu et al. (2011), Lyu et al. (2019), Saldivar et al. (2018)
macroH2A	FACT	Promotes HR factor recruitment to stressed forks and persists at fragile sites after replication stress resolution to protect from future replication stress	Kim et al. (2018)
	LSH	Promotes HR factor recruitment to stressed forks Depletion causes increased H4K20me2, which supresses fork protection	Xu et al. (2021)
	—	Protects the inactive X chromosome from replication stress	Sebastian et al. (2020)
H2A.Z (Htz1)	SWR-C	Prevents misincorporation on dNTPs and collapse of stalled replication forks	Van et al. (2015), Srivatsan et al. (2018)
CENP-A	HJURP	Suppresses formation of centromeric R-loops to prevent TRCs	Giunta et al. (2021)
H2A K13/K15 Ub	RNF168	Facilitates normal S phase progression and promotes fork restart	Schmid et al. (2018); Nakamura et al. (2021)
H2B K123Ub	Bre1 (yeast)	Stabilises nucleosomes on newly replicated DNA an facilitates fork progression Activates the intra-S phase checkpoint Maintains error-free DNA damage tolerance	Trujillo and Osley (2012), Lin et al. (2014), Northam and Trujillo (2016), Hung et al. (2017)
H3K4me1/me3	MLL3/4	Promotes MRE11-depedendent fork degradation in BRCA-deficient cells	Ray Chaudhuri et al. (2016)
	SETD1A	Promotes RAD51-mediated fork protection through H3.1 chaperoning	Higgs et al. (2018)
	Set1 (yeast)	Prevents TRCs in active regions	Chong et al. (2020)
H3K9me2/me3	Met-2, set-25 (*C. elegans*)	Represses genes in repetitive regions to prevent R-loop formation	Zeller et al. (2016)
H3K27me3	EZH2	Recruits MUS81 to facilitate fork restart	Rondinelli et al. (2017)
H4K20me1/me3	PR-Set7	Prevents replication stress by controlling fork number and velocity H4K20me0 on new histones provides binding site for TONSL-MMS22L to facilitate post-replicative repair	Jørgensen et al. (2007a), Tardat et al. (2007), Saredi et al. (2016)
H4 K5/K12 ac	HAT1	Prevents replication stress and protects stressed forks from MRE11-dependent degradation	Nagarajan et al. (2013), Agudelo Garcia et al. (2020)
H4K8ac	PCAF	Promotes MRE11- and EXO1-dependent degradation of stalled forks in BRCA2-deficient cells	Kim et al. (2020)

fork restart (García-Benítez et al., 2017). In addition, topoisomerases have been shown to suppress RNA-DNA hybrid formation during replication through modulating gene topology (Tuduri et al., 2009; Achar et al., 2020). Specifically, Top2 maintains negative supercoiling at gene boundaries, which facilitates normal transcription and suppresses hybrid formation specifically during S phase (Achar et al., 2020).

4.2 Mobility of Stressed Replication Forks by Nuclear Actin

Whilst it remains unclear whether stressed fork mobility is caused by passive diffusion or involves an active mechanism, recent studies have shown that the contractile fibre actin has roles during replication stress, suggesting fork movement is an active process. Transient polymerisation of actin, forming filamentous actin (F)-actin, occurs in the nucleus and is involved in the relocation of DSBs, particularly within heterochromatin, to facilitate HR repair (Belin et al., 2015; Caridi et al., 2018; Schrank et al., 2018). During unperturbed replication, nuclear actin polymerisation is important to allow recruitment of replication factors and S phase progression (Parisis et al., 2017). Additionally, upon replication stress, F-actin formation at stressed replication foci reversibly increases in an ATR and mTOR signalling-dependent manner (Lamm et al., 2020). Stalled forks then move along the F-actin, facilitated by myosin motor proteins, to localise with the nuclear periphery. This localisation allows replication restart, and therefore resolution of replication stress before collapse into a single ended DSB, which distinguishes this pathway from the movement of DSBs to allow HR (Schrank et al., 2018; Lamm et al., 2020) (**Figure 2**). In addition, the nuclear volume increases as a result of F-actin formation, possibly due to chromatin decompaction, and this response is not seen during DSB repair (Lamm et al., 2020). The movement of stalled forks to the nuclear lamina may allow direct interactions with the nuclear envelope and be important in the recruitment of repair factors; indeed, the laminA/C proteins has been implicated in promoting the recruitment of RPA, RAD51 and FANCD2 (Singh et al., 2013). On the other hand, DSBs located at the nuclear periphery within LADs suppress HR by inhibiting the recruitment of RAD51 (Lemaître et al., 2014). In the future, it will be therefore important to understand the roles of the nuclear lamina in the regulation of homologous-directed repair of DSBs and stalled replication forks.

5 CONCLUSION AND FUTURE PERSPECTIVES

The organisation of the genome plays roles in facilitating progression of DNA replication on multiple levels. At the higher levels of genome organisation, arrangement of genetic material into higher order structures and localisation within specific compartments play a major role in controlling the replication timing program. In addition, the composition of the nucleosome as defined by histone variants and PTMs, and the enrichment of non-histone proteins all modulate replication timing (**Figure 1**). In many cases, this replication timing is strongly linked to transcription where active regions correlate with early replication and inactive regions with late. The importance of this link between transcription and replication timing and whether there is any causality between these two processes are still unclear. Possibly, the compacted state of repressed chromatin may act as a barrier to early replication, localisation of inactive/active regions at specific loci may impact origin firing or it may be favourable to duplicate replication stress prone sequences (e.g., gene-poor repetitive regions) later in S phase.

In addition, the chromatin environment plays important roles in the resolution of replication stress. Particularly, the presence of histone variants and modifications at stressed loci play major roles in promoting or repressing specific repair pathways (**Table 1**). However, the question remains whether these variants/marks are deposited *de novo* at all genomic regions during replication stress and remain at these loci after the resolution of replication stress to protect against future challenges or whether they are present only at specific regions that are more difficult to repair.

The SUMO-directed movement of stressed replication forks to facilitate recombination-dependent repair shares some striking similarities with the mobility of DSBs to the nuclear periphery. This mobility of stalled forks is primarily important within repetitive regions such as expanded CAG repeats and telomeres, and the mobility of DSBs occurs when breaks are situated in heterochromatin (**Figure 2**). The movement of these specific regions to the nuclear periphery could be important for at least two reasons: movement may prevent detrimental recombination events between repeats and/or the environment of the nuclear periphery may provide a protective environment for the repair of these difficult sequences. Indeed, association of stressed forks with components of the nuclear pore complex is vital for repair of replicative lesions. Future studies linking the roles of nuclear actin and movement of stressed forks to the NPC would be beneficial to understand how these fork movements are regulated and propagated.

Tumours are highly prone to replication stress due to their uncontrolled proliferation and deregulated cellular signalling, and this feature is already being targeted in several therapies in clinic (Ubhi and Brown, 2019). Consequently, some of the features of the chromatin could be exploited to provide novel targeted therapies for cancer. Histone marks that regulate the protection of stalled forks form nucleolytic degradation such as methylated H3K4 and H3K27 and acetylated H4K8 have major implications in the treatment of cancers, specifically those harbouring BRCA1/2 mutations. Therefore, measuring the expression of the enzymes that deposit/remove these marks and the enrichment of the marks themselves could provide biomarkers that could be used in the future to predict resistance of tumours to certain therapies and allow greater stratification of treatment. In addition, drugs that target specific enzymes and modify the epigenome and sensitise cells to other therapies. For example, histone deacetylase (HDAC) inhibitors are already used in the clinic and combination of these with other anti-cancer drugs are being explored (Suraweera et al., 2018). Finally, the movement of stalled forks by nuclear actin is mediated through ATR and mTOR signalling, so these pathways provide attractive targets for anti-cancer drugs. Indeed, polymerisation of nuclear actin facilitates survival of cancer cells (Lamm et al., 2020). In summary, future studies elucidating the role of chromatin during replication stress are important in discovering new and more stratified cancer treatments.

AUTHOR CONTRIBUTIONS

JW and ES wrote the review.

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REVIEW
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The (Lack of) DNA Double-Strand Break Repair Pathway Choice During V(D)J Recombination

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DNA double-strand breaks (DSBs) are highly toxic lesions that can be mended via several DNA repair pathways. Multiple factors can influence the choice and the restrictiveness of repair towards a given pathway in order to warrant the maintenance of genome integrity. During V(D)J recombination, RAG-induced DSBs are (almost) exclusively repaired by the non-homologous end-joining (NHEJ) pathway for the benefit of antigen receptor gene diversity. Here, we review the various parameters that constrain repair of RAG-generated DSBs to NHEJ, including the peculiarity of DNA DSB ends generated by the RAG nuclease, the establishment and maintenance of a post-cleavage synaptic complex, and the protection of DNA ends against resection and (micro)homology-directed repair. In this physiological context, we highlight that certain DSBs have limited DNA repair pathway choice options.

Keywords: DNA double-strand break, V(D)J recombination, non-homologous end-joining, homology-directed repair, DNA end resection, DNA double-strand break repair pathway choice

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INTRODUCTORY REMARKS

The integrity of a cell's genome is continuously threatened by exogenous or endogenous factors generating DNA damage of various nature, which can impact a single nucleotide or result in lesions of the DNA backbone. Independently of the type or circumstances leading to the DNA damage, it must robustly be sensed, signaled, and repaired, ideally resulting in no or minimal alterations to the genetic code and recovery of an intact genome. Mammalian cells are equipped with several molecular tool kits warranting efficient repair of damaged DNA, where the nature of the DNA lesion largely dictates the selected repair apparatus. Nevertheless, multiple DNA repair pathways exist for a single type of damage such as the case for the mending of DNA double-strand breaks (DSBs). DNA DSBs are often considered as the most deleterious form of DNA damage for a cell, resulting in the physical separation of DNA molecules. Failure to accurately repair DSBs can lead to cell death or to DNA structural changes (*i.e.,* loss of genetic material, sequence alterations or joining of the wrong couple of DNA ends generating chromosomal translocations) potentially triggering carcinogenesis or onset of pathologies, including neurodegenerative diseases or immunodeficiencies (Mitelman et al., 2007; Jackson and Bartek, 2009; Ciccia and Elledge, 2010; Goldstein and Kastan, 2015). Maybe recklessly, chromosomal breakage has been co-opted by the immune system as an integral part of B- and T-cell development when V(D)J recombination–a programmed DNA rearrangement process–generates a vast array of antigen receptor molecules. V(D)J recombination is initiated when the lymphoid-restricted recombination-activating genes *RAG1* and *RAG2* are expressed and form a site-specific endonuclease (the RAG nuclease or RAG recombinase) that induces DSBs within T cell receptor

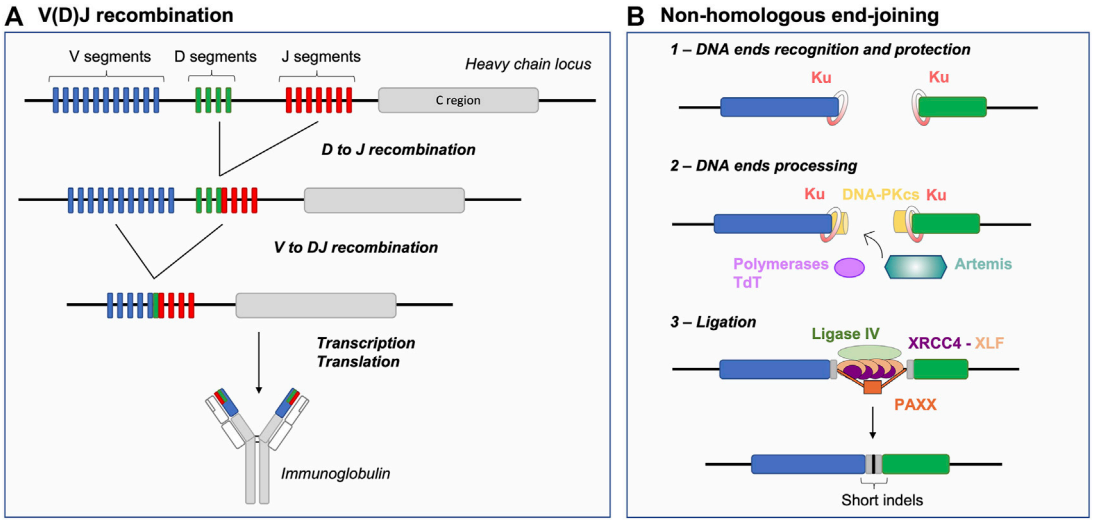

FIGURE 1 | V(D)J recombination and NHEJ Basics: Generating antigen receptor diversity. **(A)** V(D)J recombination at the immunoglobulin heavy chain locus (depicted as an example) consists in a sequential 2-step rearrangement of V, D and J segments. This combinatorial process generates the diversity of antigen receptors. **(B)** After RAG cleavage, the NHEJ repair pathway is initiated by binding of the Ku70/80 heterodimer (Ku) to DNA ends. Ku together with DNA-PKcs form the DNA-PK holoenzyme. RAG DNA ends are then processed by the endonuclease Artemis and polymerases (*e.g.,* Pol μ), specifically the terminal deoxynucleotidyl transferase (TdT), resulting in increased junctional diversity (in gray). This additional diversity is generated, prior to joining, in two forms: 1) P- palindromic sequences, produced through the endonuclease action of Artemis at RAG-induced hairpin-sealed ends and 2) N-nucleotide sequences, the addition of non-templated nucleotides by TdT. Finally, the ligation complex composed of Ligase IV, XRCC4 and XLF joins the processed ends. Joining of DNA ends via NHEJ further participates to generating indels, moreover favoring junctional diversity. NHEJ: non-homologous end-joining, indels: insertions or deletions.

(TCR, α/δ, β, γ) and Ig (h, κ, λ) gene loci. Despite the existence of multiple DSB repair pathways, including the canonical non-homologous end-joining (NHEJ) and homologous recombination (HR) pathways as well as additional (micro) homology-directed sub-pathways, RAG-initiated DSBs are "almost" exclusively repaired by NHEJ. In the following review, we address various parameters which restrict DNA DSB repair pathway choice in lymphocytes undergoing V(D)J recombination and discuss how NHEJ-mediated repair impacts on successful antigen receptor gene assembly or association to immunodeficiencies and lymphoid cancers.

V(D)J RECOMBINATION AND DOUBLE-STRAND BREAK REPAIR

V(D)J recombination is a somatic antigen receptor gene rearrangement process occurring in developing B and T cells, involving rearrangement of V (variable), D (diversity) and J (joining) gene segments located within the Ig or TCR locus (**Figure 1A**) (reviewed in (Gellert, 2002; Litman et al., 2010; Schatz and Ji, 2011; Roth, 2014; Lescale and Deriano, 2016)). This locus-specific reaction is initiated by the RAG nuclease which introduces two DSBs at recombination signal sequences (RSSs) flanking selected V, D and J segments. RAG-DSBs pose a threat to overall genome stability and thus the activity of the RAG recombinase is tightly controlled (Roth, 2003; Roth, 2014; Lescale and Deriano, 2016).

Lymphocytes, as any other cell types, possess several DSB repair pathways including HR and NHEJ, which are considered the main DNA DSB repair pathways. HR is based on the capacity of the cellular machinery to find and access an intact template (sister chromatid or chromosome homolog) used to mediate error-free repair of the break. Initiation of HR involves the identification of broken DNA end(s), a 5′-3′ nucleolytic digestion process generating 3′ single-stranded DNA (ssDNA) (end resection) permitting homology search and DNA synthesis (Elbakry and Löbrich, 2021). NHEJ is thought to be a rapid and efficient way of repairing DSBs, as it involves the identification and (quasi) direct ligation of the two DNA ends without search for (extended) homology (**Figure 1B**) (reviewed in (Chang et al., 2017)). Briefly, upon detection of a DSB, the Ku70/80 heterodimer (Ku) is loaded onto DNA ends and acts as a scaffold for recruitment of additional NHEJ factors (Gottlieb and Jackson, 1993; Nick McElhinny et al., 2000; Walker et al., 2001; Fell and Schild-Poulter, 2015; Ochi et al., 2015). Ku recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK holoenzyme that phosphorylates multiple substrates, promoting synapsis of DNA ends and facilitating the recruitment of end processing and ligation enzymes (Chen X. et al., 2021; Zha et al., 2021). The ligation complex, composed of Ligase IV-XRCC4-XLF, joins the ends together (Ahnesorg et al., 2006; Buck et al., 2006). PAXX, a paralog of XRCC4 and XLF, also contributes to end-joining during NHEJ (Ochi et al., 2015; Lescale et al., 2016b) notably by promoting accumulation of Ku at DSBs (Liu et al., 2017). This repair pathway, as opposed to HR, is sometimes defined as error-prone because it can generate small insertions and deletions (indels) (**Figure 1B**) (Stinson et al., 2020). DNA 5′ end resection is a major determining factor for the NHEJ to HR

choice in cells, as mentioned above HR involves the formation of extensive 3′ ssDNA. The chromatin-bound protein 53BP1, together with downstream effectors, counteracts DNA end resection and thus act as a pro-NHEJ regulator upon DSB injury (Mirman and de Lange, 2020). Alternative end-joining (alt-EJ) and single-strand annealing (SSA) are yet other DSB repair pathways relying on intermediate length of DNA end resection and bias towards usage of (micro)homologies (Symington, 2016). These pathways are intrinsically unfaithful as they generate deletions between microhomology tracts and as alt-EJ is associated to genomic instability, notably chromosomal translocations (Corneo et al., 2007; Soulas-Sprauel et al., 2007; Yan et al., 2007). Alt-EJ is thought to be particularly active in cells deficient for HR or NHEJ (Corneo et al., 2007; Soulas-Sprauel et al., 2007; Yan et al., 2007; Wyatt et al., 2016), although some studies indicate that this pathway is also utilized in DNA repair proficient cells (Lee et al., 2004; Corneo et al., 2007; Coussens et al., 2013; Deriano and Roth, 2013).

During V(D)J recombination, antigen receptor gene diversity is achieved by 1) unique combinations of V, D and J coding segments, so called combinatorial diversity and 2) the imprecision of the DSB repair reaction at segment joints–driven by NHEJ and the action of the terminal deoxynucleotidyl transferase (TdT), termed junctional diversity (**Figure 1B**) (Gilfillan et al., 1993; Komori et al., 1993; Ramsden, 2011). NHEJ thus offers the ideal repair pathway to permit Ig and TCR gene diversification in early lymphocytes as opposed to resection- and homology-based repair pathways that would ultimately restore germline sequences or generate genetic instability in the cell. In the following sections, we address different parameters that limit DSB repair pathway choice to NHEJ during V(D)J recombination, including 1) the nature of DSB end structures, 2) the establishment of DSB end synapsis and 3) the impediment of DNA end resection and (micro)homology-driven repair.

DNA END STRUCTURES–MEANT TO BE REPAIRED BY NON-HOMOLOGOUS END-JOINING

Broken DNA ends often cannot be directly reattached and require processing prior to mending, thus the nature of the broken ends acts as an important factor influencing repair pathway choice (Chang et al., 2017). During V(D)J recombination, the RAG complex promotes the assembly of a pre-synaptic complex that includes a 12 and a 23 RSS prior to conducting its nuclease activity (Schatz and Ji, 2011). DNA cleavage occurs in two steps and relies on RAG1, RAG2, a divalent metal ion, and the ubiquitous bending factors HMGB1 or HMGB2. RAG introduces a nick between each RSS and its flanking coding sequence, generating a free 3′-OH group which then attacks the opposite strand by transesterification. This cleavage reaction results in four broken DNA ends with specific structures: two hairpin-sealed coding ends (CE) at gene segments and two blunt signal ends (SE) at RSSs (**Figure 2A**). Upon cleavage, RAG-induced DNA breaks activate Ataxia telangiectasia mutated (ATM), an important mediator of the DNA DSB response (Helmink and Sleckman, 2012). Activated ATM phosphorylates numerous proteins that promote the G1/S checkpoint and participate in DNA end protection (see below), favoring NHEJ. Ku has a strong affinity for hairpin sealed, blunt or short overhang DNA ends (Falzon et al., 1993; Downs and Jackson, 2004), directing RAG-DSBs towards NHEJ repair. Ku not only binds avidly broken ends but also serves as a scaffold for the recruitment of DNA-PKcs, forming the DNA-PK holoenzyme, and downstream NHEJ factors that permit the processing and ligation of RAG-induced DSB ends. This second attribute of Ku is particularly important for CEs as blunt SEs can be directly ligated by XRCC4-Ligase IV to form DNA circles (*i.e.,* in the case of deletional recombination). Indeed, CEs necessitate the action of the endonuclease Artemis to open the hairpin structure (**Figure 2A**). Proper Artemis endonuclease activity requires DNA-PK and leads to the formation of protruding 3′ ends with an -OH group (Ma et al., 2002). This latter DNA end topology favors repair by NHEJ, as XRCC4-Ligase IV necessitate a -OH at both DNA ends for ligation. Additionally, the XRCC4-Ligase IV complex can stimulate the removal of few nucleotides-long overhangs–generated by Artemis–prior to ligation (Gerodimos et al., 2017). Notably, Ku also promotes the recruitment of TdT–the third lymphoid-specific protein in addition to RAG1 and RAG2 – that adds nucleotides at Artemis-opened CEs preceding the ligation by XRCC4-Ligase IV. This links NHEJ to the generation of junctional diversity at coding joins (Gilfillan et al., 1993; Komori et al., 1993; Purugganan et al., 2001; Ramsden, 2011), increasing the genetic diversity of V(D)J rearrangement outcomes. Therefore, the topology of RAG-induced DSB ends significantly biases repair towards NHEJ, by generating an NHEJ prone environment.

SYNAPSIS - KEEPING DNA ENDS TOGETHER FOR SAFE REPAIR BY NON-HOMOLOGOUS END-JOINING

Maintaining broken DNA ends in close proximity is a major parameter that influences pathway choice, notably because NHEJ requires the physical proximity of both DNA ends, while it is dispensable for certain HR reactions such as break-induced replication (Pham et al., 2021). Synapsis of DSB ends during V(D)J recombination is quite challenging as it involves the sequestration of four DSB ends (*i.e.,* two CEs and two SEs). Additionally, V(D)J recombination implicates gene segments that can be situated at considerable distances from one another; for instance the murine germline immunoglobulin heavy chain (IgH) locus spans approximately 2.75 Mbp of chromosome 12 (Lucas et al., 2015). For efficient V(D)J recombination, 1) V, D and J gene segments must be brought in vicinity of each other prior to cleavage and 2) DNA ends, specifically CEs which require processing, must be kept together for ligation.

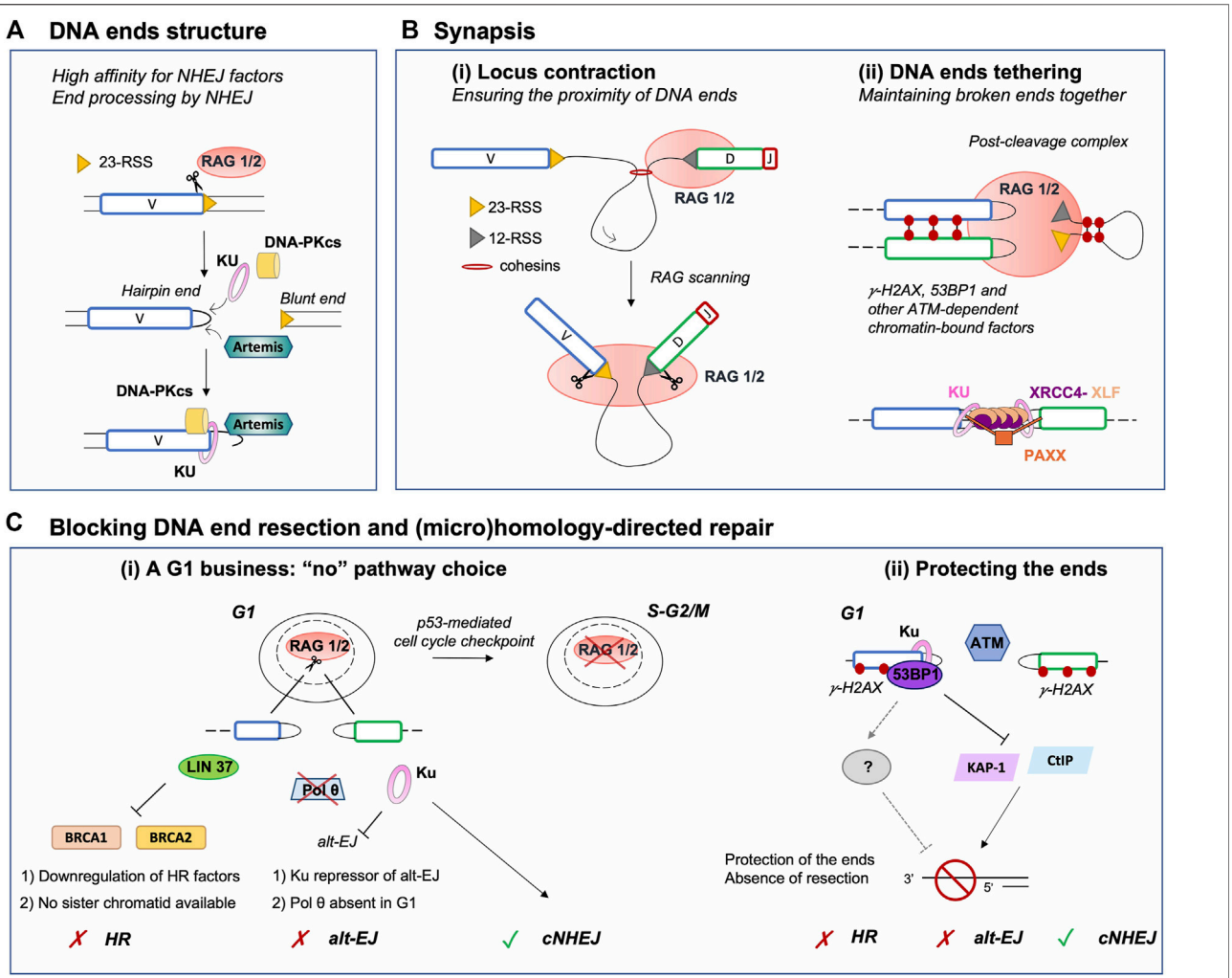

FIGURE 2 | Major parameters restricting DNA repair pathway choice to NHEJ during V(D)J recombination. **(A)** RAG-induced breakage generates a covalently sealed hairpin end (coding end) and a blunt end (signal end). This facilitates the loading of Ku, which acts as a scaffold for other NHEJ factors, as it has a high affinity for blunt or hairpin sealed ends. In addition, hairpin sealed ends require to be opened by another NHEJ factor Artemis, which renders ends compatible for ligation. Thus, this DNA end topology contributes to the establishment of a NHEJ-prone environment. **(B) (i)** Upon binding an RSS, RAG scans the adjacent chromatin by a loop extrusion mechanism. Breakage is induced only upon reaching a compatible RSS, ensuring the induction of DSBs in close proximity despite the large size of the immunoglobulin locus. **(ii)** Following DSB induction, RAG remains bound to DNA ends in a post-cleavage complex (PCC). The PCC together with NHEJ and ATM-dependent chromatin-bound DNA factors (*e.g.,* phosphorylated H2AX and 53BP1) favor DNA ends tethering and stabilization. This likely prevents the search for distant partner DNA ends and channels broken DSB ends to NHEJ for safe repair. **(C) (i)** V(D)J recombination is a G1-restricted process, as RAG is degraded upon entry in the S phase. In G1, HR cannot operate as pre-replicative cells do not harbor a sister chromatid, used as a template for repair. In addition, several factors required for HR are transcriptionally repressed in G0/G1. Similarly, Pol θ, an important factor for alt-EJ, is poorly expressed in G1 consequently limiting the use of this repair pathway. Furthermore, alt-EJ is blocked by Ku upon binding DNA ends, yet again promoting processing and repair by NHEJ. **(ii)** Chromatin DSB-response factors γH2AX, 53BP1 and possibly additional downstream effectors contribute to the protection of RAG-DSB ends by blocking the activity of nucleases such as CtIP or acting via transcriptional repressors such as KAP-1. This protection prevents DNA end resection, an essential intermediate step for (micro)homology-directed repair (*e.g.,* alt-EJ, HR, etc.), hence promoting NHEJ. NHEJ: non-homologous end-joining, RSS: Recombination Signal Sequence, Alt-EJ: alternative end-joining, HR: homologous recombination.

Contraction and spatial reorganization of antigen receptor loci during V(D)J recombination rely largely on the formation of chromatin loops through a cohesin-dependent extrusion process (Bossen et al., 2012; Ebert et al., 2015; Ba et al., 2020; Hill et al., 2020; Dai et al., 2021; Davidson and Peters, 2021), as well as transcription and subnuclear relocation (Rogers et al., 2021). Remarkably, this mechanism poises the loci for recombination independently of RAG, but also endows RSS-bound RAG with the ability to scan chromatin for a partner RSS, providing directionality and spatial restriction to RAG activity within the chromatin loop domain (**Figure 2Bi**) (Lin et al., 2018; Zhang et al., 2019; Ba et al., 2020; Hill et al., 2020; Dai et al., 2021). Additional chromatin-bound factors such as 53BP1 contribute to bringing V(D)J segments close-by, as depletion of this latter factor results in a reduction of distal V to DJ segments joins (Difilippantonio et al., 2008). Induction of two DSBs in the

vicinity of one another likely contributes to favoring rapid repair of RAG-induced DNA breaks by NHEJ, without the need to search for partner DNA ends.

After cleavage, the RAG proteins stay associated with the DNA ends in a post-cleavage complex (PCC) (**Figure 2Bii**). Mutations resulting in RAG-PCC destabilization were shown to increase repair of RAG-mediated DSBs via HR and alt-EJ (Lee et al., 2004; Corneo et al., 2007; Coussens et al., 2013; Deriano and Roth, 2013), pathways considered as unconventional for V(D)J recombination. These observations suggested that the RAG-PCC might contribute to shepherding DNA ends to the NHEJ machinery for repair, thus protecting them from error prone end-joining pathways and aberrant recombination events (Roth, 2003; Lee et al., 2004; Deriano and Roth, 2013). Indeed, a RAG2 mutant - possessing deletion of C-terminal residues 352–527 (core RAG2) - destabilizes the RAG-PCC and is associated with an increased rate of aberrant recombination outcomes *in vitro* and to inter-chromosomal translocations involving the V(D)J loci *in vivo* (Sekiguchi et al., 2001; Talukder et al., 2004; Corneo et al., 2007; Curry and Schlissel, 2008; Deriano et al., 2011; Coussens et al., 2013). Additionally, core RAG2/p53-deficient mice present increased genomic instability and accelerated lymphomagenesis via alt-EJ, generating tumors bearing a complex landscape of chromosomal rearrangements (Deriano et al., 2011; Mijušković et al., 2012; Mijušković et al., 2015). Strikingly, the lymphomas and translocations observed in the latter animals resemble those of ATM-deficient mice, suggesting that a similar DNA end destabilization mechanism might underlie genomic instability and lymphomagenesis in both mouse models (Deriano et al., 2011). Consistent with this, ATM - beyond its role in activating checkpoints - is important for the stability of RAG-PCCs *in vivo* (Bredemeyer et al., 2006). Upon DSB damage, ATM phosphorylates chromatin- and DNA-associated proteins, including the histone variant H2AX (forming γH2AX), 53BP1, MDC1 and factors of the MRN complex (MRE11, RAD50, and NBS1) that assemble on both sides of DNA breaks forming so-called nuclear DNA repair foci. The stabilization function of ATM depends on its kinase activity. Thus, formation of ATM-dependent DNA repair foci has been proposed to tether DNA ends for proper joining via NHEJ (**Figure 2Bii**). In ATM-deficient cells undergoing V(D)J recombination, the fraction of CEs which evade the PCC are occasionally joined aberrantly, forming chromosomal deletions, inversions, and translocations (Bredemeyer et al., 2006; Helmink and Sleckman, 2012). Altogether, these results indicate that RAG2 (by extension the RAG-PCC) and ATM share mechanistic properties during V(D)J recombination, via the stabilization of broken DNA-ends consequently avert the use of alternative repair pathways.

Additional insights into the mechanisms responsible for the stabilization of RAG-cleaved DNA ends come from the analysis of animal models double-deficient for XLF and ATM or core RAG2. XLF and XRCC4 are two distantly related members of the same protein family and share structural similarity (Callebaut et al., 2006; Andres et al., 2007; Li Y. et al., 2008). Together, they form long filaments, thought to help DNA end tethering and synapsis during repair (**Figure 2Bii**) (Tsai et al., 2007; Riballo et al., 2009; Hammel et al., 2011; Ropars et al., 2011; Reid et al., 2015; Chen S. et al., 2021). In contrast to other NHEJ-deficient mice, XLF-deficient mice are not markedly immune-deficient and early lymphoid cells from these animals perform nearly normal V(D)J recombination. These observations suggest that other factors or pathways compensate for XLF function during V(D)J recombination (Li G. et al., 2008). In fact, cells deficient for both XLF and ATM-dependent DSB response (*e.g.,* XLF and ATM, 53BP1, or H2AX double mutants) display severe block in lymphocyte development and a significant defect in the repair of RAG-mediated DSBs. This reveals functional redundancy between XLF and ATM-DSB response factors during V(D)J recombination (Li G. et al., 2008; Zha et al., 2011; Liu et al., 2012; Oksenych et al., 2012; Oksenych et al., 2013; Vera et al., 2013; Kumar et al., 2014). Similarly, core RAG2/XLF double deficiency leads to a profound lymphopenia associated with a severe defect in joining of RAG-cleaved DNA ends (Lescale et al., 2016a). These findings are consistent with a two-tier model in which the RAG proteins, together with the ATM chromatin DSB-response, collaborate with NHEJ factors to promote functional V(D)J recombination and emphasize the importance of DNA end tethering for proper repair.

BLOCKING DNA END RESECTION AND (MICRO)HOMOLOGY-DRIVEN DOUBLE-STRAND BREAK REPAIR

A G1-Phase Business

RAG-induced V(D)J recombination is limited to the G1 phase of the cell cycle, which offers an additional level of restriction to NHEJ-driven repair. This is due to the specific destruction of RAG2 during the G1-to-S transition that is triggered by phosphorylation of the T490 residue (Li et al., 1996). Additionally, RAG-induced DSBs trigger an ATM/p53-dependent DSB response that promotes G1/S cell cycle arrest and eventually cell death (**Figure 2Ci**). Finally, RAG-DSBs activate a specific checkpoint that opposes the pre-B cell receptor proliferative signals and prevent cells from entering into S phase before resolving the damage (Bredemeyer et al., 2008; Bednarski and Sleckman, 2012; Bednarski et al., 2016).

As the ideal template for HR is the sister chromatid, HR is restricted to the S and G2 phases of the cell cycle and cannot fully operate in G1-phase cells. Alt-EJ (and SSA) do not possess this constraint, thus could potentially serve as alternatives to NHEJ for repair of DSBs in non-dividing cells. Using high-throughput sequencing techniques, it was recently shown that end joining of RAG-induced DSBs is virtually null in G0/G1-arrested progenitor (pro-) B cells deficient for XRCC4 (Yu et al., 2020). Within the same setting, Cas9-induced DSBs are also poorly repaired, suggesting that additional factors, other than RAG, limit the access of broken DNA ends to alt-EJ pathways in G0/G1-phase cells. Similarly, DSBs generated by RAG, Cas9 or zinc finger endonucleases in G0/G1-arrested pro-B cells remain unjoined in the absence of Ligase IV (Liang et al., 2021). However, Ku70-deficient or Ku70/Ligase IV-deficient G0/G1-arrested pro-B cells

perform quite robust end-joining, albeit at lower levels than wild type cells, indicating that Ku acts as a strong repressor of alt-EJ in G0/G1-phase cells (**Figure 2Ci**) (Frock et al., 2021; Liang et al., 2021). Cells might also not be fully equipped to perform resection- and homology-dependent repair in G0/G1. For instance, LIN37, a component of the DREAM transcriptional repressor, inhibits resection and HR in G0/G1-blocked pro-B cells by repressing the expression of HR proteins such as BRCA1, BRCA2, PALB2 and RAD51 (Chen B. R. et al., 2021). Similarly, DNA polymerase theta (Pol θ, encoded by *Polq* in mice), implicated in alt-EJ (Ramsden et al., 2021), is not expressed in G0/G1-arrested pro-B cells (**Figure 2Ci**) (Yu et al., 2020).

Nevertheless, analysis of mice harboring combined deficiency in p53 and in NHEJ (*i.e.,* Ku, XRCC4 or Ligase IV) irremediably develop aggressive pro-B lymphomas displaying RAG-dependent translocations and amplifications between *Igh* and *c-Myc* by alt-EJ (Nussenzweig and Nussenzweig, 2010; Gostissa et al., 2011; Ramsden and Nussenzweig, 2021). It was suggested that p53 deficiency enables cells to move inappropriately into S phase and acquire DSBs that initiate chromosomal translocations and amplifications (Paulson et al., 1998; Zhu et al., 2002). In fact, an *in vitro* study using XRCC4/p53-deficient pro-B cell lines shows that the transition from G0/G1-phase to S-G2/M-phases of the cell cycle enables alt-EJ repair, promoting massive genetic instability in the form of chromosomal deletions and translocations (Yu et al., 2020). It is tempting to speculate that unrepaired G1-DNA breaks progressing to S-G2/M get lost in the cellular space with unprotected DNA ends being subjected to repetitive nuclease attacks until (micro)homology-driven alt-EJ stabilizes them in *cis* or *trans*. In that regard, although multiple homology-directed sub-pathways would theoretically be able to process these lost DNA ends (Elbakry and Löbrich, 2021), the repair of G0/G1-DSBs in S-G2/M would strictly depend on Pol θ. Indeed, in XRCC4/Pol θ/p53-triple deficient pro-B cells, DSBs induced in G1 accumulate in the form of chromosomal breaks resulting in lethality at the next mitosis (Yu et al., 2020). Whether Pol θ contributes to the development of pro-B cell lymphomas, carrying *Igh/c-Myc* translocations, in NHEJ/p53-deficient animals remains to be addressed.

DNA End Protection

The extent of resection is actively regulated by the protection of DNA ends, which limits the access of nucleases to the break sites. In addition to the above-mentioned parameters (*i.e.,* DNA end structures and synapsis), several DSB response chromatin-bound factors localize at RAG-DSBs and are thought to protect DNA ends against resection, including γH2AX and 53BP1 (Difilippantonio et al., 2008; Helmink et al., 2011; Zha et al., 2011; Dorsett et al., 2014; Lescale et al., 2016a; Chen B. R. et al., 2021). In G1, γH2AX prevents CtIP-mediated nucleolytic resection (Helmink et al., 2011). Similarly, KAP-1, a transcriptional repressor modulating chromatin structure, was shown to promote resection in G1 lymphocytes in the absence of γH2AX and 53BP1 (Tubbs et al., 2014). Moreover, depletion of 53BP1 in Ligase IV-deficient G0/G1-blocked pro-B cells results in increased levels of resection at irradiation-induced DSB ends, demonstrating that 53BP1 is crucial for DNA end protection in this cell-cycle phase (**Figure 2Cii**) (Chen B. R. et al., 2021). The Shieldin complex, composed of SHLD1, SHLD2, SHLD3 and MAD2L2/REV7, acts downstream of 53BP1-RIF1 to antagonize DNA end resection and favor NHEJ over HR (Greenberg, 2018; Setiaputra and Durocher, 2019; Mirman and de Lange, 2020; de Krijger et al., 2021). It acts in a paradoxical manner as it requires to bind >50 nt-long ssDNA ends in order to hinder DNA end resection (Dev et al., 2018; Findlay et al., 2018; Gao et al., 2018; Noordermeer et al., 2018). Mechanistically, it is thought to directly inhibit resection by physically blocking access of nucleases to the free ssDNA-dsDNA ends. Additionally, this complex promotes the recruitment and coordination of additional factors leading to the processing of ssDNA-dsDNA intermediates prior to NHEJ repair such as ASTE1, which cleaves the protruding ssDNA, and the CST (CTC1–STN1–TEN1)–DNA polymerase-α–primase complex, to fill in the residual ssDNA (Mirman et al., 2018; Zhao et al., 2021). Although the Shieldin complex counteracts HR in BRCA1-deficient cells and is important for NHEJ-driven repair during class switch recombination or in the fusion of unprotected telomeres, it seems dispensable for V(D)J recombination (Dev et al., 2018; Ghezraoui et al., 2018; Mirman et al., 2018; Ling et al., 2020). Indeed, SHLD2 or REV7 deficiencies in mice do not significantly alter lymphocyte development and V(D)J recombination (Ghezraoui et al., 2018; Ling et al., 2020). It is to note that in wild-type cells, the processing of RAG-induced DNA ends does not generate >50 nt-long ssDNA intermediates, thus potentially explaining why Shieldin-mediated protection prior to joining seems dispensable during V(D)J recombination. However, whether the Shieldin complex plays a role in protecting RAG-generated DNA ends against resection in the context of crippled NHEJ remains to be investigated. In XLF-deficient mice (impaired NHEJ), 53BP1 plays an essential role in counteracting resection at RAG-DSB ends, promoting V(D)J recombination and lymphocyte differentiation (Liu et al., 2012; Oksenych et al., 2012). Nonetheless, it is unclear if this DNA end protection is mediated through 53BP1 downstream effectors (*e.g.*, Shieldin complex) or via intrinsic properties of 53BP1 (**Figure 2Cii**). Notably, Ku also antagonizes DNA end resection through at least two distinct mechanisms 1) by blocking the access of nucleases to DSB ends (Zahid et al., 2021) and 2) by recruiting TdT which promotes template-independent and -dependent synthesis prior to ligation (Loc'h and Delarue, 2018). Taken together, in the context of V(D)J recombination the downregulation of the DSB end resection machinery and the protection of DNA ends by chromatin-bound factors and Ku seem to act as forefront anti-resection barriers, promoting repair via NHEJ but not HR or alt-EJ (**Figure 2C**).

CONCLUSION AND PERSPECTIVES

In this review, we present V(D)J recombination as a relevant biological setting to investigate factors influencing DSB repair pathway choice, specifically those constraining the repair of DSBs to NHEJ. This repair pathway is essential for V(D)J combinatorial

rearrangement as well as for the generation of diversity at V(D)J junctions, two pre-requisites for antigen receptor gene diversification and the establishment of a primary immune repertoire. During V(D)J recombination, several factors prime for repair via NHEJ, including the spatial organization of the genomic loci subjected to these rearrangement events. Additionally, the RAG nuclease, the type of generated DSBs, the G1 phase-specific environment and the dedicated DSB response predispose (arguably dictate) repair through NHEJ. Albeit numerous the studies which shed light onto the mechanisms through which DSBs generated during V(D)J recombination are biased towards repair by NHEJ, several questions remain unanswered. For instance, while the RAG-PCC plays a role in favoring repair via NHEJ, possibly by stabilizing DNA ends, it remains unclear if the RAG proteins directly interact with certain NHEJ factors and whether such interaction(s) would contribute to NHEJ pathway choice. We also discussed the importance of blocking DNA end resection during V(D)J recombination through the action of specific chromatin DSB response factors, most notably 53BP1. Whether this end protection only relies on the capacity of the chromatin-bound factors to maintain a stable PCC or whether it also requires specific downstream effectors to act at the DSB ends is unclear. In that regard, it is interesting to note that the mode of action of the Shieldin complex on DSB ends generated by AID during IgH class switch recombination is somewhat reminiscent to that of Ku during V(D)J recombination. Both Ku and the Shieldin complex have the capacity to physically obstruct resection at DSB ends and to actively recruit factors implicated in DNA end modifications (*i.e.,* action of Ku and TdT/Artemis versus Shieldin-complex and ASTE1/CST-DNA polymerase α). In Ku-deficient G0/G1-arrested pro-B cells (and to a much lesser extent in XRCC4- or Ligase IV-deficient cells), V(D)J joints harbor rather short resection tracks (typically less than 100 nucleotides) (Yu et al., 2020; Liang et al., 2021). Could RAG-DSB ends benefit from Shieldin complex protection against resection in such circumstances? Additionally, the nature of alt-EJ and the factors implicated in alt-EJ in G0/G1-phase cells as opposed to Pol θ-mediated alt-EJ in S-G2/M remain to be established. The role of these sub-pathways in the onset of pro-B cell lymphomas in NHEJ/p53-deficient animals also remains to be investigated. Finally, antigen receptor loci relocate from the nuclear periphery to permissive euchromatin in the nuclear interior before V(D)J recombination (Rogers et al., 2021). This subnuclear relocation likely provides specific local chromatin environments that might influence downstream DSB repair events (Mitrentsi et al., 2020). Recent studies have also highlighted the importance of 3D genome (re)organization and dynamics in DSB repair, for instance through the establishment of γH2AX/53BP1 DSB response foci (Arnould et al., 2021) or the restriction of homology search during HR (Piazza et al., 2021), two crucial chromatin events influencing DSB repair outcome and pathway choice. How such chromosome dynamics contributes to (lack of) DNA DSB pathway choice and overall genome integrity maintenance during V(D)J recombination remains a question for future studies.

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REVIEW
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DNA Double Strand Break Repair and Its Control by Nucleosome Remodeling

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DNA double strand breaks (DSBs) are repaired in eukaryotes by one of several cellular mechanisms. The decision-making process controlling DSB repair takes place at the step of DNA end resection, the nucleolytic processing of DNA ends, which generates single-stranded DNA overhangs. Dependent on the length of the overhang, a corresponding DSB repair mechanism is engaged. Interestingly, nucleosomes—the fundamental unit of chromatin—influence the activity of resection nucleases and nucleosome remodelers have emerged as key regulators of DSB repair. Nucleosome remodelers share a common enzymatic mechanism, but for global genome organization specific remodelers have been shown to exert distinct activities. Specifically, different remodelers have been found to slide and evict, position or edit nucleosomes. It is an open question whether the same remodelers exert the same function also in the context of DSBs. Here, we will review recent advances in our understanding of nucleosome remodelers at DSBs: to what extent nucleosome sliding, eviction, positioning and editing can be observed at DSBs and how these activities affect the DSB repair decision.

Keywords: nucleosome remodeling, double strand break, DNA repair, DNA end resection, cell cycle, genome stability

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INTRODUCTION

DNA double strand breaks are a highly toxic form of DNA damage, arising from intrinsic and extrinsic sources (Ciccia and Elledge, 2010). Eukaryotes are equipped with several mechanisms to repair DSBs, including non-homologous end joining (NHEJ), alternative end joining (alt-EJ), homologous recombination (HR) and single strand annealing (SSA) (Chang et al., 2017; Ranjha et al., 2018). Notably, these pathways do not only have different prerequisites (for example HR requiring a homologous donor sequence), but they also differ in the repair outcome and the potential to introduce genetic changes (such as mutations and chromosomal rearrangements). The cellular repair pathway decision is therefore critical for the survival of the affected cell or organism as well as for the stability of its genome (Symington and Gautier, 2011). Moreover, the fact that DSB repair is controlled by endogenous factors is a major limitation for genome editing strategies, which can nowadays involve efficient delivery of DSBs at the gene of interest, but often lead to a heterogenous outcome of the genome editing reaction across cell populations.

The cellular DSB repair pathway decision is made at the step of DNA end resection, the nucleolytic processing of DSB ends (Symington and Gautier, 2011; Cejka, 2015; Daley et al., 2015; Symington, 2016; Bonetti et al., 2018). Resection involves endo- and exonucleolytic cleavage of DNA ends that reveals 3′ single-stranded DNA overhangs. Notably, resection destroys the substrate for repair by NHEJ and increasing amounts of 3′ single-stranded DNA (ssDNA) predisposes for repair

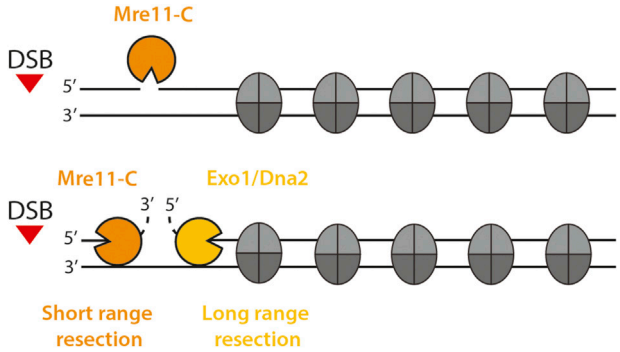

FIGURE 1 | Eukaryotic DNA end resection in the chromatin context. DNA end resection is a two step process that can be divided into short-range resection (orange) and long-range resection (yellow). Mre11-C initiates short-range resection by nicking the 5′ terminated strand in proximity to the DSB via its endonuclease activity. Then, Mre11-C generates a short 3′ ssDNA overhang close to the DSB using its 3′-5′ exonuclease function. Exo1 and STR-Dna2 carry out long-range resection and extend the length of the resected ssDNA tract through chromatin.

by different mechanisms (alt EJ < HR < SSA, Blier et al., 1993; Falzon et al., 1993; Ira et al., 2004). The enzymatic process of resection has been subject of excellent reviews in this issue and elsewhere (Symington and Gautier, 2011; Cejka and Symington, 2021; Elbakry and Löbrich, 2021; Sanchez et al., 2021). Here we focus on how resection and thereby the repair pathway decision is regulated by nucleosomes and nucleosome remodelers, enzymes that can evict, position and edit nucleosomes. For general reviews on how DNA damage triggers post-translational histone modifications, we refer to the following articles (Smeenk and van Attikum, 2013; Van and Santos, 2018).

Nucleosomes form obstacles to the resection nucleases (**Figure 1**). Initial short-range resection is carried out by the Mre11-complex (Mre11-C in the following, consisting of Mre11-Rad50-Xrs2 with the Sae2 activator in budding yeast, and analogously of MRE11-RAD50-NBS1 with CtIP in human) (Symington and Gautier, 2011; Cejka and Symington, 2021; Elbakry and Löbrich, 2021; Sanchez et al., 2021). Endonucleolytic cleavage by Mre11-C occurs preferentially within nucleosome-free linker DNA, suggesting that nucleosomal DNA is protected and/or that chromatin binding of Mre11-C is guided by nucleosomes (Mimitou et al., 2017; Wang et al., 2017). Moreover, the nucleases that carry out long-range resection are directly inhibited by the presence of nucleosomes: biochemical studies with yeast proteins have shown that the Exo1 exonuclease is unable to act on a nucleosome substrate and the combined helicase-endonuclease STR-Dna2 (Sgs1-Top3-Rmi1-Dna2) can only process nucleosomal DNA, if sufficient nucleosome-free DNA is present (Adkins et al., 2013). Therefore, nucleosomes are a barrier to the resection process and resection control factors are expected to modify the permeability of this barrier.

Nucleosome remodelers have received attention as regulators of DNA end resection and DSB repair pathway choice. These nucleosome remodelers are enzymes that in ATP-dependent fashion catalyze the breakage of histone-DNA-contacts within the nucleosome and translocate the DNA relative to histone proteins (Clapier and Cairns, 2009; Clapier et al., 2017). All eukaryotes possess several nucleosome remodelers - often in the form of multi-protein complexes - which are grouped into several sub-families according to the conservation of their ATPase subunit (Flaus et al., 2006). Biochemical and structural data suggest that the overall enzymatic mechanism of DNA translocation and breakage of DNA-histone contacts is highly related (Clapier and Cairns, 2009; Clapier et al., 2017) (with the potential exception of Fun30/SMARCAD1, see below). Nonetheless, studies on gene transcription and general chromatin organization have revealed that specific remodelers appear to have specific enzymatic activities, by which they slide, evict, position or edit nucleosomes (Clapier and Cairns, 2009; Clapier et al., 2017). Here, we will investigate whether such distinct roles can also be found in the context of DNA double strand breaks and how these activities may affect DSB repair. A natural focus of this review will be the budding yeast system, where remodelers have been studied comprehensively also in the context of DSBs, but we will additionally address whether the picture emerging from these studies is conserved in higher eukaryotes.

RESECTION IS AFFECTED BY NUCLEOSOMES

In many eukaryotes, DNA end resection is carried out by three resection enzymes, Mre11-C, STR-Dna2 and Exo1, which act specifically at one of the two stages of the resection process (short-range resection/resection initiation and long-range resection/resection elongation, **Figure 1**). Notably, all three act by distinct molecular mechanisms and it is therefore unsurprising that nucleosomes have distinct effects on each of them. Mre11-C recognizes the DSB end either directly or through a DSB end-binding protein (most likely the end-binding factor Ku) and, after activation by Sae2/CtIP, induces a single-strand break on the 5′-strand (Sartori et al., 2007; Cannavo and Cejka, 2014; Anand et al., 2016; Deshpande et al., 2016; Reginato et al., 2017; Wang et al., 2017). From this point, bidirectional resection occurs: Mre11-C catalyzes 3′-5′ exonucleolytic resection towards the break, while Dna2 and Exo1 exonucleases catalyze long-range resection with 5′-3′ polarity into undamaged chromatin (Mimitou and Symington, 2008; Zhu et al., 2008; Cejka et al., 2010; Niu et al., 2010; Garcia et al., 2011; Shibata et al., 2014).

Preferential cleavage of linker DNA indicates that nucleosomal DNA may be refractory to endonucleolytic clipping by Mre11-C (Mimitou et al., 2017; Wang et al., 2017). However, nucleosomes per se are not a barrier to Mre11-C. Rather, it can slide or reach over nucleosomes (Myler et al., 2017; Wang et al., 2017). In cases where such bypass occurs, the nucleosome located between DSB and incision site could then potentially constitute a barrier to the 3′-5′ exonuclease activity of Mre11-C. Given the dual

endo- and exonucleolytic activities of Mre11-C, this question has so far been difficult to address.

Long-range resection enzymes are even more strongly affected by the presence of nucleosomes. For example, *in vitro* studies have shown that Exo1 cannot resect through nucleosomes (Adkins et al., 2013), suggesting that additional activities are needed to overcome the chromatin barrier. Interestingly, changing nucleosome composition may be sufficient to allow Exo1-mediated resection. Incorporation of the H2A-variant H2A.Z decreases nucleosome stability and increases accessibility of nucleosomal DNA (Abbott et al., 2001; Zhang et al., 2005; Jin and Felsenfeld, 2007; Adkins et al., 2013; Watanabe et al., 2013; Lewis et al., 2021), which may allow Exo1 to bypass the nucleosomal barrier (Adkins et al., 2013).

In contrast to Exo1, the other long-range resection enzyme STR-Dna2 is in principle able to bypass nucleosomes. This may be due to a different enzymatic mechanism. While during long-range resection STR-Dna2 has the net effect of an exonuclease, STR-Dna2 utilizes the combined action of the Sgs1 helicase that unwinds DNA, followed by endonucleolytic cleavage of the emerging flap structure by Dna2 (Cejka et al., 2010; Niu et al., 2010). Apparently, the Sgs1 helicase motor is powerful enough to disrupt nucleosomes, allowing STR-Dna2 to resect nucleosomal DNA (Adkins et al., 2013). However, in order to carry out resection of nucleosomal DNA, STR-Dna2 will need as much as 300 bp of free DNA to be able to traverse through nucleosomes (Adkins et al., 2013). This distance is greater than the nucleosomal linker DNA-length and, consistently, STR-Dna2 is effectively inhibited by a nucleosomal array (Adkins et al., 2013).

Therefore, both long-range resection enzymes are blocked by chromatin and will require the activity of additional factors. One factor that could help to overcome the nucleosomal barrier is Mre11-C. Speculatively, Mre11-C could catalyze further endonucleolytic incisions downstream of the nucleosome from which long-range nucleases could (re-)initiate and thereby allow to bypass the nucleosome barrier. Currently, such an auxiliary role of Mre11-C in long-range resection lacks experimental support, but recent data suggest that short-range and long-range resection nucleases work in a coordinated fashion (Ceppi et al., 2020).

Alternatively, resection enzymes will need assistance by chromatin remodelers to get past nucleosomes and it is therefore important to consider how these enzymes may be able to modify the nucleosome barrier.

REMODELERS HAVE DISTINCT ROLES IN CHROMATIN ORGANIZATION

Eukaryotes express several nucleosome remodelers (Flaus et al., 2006) and chromatin immunoprecipitation (ChIP) and related techniques have localized several of them to DSBs (Bantele et al., 2017; Bennett and Peterson, 2015; Bennett et al., 2013; Bird et al., 2002; Chai et al., 2005; Chen et al., 2012; Costelloe et al., 2012; Downs et al., 2004; Eapen et al., 2012; Gnugnoli et al., 2021; Lademann et al., 2017; Morrison et al., 2004; Shim et al., 2005; 2007; Tsukuda et al., 2005; van Attikum et al., 2004; 2007). This raises the question, whether these remodelers have distinct functions at DSBs or whether they act redundantly.

Nucleosome remodelers are found to be either single protein enzymes or multi-protein complexes. Historically, four major sub-families of remodelers have been proposed (Clapier and Cairns, 2009), but phylogenetic analysis based on sequence conservation of the catalytic ATPase subunits showed the existence of additional sub-families (Flaus et al., 2006). Five sub-families are found throughout eukaryotes – ISWI, SWI/SNF, CHD1, INO80 and Fun30/ETL. In contrast, ALC1, CHD7 and Mi2/NURD sub-families are not found throughout eukaryotes, with ALC1 and CHD7 orthologues specifically found in metazoans (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998; Ma et al., 2008; Bouazoune and Kingston, 2012). **Table 1** summarizes the different remodeler sub-families with their putative catalytic activities and involvement in DSB repair.

It seems expedient to group remodelers not only by evolutionary conservation, but also by functional similarity (**Figure 2**, **Table 1**). *In vitro* and *in vivo* we can discriminate at least three activities of nucleosome remodelers: 1) sliding/eviction leads to movement of nucleosomes along DNA that can even result in the removal of the entire nucleosome (**Figure 2A**); 2) positioning involves movement of nucleosomes to form regularly spaced nucleosomal arrays (**Figure 2B**); 3) editing involves the exchange of histones (commonly H2A-H2B dimers) to alter the composition of nucleosomes (Clapier and Cairns, 2009; Clapier et al., 2017). Based on studies of genome-wide chromatin organization, we currently think that SWI/SNF sub-family complexes (SWI/SNF and RSC in yeast) act as major sliding/eviction enzymes, that ISWI and CHD1 sub-family remodelers as well as INO80-C act as positioning enzymes and that INO80 sub-family complexes (SWR1 and INO80 in yeast) catalyze editing (**Table 1**, Clapier and Cairns, 2009; Clapier et al., 2017). In the following, we will investigate whether nucleosome remodelers carry out the same activities at DSBs.

NUCLEOSOME EVICTION AND RESECTION ARE COUPLED

With nucleosomes forming a barrier to resection, nucleosome eviction is the most straight-forward solution to allow spreading of resection into chromatin (**Figure 3**). Indeed, nucleosomes are lost around DSBs in the region where resection occurs (Bantele and Pfander, 2019; Chen et al., 2008; Mimitou et al., 2017; Tsukuda et al., 2005; 2009; van Attikum et al., 2007). While it was proposed that nucleosomes may associate in some form with resected, single-stranded DNA to form single-stranded nucleosomes (Adkins et al., 2017; Huang et al., 2018), a dedicated study did not find evidence to support wide-spread association of nucleosomes with single-stranded DNA *in vivo* (Peritore et al., 2021). But how do nucleosomes become evicted and how do sliding/evicting nucleosome remodelers of the SWI/SNF sub-family facilitate this eviction (**Figure 3A**)? In budding yeast, the SWI/SNF and RSC complexes are specifically recruited to DSBs (Chai et al., 2005; Shim et al., 2005, 2007; Kent et al.,

TABLE 1 | Overview of nucleosome remodeler sub-families and their members.

Family	Sub-family	Putative activity	*S. cerevisiae*	*H. sapiens* orthologues	Function at DSBs
Snf2-like	SWI/SNF	Nucleosome sliding/ eviction	SWI/SNF RSC	BAF PBAF	Delamarre et al. (2020), Hays et al. (2020), Hu et al. (2020), Kakarougkas et al. (2014), Kent et al. (2007), Lee et al. (2010), Meisenberg et al. (2019), Ogiwara et al. (2011), Peng et al. (2009), Peritore et al. (2021), Qi et al. (2015), Shim et al. (2005), Shim et al. (2007), Ui et al. (2014), Watanabe et al. (2014), Wiest et al. (2017)
	ISWI	Nucleosome positioning	Isw1a Isw1b Isw2	ACF CHRAC NoRC RSF WICH NURF CERF	Casari et al. (2021), Delamarre et al. (2020), Helfricht et al. (2013), Lan et al. (2010), Nakamura et al. (2011), Pessina and Lowndes, (2014), Sánchez-Molina et al. (2011), Sheu et al. (2010), Smeenk et al. (2012), Toiber et al. (2013), Vidi et al. (2014), Xiao et al. (2009)
	CHD-I	Nucleosome positioning	Chd1	CHD1, CHD2	Delamarre et al. (2020), Gnugnoli et al. (2021), Kari et al. (2016), Luijsterburg et al. (2016), Zhou et al. (2018)
	CHD-II	?	-	Mi-2/NuRD	Chou et al. (2010), Goodarzi et al. (2011), Larsen et al. (2010), Luijsterburg et al. (2012), Pan et al. (2012), Polo et al. (2010), Qi et al. (2016), Smeenk et al. (2010), Smith et al. (2018), Spruijt et al. (2016)
	CHD-III	?	-	CHD6, CHD7, CHD8, CHD9	Rother et al. (2020)
	ALC1	?	-	ALC1	Ahel et al. (2009), Blessing et al. (2020), Juhász et al. (2020), Sellou et al. (2016)
Swr1-like	INO80	Nucleosome editing Nucleosome positioning	INO80 SWR1	INO80 SRCAP TRAPP/Tip60	Adkins et al. (2013), Alatwi and Downs, (2015), Bennett et al. (2013), Brahma et al. (2017), Chen et al. (2012), Downs et al. (2004), Kalocsay et al. (2009), Lademann et al. (2017), Morillo-Huesca et al. (2010), Morrison et al. (2004), Oberbeckmann et al. (2021b), Papamichos-Chronakis et al. (2006), Tsukuda et al. (2005), van Attikum et al. (2004), van Attikum et al. (2007)
	Fun30/ ETL	?	Fun30	SMARCAD1	Bantele et al. (2017), Chen et al. (2012), Costelloe et al. (2012), Densham et al. (2016), Eapen et al. (2012)

Nucleosome remodelers are grouped into two families based on conservation of the ATPase subunit: Snf2-like and Swr1-like. Both families have several sub-families.
Snf2-like: The SWI/SNF (switch/sucrose non-fermentable) sub-family consists of two members in budding yeast - SWI/SNF and RSC (remodels the structure of chromatin) – as well as in human – BAF and PBAF. For human BAF variant complexes can be found harbouring ATPase subunit paralogs (Mittal and Roberts, 2020). The ISWI (imitation switch) sub-family in yeast contains 3 active complexes – Isw1a, Isw1b, Isw2 - that combine 2 different catalytic subunits - Isw1 and Isw2 - with different sets of proteins. For humans the setup with 2 catalytic subunits is similar, but with a higher number of different complexes: ACF, CHRAC, NoRC, RSF, WICH, NURF, CERF (Aydin et al., 2014). The CHD (chromodomain helicase DNA-binding) sub-family has a single member in yeast – Chd1 - and 3 subfamilies with in total 9 members in human: CHD1-2, CHD3-5 – forming NuRD/Mi-2 complex and CHD6-9 (Marfella and Imbalzano, 2007). The ALC1 sub-family carries a macrodomain for poly(ADP-ribose)-binding instead of a chromodomain and is found in human (Ahel et al., 2009).
Swr1-like: The INO80 (inositol requiring) sub-family has two members in yeast: INO80 and SWR1. In humans again there is additional complexity of this sub-family with INO80, SRCAP and TRAPP/Tip60 complexes (Willhoft and Wigley, 2020). The Fun30/ETL sub-family contains Fun30 in yeast and SMARCAD1 in human (Bantele and Pfander, 2019). Even though nucleosome remodelers appear to follow a highly similar enzymatic mechanism, they appear to exhibit distinct activities in chromatin organization. These putative activities are given along studies showing possible functions at DNA double strand breaks.

2007; Liang et al., 2007; Bennett et al., 2013; Bennett and Peterson, 2015; Wiest et al., 2017), suggesting that they may act during DSB repair or signaling. To interrogate the function of SWI/SNF and RSC, deletion of non-essential subunits or conditional depletion of the essential catalytic subunits have been used. Interestingly, interfering with either SWI/SNF or RSC function induced a defect already in the association of Mre11-C with DSBs (Shim et al., 2007; Wiest et al., 2017), suggesting that these remodelers could act at an early stage of DSB repair. Notably, under single-mutant conditions, resection and DSB repair were found to be delayed or reduced, but not abolished. Recently, experimental conditions were established that allowed to simultaneously induce the degradation of the ATPase subunits of both SWI/SNF and RSC (Peritore et al., 2021). Under these double mutant conditions, we find that nucleosome eviction and resection are both blocked (Peritore et al., 2021). This indicates that 1) SWI/SNF and RSC are redundantly required for DNA end resection and that 2) resection and nucleosome eviction are intrinsically coupled. Altogether, these data are consistent with SWI/SNF and RSC complexes playing a major role as nucleosome evictors also in the context of DSBs. The single mutant data (Shim et al., 2007; Wiest et al., 2017), which showed defects in recruitment of Mre11-C, suggest that both complexes play an early role and evict or move DSB-proximal nucleosomes to allow binding of Mre11-C as well as resection initiation. Whether SWI/SNF or RSC influence Mre11-C activity, endonucleolytic clipping in particular, remains to be tested.

These findings raise the question of how these nucleosome remodelers sense the presence of a DSB and become recruited to DSB-proximal chromatin at such an early stage. Notably, RSC and SWI/SNF localize to the proximity of DSBs independently of each other and follow different recruitment kinetics, suggesting that they recognize DSBs by different mechanisms. RSC is recruited to a DSB within 10 min and thereby precedes resection initiation (Chai et al., 2005). While its recruitment kinetics are therefore similar to those of Mre11-C (Shim et al., 2007), we currently do not understand which signal is being recognized by RSC. SWI/SNF in contrast shows significantly slower recruitment (Chai et al., 2005) that depends on nucleosome modifications. Specifically, histone

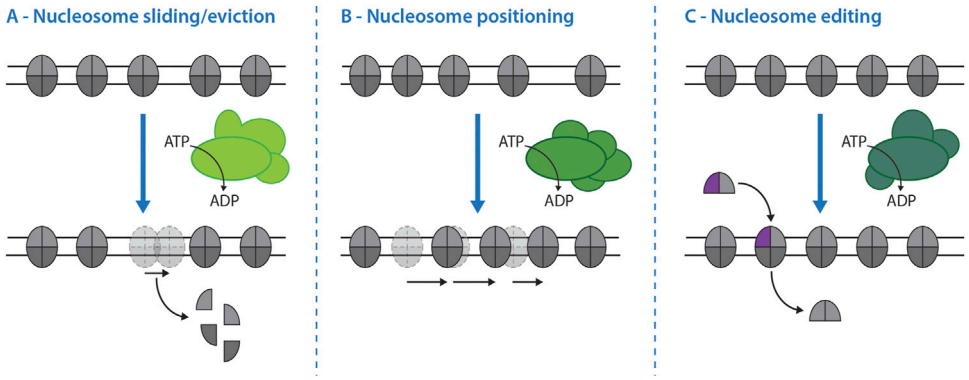

FIGURE 2 | Nucleosome remodeler activities and their effects on chromatin. The activity of different nucleosome remodelers (shades of green) can result in three principal effects on nucleosomes. **(A)**–Nucleosome sliding and eviction. While all remodelers have the propensity to slide nucleosomes, eviction of nucleosomes from double-stranded DNA is catalyzed mainly by the SWI/SNF sub-family of nucleosome remodelers. **(B)**–Nucleosome positioning. Some nucleosome remodelers have the ability to slide and position nuclesomes on DNA in a controlled fashion that leads to the formation of regularly spaced arrays. In the budding yeast system this activity is catalyzed mainly by ISW1a-, ISW1b-, Chd1 and INO80-complexes. **(C)**–Nucleosome editing. Nucleosome editing is defined as the exchange of canonical histones (grey) for non-canonical histone variants, like H2A.Z (purple), within the nucleosome and vice versa. In budding yeast H2A/H2A.Z exchange is performed by the INO80 sub-family of remodelers: the SWR1-complex catalyzes the incorporation of H2A.Z-H2B dimers, while the INO80-C is thought to catalyze the reverse reaction.

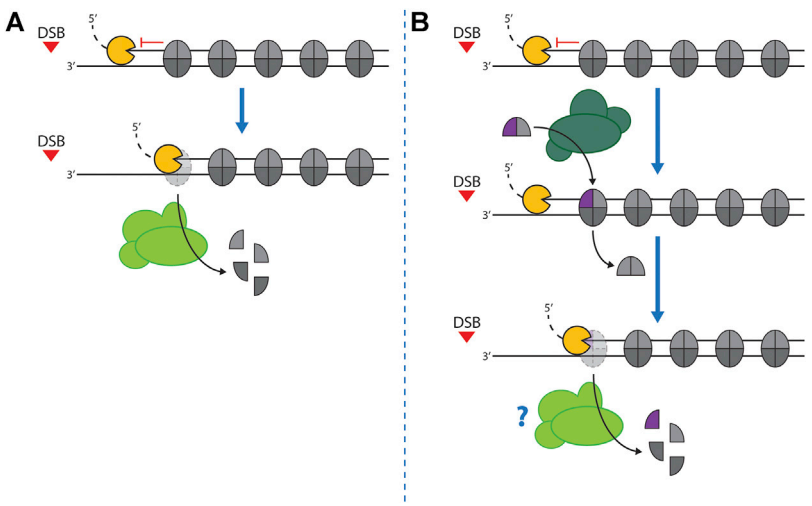

FIGURE 3 | Nucleosome eviction at DSBs. **(A)**–Resection nucleases (Exo1/Dna2) are inhibited by the presence of nucleosomes. Thus, eviction of nucleosomes from dsDNA is required to facilitate resection. This reaction may be catalyzed by nucleosome remodelers with evicting activity (light green). Moreover, binding of the Mre11-C to the DSB ends might be inhibited by nucleosomes (not shown). Therefore, eviction by nucleosome remodelers might be additionally required also for resection initiation. **(B)**–Incorporation of H2A.Z (purple) into nucleosomes by nucleosome remodelers with editing activity (dark green) leads to a reduced stability of nucleosomes. H2A.Z-containing nucleosomes may therefore be directly evicted by long-range resection nucleases, but nucleosome remodelers with evicting activity (light green) may be additionally involved (see "?").

acetylation is recognized by SWI/SNF and appears to lead to its DSB recruitment, consistent with the presence of several acetylation-binding bromodomains in the SWI/SNF complex (Bennett and Peterson, 2015; Cheng et al., 2021). Notably, the histone acetyltransferase NuA4 is specifically recruited to DSBs and this recruitment was shown to depend on Mre11-C (Cheng et al., 2021). The fact that SWI/SNF was found to be required for recruitment of Mre11-C, but at the same time also dependent on Mre11-C activity (Shim et al., 2005, 2007; Wiest et al., 2017) is not necessarily a contradiction, but could suggest the presence of a positive feedback loop that promotes resection initiation.

Biochemical data suggest that long-range resection should be particularly dependent on nucleosome eviction (Adkins et al., 2013). Consistently, SWI/SNF appears to stimulate long range resection (Wiest et al., 2017), but this has yet to be correlated with nucleosome eviction. Altogether, these data show that RSC and SWI/SNF complexes promote DNA end resection in budding yeast and likely do so by acting as nucleosome evictors. Detailed biochemical and cell biological analysis will however be needed to

pinpoint exactly at which steps of DNA end resection these nucleosome remodelers act. These studies also need to account for the fact that RSC and SWI/SNF may also influence the long-range chromatin response to DSBs (on the 10 kb–1 Mb range). Indeed, γH2A - the long-range chromatin mark of DSBs - was found to be reduced in RSC mutants (Kent et al., 2007; Shim et al., 2007), but it is unclear whether this effect relates to nucleosome eviction.

Lastly, SWI/SNF is also required later during HR, as SWI/SNF mutants show defects in synapsis and strand invasion (Chai et al., 2005). It is currently unclear whether this is due to defects in resection, due to a second "late" role in HR or due to long-range chromatin changes on the broken chromosome.

Nucleosome eviction appears to be conserved in human remodeler complexes. Human BAF and PBAF complexes are recruited to sites of DSBs (Park et al., 2006; Hays et al., 2020). Moreover, they appear to promote resection, possibly by acting on the Mre11-C activator CtIP (Hays et al., 2020). This suggests an early role in resection and it will be interesting to investigate whether this function is linked to nucleosome eviction.

While SWI/SNF and RSC are the major players in nucleosome eviction, it could be possible that also other nucleosome remodelers evict nucleosomes during DSB repair and resection. In particular, the INO80 complex has been linked to the eviction of nucleosomes at sites of transcription and DSBs as well (Tsukuda et al., 2005; van Attikum et al., 2007; Qiu et al., 2020), but given several functions of INO80 during DSB repair (see below) this activity is particularly challenging to ascertain. Additionally, whatever this INO80 complex function is, it appears to act differently from SWI/SNF and RSC complexes (Peritore et al., 2021). In all, we therefore conclude that 1) histone eviction occurs at DSBs, that 2) it is critical for DSB resection and repair and that 3) it appears to be mediated by the major cellular eviction activities of the SWI/SNF sub-family complexes.

THE ROLE OF NUCLEOSOME POSITIONING AT DOUBLE STRAND BREAKS REMAINS TO BE DETERMINED

Nucleosomes are positioned in a non-random fashion throughout the genome. In particular, a specific organization is seen at sites of transcribed genes, where a nucleosome-free region marks or neighbors the transcription start site, followed by regularly spaced nucleosomal arrays (Yuan et al., 2005; Weiner et al., 2010; Baldi et al., 2020). Positioning remodelers are responsible for the characteristic spacing of nucleosomes within such nucleosome arrays (Baldi et al., 2020). To generate the specific spacing of nucleosomes within the array, positioning remodelers use intrinsic ruler mechanisms as well as sensing of DNA shapes (Yamada et al., 2011; Krietenstein et al., 2016; Oberbeckmann et al., 2021a; Oberbeckmann et al., 2021b). The generation of nucleosome arrays has been extensively studied in budding yeast, where a combination of *in vitro* and *in vivo* studies suggests that four remodelers – Chd1, ISW1a, ISW2 and INO80 – can specifically position nucleosomes to form nucleosome arrays (Gkikopoulos et al., 2011; Krietenstein et al., 2016; Ocampo et al., 2016; Kubik et al., 2019; Oberbeckmann et al., 2021b). Importantly, these remodelers also sense the presence of barrier-factors bound at specific sites in the genome to which the array is aligned to or "phased" (Eaton et al., 2010; Li et al., 2015; Krietenstein et al., 2016; Kubik et al., 2018; Rossi et al., 2018). Typical barrier factors are DNA-binding factors, like the abundant general regulatory factors Abf1, Rap1 or Reb1 in budding yeast or genome organizing factors like CTCF in mammals or Phaser in flies (Fu et al., 2008; Wiechens et al., 2016; Baldi et al., 2018). Importantly, recent *in vitro* work suggests that also DSBs are sensed as a barrier-factor by nucleosome remodelers and guide the formation of nucleosome arrays (Oberbeckmann et al., 2021a).

The finding that regularly spaced nucleosome arrays can form around DSBs in *in vitro* systems raises two questions: do remodelers position nucleosomes to form arrays around DSBs also *in vivo* and would such arrays promote DNA end resection? Experimentally, nucleosome positioning is typically investigated using micrococcal nuclease (MNase), which cleaves preferentially non-nucleosomal DNA. Several studies that used MNase to investigate nucleosome localization around a single DSB showed eviction of DSB-proximal nucleosomes, but came to different conclusions as to whether DSB-distal nucleosomes would shift their position (Kent et al., 2007; Shim et al., 2007; Tsabar et al., 2016). While these results are seemingly contradictory, this may simply be due to the fact that results from a single DSB are difficult to interpret. For example the newly formed array can be indistinguishable from the initial nucleosome positions, if the DSB and initial barrier factor are located at the same position. To overcome these limitations, a recent study utilized the *PHO5* gene, with its well characterized nucleosomal array and found evidence for eviction of the break-proximal nucleosome as well as repositioning of further distal nucleosomes (Tripuraneni et al., 2021). Further studies will need to show whether repositioned nucleosomes are indeed aligned to the DSB and whether the DSB itself or DSB-associated proteins serve as barrier. Furthermore, studies need to identify, if arrays are generated by positioning remodelers Chd1, ISW1a, ISW2 or INO80.

Interestingly, several studies in both yeast and human cells, point towards a function of these specific remodelers in promoting homologous recombination (Lan et al., 2010; Nakamura et al., 2011; Smeenk et al., 2012; Toiber et al., 2013; Kari et al., 2016; Zhou et al., 2018; Rother et al., 2020; Casari et al., 2021; Gnugnoli et al., 2021). In particular, remodelers of ISWI, CHD1 and CHD7 sub-families appear to be recruited to sites of DNA damage and to stimulate resection (Smeenk et al., 2012; Toiber et al., 2013; Kari et al., 2016; Delamarre et al., 2020; Rother et al., 2020; Gnugnoli et al., 2021). The precise mechanism by which these remodelers promote resection and HR is however uncertain. Moreover, even if these remodelers established nucleosome arrays around DSBs, it is at this point entirely unclear whether such arrays will have a positive function in DSB repair or whether they are simply a consequence of the enzymatic mechanism of positioning remodelers (Baldi et al.,

2018). Therefore, despite first hints that nucleosome arrays could form in the proximity of DSBs, the role of positioning remodelers in DSB repair still needs to be determined.

NUCLEOSOME EDITING AND H2A.Z EXCHANGE GUIDE DOUBLE STRAND BREAK REPAIR

Nucleosome editing describes the activity of exchanging canonical histone subunits with non-canonical histone variants and vice versa (Das and Tyler, 2013; Venkatesh and Workman, 2015). Nucleosome remodelers can facilitate editing by catalyzing the exchange of histone dimers. In eukaryotes, several histone variants exist primarily for H2A and H3 (Draizen et al., 2016; Talbert and Henikoff, 2010; 2017; 2021). While the principal mechanism for the incorporation of H3 and its variants is via *de novo* assembly of nucleosomes, H2A and its variants can be incorporated into existing nucleosomes by H2A-H2B dimer exchange (reviewed in Luger et al., 2012). In this chapter, we will therefore concentrate on nucleosome editing of H2A. Nucleosome editing has been extensively studied in budding yeast, where two H2A variants exist: H2A (which includes features of H2A.X) and H2A.Z (Santisteban et al., 2000). The SWR1 complex catalyzes the incorporation of H2A.Z-H2B dimers (Krogan et al., 2003; Mizuguchi et al., 2004). Furthermore, the INO80 complex is thought to catalyze the reverse reaction, the exchange of H2A.Z-H2B with H2A-H2B dimers (Papamichos-Chronakis et al., 2011; Brahma et al., 2017). This model of INO80 function is based on the principal finding that deletion of the H2A.Z gene *HTZ1* genetically suppresses many phenotypes of mutants deficient in INO80 function (Lademann et al., 2017; Papamichos-Chronakis et al., 2006; 2011).

The SWR1 complex is the prototypical nucleosome editing remodeler: mechanistically, it is able to translocate short stretches of DNA with no changes in nucleosome position, which then allows H2A-H2B dimers to be exchanged for H2A.Z-H2B dimers (Wu et al., 2009; Luk et al., 2010; Ranjan et al., 2015; Willhoft et al., 2018; Singh et al., 2019). In budding yeast, the SWR1 complex incorporates H2A.Z into chromatin around DSBs, as indicated by 1) the recruitment of SWR1 to DSB sites (van Attikum et al., 2007; Morillo-Huesca et al., 2010) and 2) a transient increase in H2A.Z occupancy in the DSB-surrounding chromatin shortly after DSB induction (Kalocsay et al., 2009). A transiently increased incorporation of H2A.Z into DSB-proximal chromatin was observed also in human cells (Xu et al., 2012; Nishibuchi et al., 2014; Alatwi and Downs, 2015; Gursoy-Yuzugullu et al., 2015). Compared to canonical nucleosomes, H2A.Z-containing nucleosomes are more labile (Abbott et al., 2001; Zhang et al., 2005; Jin and Felsenfeld, 2007) suggesting that their presence will promote DNA end resection. Consistently, yeast cells lacking H2A.Z show a pronounced resection defect (Kalocsay et al., 2009; Lademann et al., 2017). In contrast, the absence of SWR1 causes a much milder resection phenotype (van Attikum et al., 2007; Chen et al., 2012; Adkins et al., 2013). These data suggest that either 1) H2A.Z becomes incorporated at DSB sites by an SWR1-independent mechanism or that 2) H2A.Z-incorporation into DSB-surrounding chromatin is not a major regulator of resection and that H2A.Z regulates resection by means independent from its incorporation in DSB-surrounding chromatin.

If H2A.Z-incorporation into DSB-proximal chromatin promotes resection, there are two putative mechanisms by which it could do so. First, the aforementioned reduction of nucleosome stability may allow remodelers or even resection nucleases to bypass and evict H2A.Z-containing nucleosomes (Adkins et al., 2013). Second, H2A.Z could serve as binding platform for associated factors (Xu et al., 2012) as has been shown for nucleotide excision repair (Yu et al., 2013). Binding of factors to H2A.Z or SUMO-modified H2A.Z is for example thought to lead to relocalization of DSBs to the nuclear periphery (Nagai et al., 2008; Kalocsay et al., 2009; Oza et al., 2009; Horigome et al., 2014). Relocalization of DSBs is also observed in Drosophila, where heterochromatic DSBs are first brought to the periphery of the heterochromatic domain (Chiolo et al., 2011) and then to the nuclear pore complex (Ryu et al., 2015). Similarly, in mammalian cells DSB relocation to discrete clusters in the periphery of heterochromatin has been observed (Jakob et al., 2011; Tsouroula et al., 2016; Schrank et al., 2018), but a connection between DSB relocation and H2A.Z has not been shown so far. Therefore, nucleosome editing and H2A.Z incorporation are used to regulate DSB repair, but the underlying molecular mechanisms warrant further investigation.

The importance of nucleosome editing for DSB repair raises the question whether H2A.Z incorporation becomes reversed at some point. Indeed, studies in budding yeast have shown that the INO80 complex is not only recruited to DSBs (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004; Bennett et al., 2013), but that it also counteracts H2A.Z incorporation (Papamichos-Chronakis et al., 2011). Also in human cells H2A.Z is removed from chromatin surrounding DSB sites (Xu et al., 2012; Nishibuchi et al., 2014; Alatwi and Downs, 2015; Gursoy-Yuzugullu et al., 2015; Clouaire et al., 2018). While INO80's role as nucleosome editing and H2A.Z removal enzyme was initially controversial (Papamichos-Chronakis et al., 2011; Watanabe et al., 2013; Jeronimo et al., 2015; Tramantano et al., 2016; Wang et al., 2016; Watanabe and Peterson, 2016), recent structural work showed that besides its nucleosome positioning activity, the INO80 complex may be able to catalyze translocation of short stretches of DNA without nucleosome sliding, consistent with histone dimer exchange activity (Ayala et al., 2018; Eustermann et al., 2018). This suggests that at DSBs INO80 may have at least two activities: 1) a nucleosome positioning activity (see above) and 2) a nucleosome editing activity (Papamichos-Chronakis et al., 2006; Alatwi and Downs, 2015; Brahma et al., 2017; Lademann et al., 2017). Consistent with INO80 antagonizing the SWR1 complex and removing H2A.Z from chromatin, mutants deficient in INO80 complex function accumulate H2A.Z around DSBs (Papamichos-Chronakis et al., 2006; Alatwi and Downs, 2015; Lademann et al., 2017). The dual remodeling activity of the INO80 complex complicates the interpretation of *ino80* mutant phenotypes. To overcome this issue, deletion of the H2A.Z gene *HTZ1* has been used, because it suppresses phenotypes arising from an H2A.Z removal defect. Using this approach, an H2A.Z removal function of the INO80 complex was found to promote the formation of the Rad51 nucleo-protein filament downstream of resection

(Lademann et al., 2017). In contrast, a resection-promoting function of the INO80 complex was found to be independent of H2A.Z (Lademann et al., 2017) and therefore unrelated to nucleosome editing. Moreover, also in human cells, nucleosome editing by the INO80 complex is important for DSB repair and acts after DNA end resection (Alatwi and Downs, 2015). Taken together, a picture emerges whereby nucleosome editing and H2A.Z incorporation by the SWR1 complex is involved in regulation of DNA end resection in yeast, while generally and throughout eukaryotes H2A.Z removal in DSB-surrounding chromatin is important for DSB repair, but likely acts only after resection.

FUN30/SMARCAD1 PROMOTE RESECTION BY ANTAGONIZING RESECTION-INHIBITORY FACTORS

Fun30 (from budding yeast), ETL1 (from mouse) and SMARCAD1 (from human) are the prototypical members of a sub-family of nucleosome remodelers that is evolutionary conserved throughout eukaryotes (Clark et al., 1992; Adra et al., 2000; Flaus et al., 2006). Historically they have not been considered major nucleosome remodelers and their molecular mechanisms have not yet been entirely elucidated (Bantele and Pfander, 2019). Recently, a study by the Luger lab suggested that SMARCAD1 evicts and also assembles entire nucleosomes by a mechanism that involves unique contacts between remodeler and nucleosome (Markert et al., 2021). Work with yeast Fun30 suggests that it can slide nucleosomes and mediate histone dimer exchange (Awad et al., 2010).

A key function of yeast Fun30 and human SMARCAD1 appears to be the stimulation of long-range resection (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). For example, in budding yeast cells lacking Fun30, long-range resection of a non-repairable DSB is 2-3-fold slower than in WT cells (Eapen et al., 2012; Bantele et al., 2017). Accordingly, *fun30* mutants scored similarly to mutants deficient in the long-range resection nucleases, when they were initially found in screens for resection-dependent repair of DSBs (Chen et al., 2012; Costelloe et al., 2012). Moreover, an evolutionary conserved pathway facilitates recruitment of Fun30 to sites of DNA end resection. This pathway requires the 9-1-1 complex as recruitment platform at the ssDNA-dsDNA junction and is activated during cell cycle phases (S-M phase), when also resection is activated (Chen et al., 2016; Bantele et al., 2017).

In contrast, Fun30 did not stimulate Exo1's ability to resect through a nucleosome in an *in vitro* system (Adkins et al., 2013). This finding raises the possibility that a crucial factor was missing from these reconstituted systems. Consistently, *fun30* mutant phenotypes can be suppressed by the additional depletion of the resection inhibitor Rad9 from yeast cells (Chen et al., 2012; Bantele et al., 2017). These data indicate a functional antagonism between Fun30 and Rad9. Notably, also in human cells SMARCAD1 acts as resection activator, while the Rad9 orthologue 53BP1 is a resection inhibitor (Lazzaro et al., 2008; Bunting et al., 2010; Bothmer et al., 2011; Costelloe et al., 2012; Densham et al., 2016), suggesting that the antagonism of both factors is conserved throughout eukaryotic evolution (please see (Sanchez et al., 2021)) in this issue for a detailed review on the interaction between 53BP1 and BRCA1 in the DSB repair decision). Notably, Rad9, 53BP1, as well as the fission yeast orthologue Crb2 associate with chromatin and have all been shown to bind to nucleosomes, where they recognize specific histone modifications (Huyen et al., 2004; Nakamura et al., 2004; Sanders et al., 2004; Wysocki et al., 2005; Botuyan et al., 2006; Du et al., 2006; Toh et al., 2006; Grenon et al., 2007; Hammet et al., 2007; Fradet-Turcotte et al., 2013; Wilson et al., 2016; Hu et al., 2017; Kilic et al., 2019). Rad9 orthologues appear to recognize distinct histone marks, but in each case two or more histone marks are bound (reviewed in Marini et al., 2019; Panier and Boulton, 2014), suggesting that Rad9 orthologues are multivalent histone binders. We therefore hypothesize that both Fun30 and Rad9 influence DSB-surrounding chromatin in an antagonistic fashion and that Fun30 specifically acts on Rad9-bound nucleosomes (Bantele and Pfander, 2019).

In budding yeast cells lacking both Fun30 and Rad9, resection and nucleosome eviction are fully functional (Peritore et al., 2021), suggesting that Fun30 is not required to overcome the general nucleosome barrier and that it is not the essential nucleosome evictor at DSBs. Alternatively, Fun30 may rather catalyze the direct removal of Rad9 from nucleosomes (**Figure 4A**) or it may counteract Rad9 association with nucleosomes by catalyzing histone dimer exchange which may remove one or more binding site(s) for Rad9 (**Figure 4B**). Lastly, it is possible that Fun30 slides or even entirely evicts Rad9-bound nucleosomes (**Figure 4C**). Given that Rad9 and Fun30 antagonize each other on multiple levels, including also the competition for binding to the scaffold protein Dpb11 (Granata et al., 2010; Pfander and Diffley, 2011; Bantele et al., 2017), future biochemical and structural studies will be needed to reveal the mechanism by which Fun30 promotes DNA end resection.

Also human SMARCAD1 antagonizes 53BP1. Depletion of SMARCAD1 stabilizes 53BP1 around DSB sites (Densham et al., 2016). However, resection regulation in human cells is more complex compared to yeast as besides SMARCAD1 a second resection promoting factor exist, the BRCA1-BARD1 complex (reviewed in Densham and Morris, 2019; Sanchez et al., 2021). BRCA1-BARD1 form an E3 ubiquitin-ligase complex that mediates ubiquitylation of H2A (Kalb et al., 2014; Densham et al., 2016; Leung et al., 2017; Nakamura et al., 2019). BRCA1-BARD1 is likely to act upstream of SMARCAD1, as ubiquitin-modified H2A promotes SMARCAD1 binding to nucleosomes around DSBs (Densham et al., 2016). Therefore, SMARCAD1 function has to be seen in the context of post-translational histone modifications, which affect DSB-surrounding chromatin. DSB-localized SMARCAD1 may also become post-translationally modified itself, including phosphorylation by the ATM kinase and ubiquitylation by the RING1 ubiquitin ligase (Chakraborty et al., 2018), which appears to activate the pro-resection function of SMARCAD1. These factors need to be taken into consideration for biochemical studies that ultimately will allow to understand whether the Fun30/SMARCAD1 sub-family remodelers facilitate resection by nucleosomes sliding and eviction, positioning or editing and whether it acts on nucleosomes or rather on nucleosome-associated proteins.

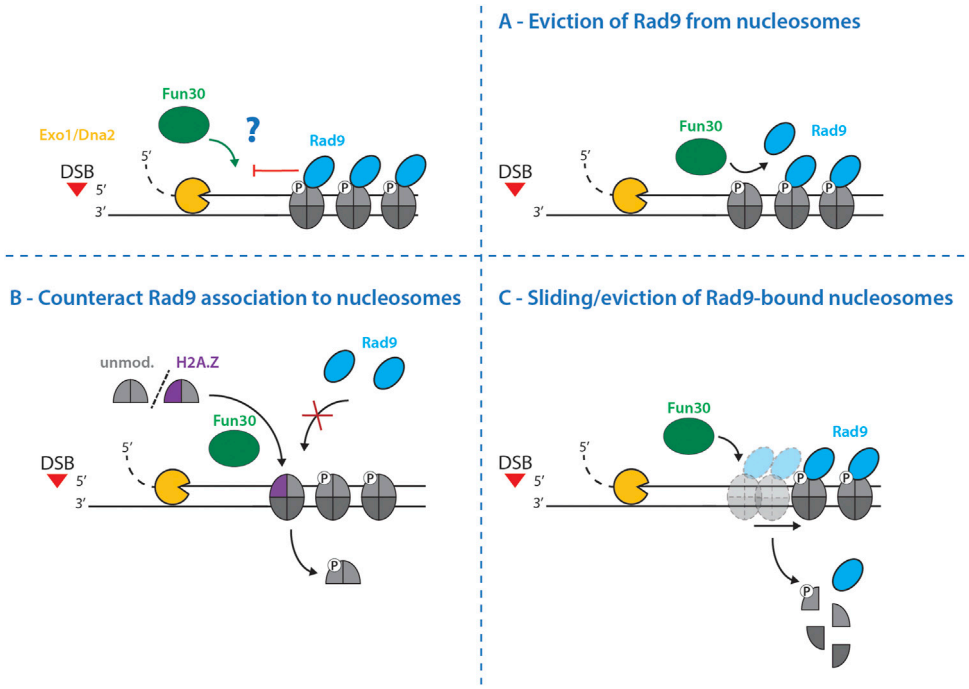

FIGURE 4 | Potential mechanisms by which Fun30 may promote resection. Long-range resection is controlled by the antagonism between the resection-promoting nucleosome remodeler Fun30 and the resection-inhibiting nucleosome binder Rad9. The precise mechanism of this antagonistic relationship is still elusive, but the following models are possible: **(A)**–Fun30 directly removes Rad9 from nucleosomes thereby removing the factor inhibiting resection. **(B)**–Fun30 counteracts Rad9 association with nucleosomes by exchanging histone dimers. It either incorporates histones lacking modifications necessary for Rad9 association – for example unmodified H2A, missing phosphorylation on S129 (γH2A), or the histone variant H2A.Z; both of which eliminate Rad9 binding sites. **(C)**–Fun30 slides and/or evicts Rad9-bound nucleosomes, freeing the DNA from the resection-inhibitory effects of Rad9 to allow the subsequent resection.

CONCLUSION

In all, we think that previous studies collectively indicate that nucleosome remodelers may serve similar roles during DSB repair as during gene transcription with nucleosome eviction, editing and potentially even positioning taking place at DSBs. Knowledge of the specific activities of individual nucleosome remodelers and of their redundancies thereby offers the potential to get to grips with chromatin changes occurring at DSBs. Moreover, we think that studies of DSB resection and repair may be generally inspired by analogies to gene transcription. Both processes appear to be similarly affected by the presence of chromatin, with nucleosomes forming a dynamic barrier and nucleosome remodelers facilitating its bypass.

Importantly, while nucleosomes clearly form a barrier to the resection nucleases, nucleosome remodelers equip cells with multiple ways to overcome this barrier. In this review, we have outlined several putative mechanisms of how bypass may occur. These include eviction, sliding and editing of nucleosomes. While we are still only beginning to understand how the nucleosome barrier is overcome, a key future question will be which bypass mechanism is chosen in which cellular scenario. Importantly, the nucleosome barrier and its dynamic nature offers additional possibilities to regulate resection and DSB repair. Moreover, critical factors of the DSB repair decision, such as 53BP1 and BRCA1, are proteins that bind and modify nucleosomes. Therefore, we propose that convergence of resection-regulatory pathways on nucleosomes is a central part of the cellular DSB repair decision.

AUTHOR CONTRIBUTIONS

LAK, MP, and LG contributed equally to writing of the manuscript. LAK, MP, LG and BP developed concepts presented here and wrote the manuscript.

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Immediate-Early, Early, and Late Responses to DNA Double Stranded Breaks

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Loss or rearrangement of genetic information can result from incorrect responses to DNA double strand breaks (DSBs). The cellular responses to DSBs encompass a range of highly coordinated events designed to detect and respond appropriately to the damage, thereby preserving genomic integrity. In analogy with events occurring during viral infection, we appropriate the terms Immediate-Early, Early, and Late to describe the pre-repair responses to DSBs. A distinguishing feature of the Immediate-Early response is that the large protein condensates that form during the Early and Late response and are resolved upon repair, termed foci, are not visible. The Immediate-Early response encompasses initial lesion sensing, involving poly (ADP-ribose) polymerases (PARPs), KU70/80, and MRN, as well as rapid repair by so-called 'fast-kinetic' canonical non-homologous end joining (cNHEJ). Initial binding of PARPs and the KU70/80 complex to breaks appears to be mutually exclusive at easily ligatable DSBs that are repaired efficiently by fast-kinetic cNHEJ; a process that is PARP-, ATM-, 53BP1-, Artemis-, and resection-independent. However, at more complex breaks requiring processing, the Immediate-Early response involving PARPs and the ensuing highly dynamic PARylation (polyADP ribosylation) of many substrates may aid recruitment of both KU70/80 and MRN to DSBs. Complex DSBs rely upon the Early response, largely defined by ATM-dependent focal recruitment of many signalling molecules into large condensates, and regulated by complex chromatin dynamics. Finally, the Late response integrates information from cell cycle phase, chromatin context, and type of DSB to determine appropriate pathway choice. Critical to pathway choice is the recruitment of p53 binding protein 1 (53BP1) and breast cancer associated 1 (BRCA1). However, additional factors recruited throughout the DSB response also impact upon pathway choice, although these remain to be fully characterised. The Late response somehow channels DSBs into the appropriate high-fidelity repair pathway, typically either 'slow-kinetic' cNHEJ or homologous recombination (HR). Loss of specific components of the DSB repair machinery results in cells utilising remaining factors to effect repair, but often at the cost of increased mutagenesis. Here we discuss the complex regulation of the Immediate-Early, Early, and Late responses to DSBs proceeding repair itself.

Keywords: DNA repair, double strand breaks (DSBs), Immediate-early response, Early response, Late response, pre-repair responses, non-homologous end joining (NHEJ), homologous recombination (HR)

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INTRODUCTION TO DNA DAMAGE RESPONSES

The DNA damage response (DDR) encompasses a network of biological pathways that detect and respond to various forms of DNA damage using a multitude of distinct and overlapping cellular mechanisms (**Figure 1** and Lindahl and Barnes, 2000; Jackson, 2002). As an estimated 10^5 lesions occur per cell per day in human cells, proper coordination of the DDR is essential to preserving genomic integrity (Lindahl, 1993; Hoeijmakers, 2009). Repair, cell cycle arrest, senescence, and apoptosis all represent biological responses to DNA damage that are dependent upon cell type and severity of damage (Jackson and Bartek, 2009). Correct coordination of the DDR protects the genome from the accumulation of mutations, ranging from simple nucleotide changes to complex chromosomal alterations such as those generated during chromothripsis (Jackson, 2002; Jackson and Bartek, 2009; Forment et al., 2012).

Interestingly, endogenous sources of DNA damage due to normal metabolism (e.g., errors during DNA metabolism or chemical attack by indigenous metabolites), rather than exogenous sources (e.g., radiation and environmental chemicals), are more important with respect to generating mutations that drive the cancerous phenotype (Tomasetti and Vogelstein, 2015). DNA damage comes in many forms, including incorrect hydrogen-bonding, bulky adducts, base damage, intrastrand cross-links, and damage to the sugar phosphate backbone (**Figure 1A** and Lindahl, 1993; Lindahl and Barnes, 2000; Jackson and Bartek, 2009). Incorrect pairing of bases is corrected by mismatch repair (MMR), bulky adducts by nucleotide excision repair (NER), and base damage by base excision repair (BER) (Friedberg, 2001; Jiricny, 2006; Krokan and Bjørås, 2013). The two strands of DNA can also be chemically cross-linked together and resolved by interstrand crosslink (ICL) repair, a highly complex pathway utilising proteins involved in other DNA repair pathways, as well as others identified as deficient in Fanconi anaemia (FA) (Deans and West, 2011; Semlow and Walter, 2021). Interestingly, in addition to having a dedicated FA core complex regulating E3 ubiquitin ligase activity, many of the other associated FANC proteins overlap with downstream double strand break (DSB) repair proteins, as a DSB forms transiently during the unhooking step required for repair of the crosslinks. These proteins include BRCA1, BRCA2, PALB2, RAD51, XRCC2, XPF, and REV7. Any of the lesions to the nucleotide, be it a simple base damage or a more complex event, can become strand breaks within the sugar-phosphate backbone if not repaired correctly (**Figure 1A**).

Single strand break (SSB) repair is often considered a 'specialised' form of BER, as most SSB repair proteins are also involved in either short-patch or long-patch repair, despite the damage being to the sugar-phosphate backbone instead of the base (Caldecott, 2014a). In BER, distinct DNA glycosylases recognise specific types of base damage, excise the damaged base, cleave the resulting abasic site, fill in the single nucleotide gap (or multiple nucleotides in long-patch BER), and ligate the nick (Krokan and Bjørås, 2013). If the ligation step does not occur, or if the damage escapes detection prior to DNA synthesis, it can result in the indirect formation of SSBs (Caldecott, 2003). SSB are detected by poly(ADP-ribose) polymerases (PARPs), which are activated in response to both direct and indirectly formed SSBs (Caldecott, 2014a). Active PARPs and poly(ADP)-ribosylation (PARylation) recruit SSB repair proteins for efficient repair.

Because SSBs and DSBs are repaired through different mechanisms, these damage pathways are often viewed separately. However, DSBs can be thought of as two closely-spaced SSBs on opposite strands that cannot be repaired by SSB repair (Jeggo and Löbrich, 2007), while SSBs can become DSBs through both polymerase run-off or from replication fork collapse resulting from replication stress. Therefore, it is possible that there is some overlap between the initial cellular responses, for example, some DSBs might be directly sensed by PARP enzymes. It is important to note that despite the huge body of work, there is no definitive consensus on a universal DDR signalling mechanism(s) generally accepted in the field. However, emerging evidence supports PARP and PAR-dependent signalling in some DSB responses (see below). However, it is still unclear whether PARP-dependent PARylation, vital for SSB repair, must be removed prior to DSB repair, or if it plays an active role in DSB response (Caldecott, 2014b; Chaudhuri and Nussenzweig, 2017). In addition, the recruitment of other DNA damage sensors, including KU70/80 and MRN, is also highly complex. The non-focal response to DSB that occurs within seconds of DSB formation constitutes the Immediate-Early response (**Figure 1B**), which also includes 'fast-kinetic' canonical non-homologous end joining (cNHEJ), which does not require further signal transduction.

The signal transduction inherent to the Early response to many DSBs is largely carried out by ataxia-telangiectasia mutated (ATM) protein kinase (see below, **Figure 1C**, and Savitsky et al., 1995; Ziv et al., 1997; Khanna et al., 2001; Shiloh, 2003; Falck et al., 2005; Maréchal and Zou, 2013). At DSBs, ATM is the apical kinase, phosphorylating many substrates and triggering complex downstream post translational modifications (PTMs), including additional phosphorylation events, as well as methylation, ubiquitination (also known as ubiquitylation), neddylation, fatylation, ufmylation, and sumoylation of substrates (Matsuoka et al., 2007; Mu et al., 2007; Bensimon et al., 2010; Dou et al., 2011; Brown and Jackson, 2015; Yu et al., 2020).

The signal cascade of the Immediate-Early and Early responses to DSBs leads to the recruitment of the scaffolding proteins 53BP1 and BRCA1 in the Late response, which is characterised by the precisely regulated balance between end resection and end protection promoted by these complexes (**Figure 1D**). After 53BP1 and BRCA1 are recruited, a DSB is committed to a specific repair pathway by mechanisms that are under intense study (**Figure 1E–I**). There are then two major pathways for DSB repair. One, termed 'slow-kinetic' canonical non-homologous end joining (slow-kinetic cNHEJ), directly aligns and ligates the broken DNA ends, with minimal or no DNA polymerase activity required (**Figure 1E** and Chang et al., 2017; Ronato et al., 2020; Zhao et al., 2020). It is active throughout the cell cycle and requires highly limited resection (0–5 nt) by the nuclease Artemis. The other, termed homologous recombination

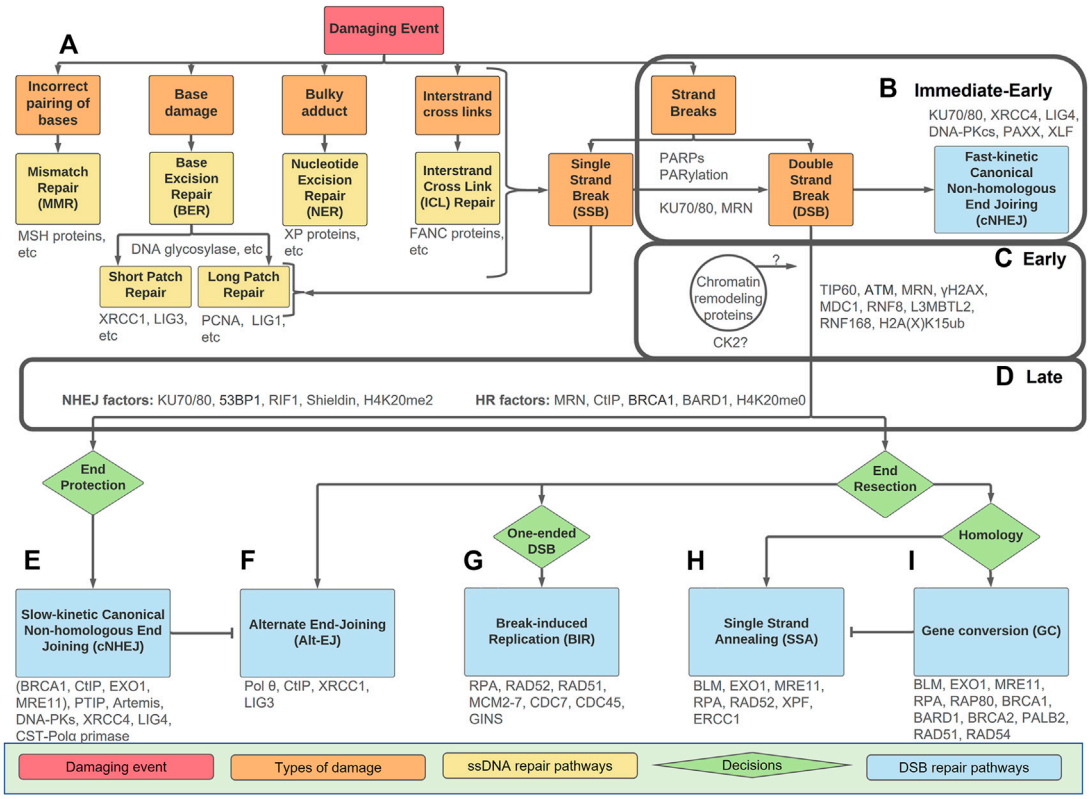

FIGURE 1 | An overview of types of DNA damage and repair pathways with the Immediate-Early, Early, and Late stage indicated. The Immediate-Early, Early, and Late stages of the DSB response are indicated in the boxes. **(A)** Mismatched nucleotides or DNA damage (orange boxes) can be divided into five categories and directed towards the repair pathways as indicated (yellow). Any form of damage can become SSBs or DSBs if not repaired correctly. **(B)** Strand breaks, either SSBs or DSB, have a PARP/PARylation response. In the Immediate-Early response, the PARP/PARylation can facilitate recruitment of KU70/80 and MRN to some DSBs. If the break is easily ligatable, fast-kinetic cNHEJ (blue) repairs the damage within the Immediate-Early response without any requirement for PARylation, processing or ATM-dependent signalling. **(C)** If the break requires processing prior to repair, the Early response is activated. This includes ATM-dependent signalling which requires dynamic chromatin remodelling. The Early response culminates in the ubiquitination of H2A(X)K15. How chromatin events and CK2 activation tie into these processes remains unclear. **(D)** The Late response includes 53BP1 and BRCA1-BARD1 as 'readers' of the H2A(X) ubiquitin mark as well as the methylation state of H4K20. The Late response occurs prior to pathway choice and includes an intricate balance of end-resection vs. end-protection machinery. **(E–I)** Downstream repair pathways (blue) with decision points between pathways (green). Slow-kinetic cNHEJ and GC are high-fidelity repairs, while Alt-EJ, BIR, and SSA, are mutagenic and result when repair machinery is not available. Key proteins discussed are in grey.

(HR), requires a homologous sequence for templated DNA synthesis to effect DSB repair (**Figure 1I** and Li and Heyer, 2008; Ronato et al., 2020). For non-repetitive DNA this is typically the sister chromatid, which is only available after DNA synthesis has occurred. For repetitive sequences, homologous sequences are available *in cis* for HR repair throughout the cell cycle. A good example of this is repair of DSBs within ribosomal DNA (van Sluis and McStay, 2017), and more recently with centromeric DNA (Yilmaz et al., 2021).

While DSB repair pathways have been extensively reviewed, particularly with a focus on the repair mechanisms (Panier and Boulton, 2013; Ceccaldi et al., 2016; Krenning et al., 2019; Scully et al., 2019; Mirman and de Lange, 2020; Ronato et al., 2020), here we use the terminology Immediate-Early, Early, and Late responses, borrowed from viral regulatory proteins (Everett, 1987), to facilitate an integrated description of the cell's complex responses to DSBs prior to repair itself. 'Immediate-Early' responses include initial DSB sensing, while the 'Early' response is characterised by chromatin changes and ATM signalling, and the 'Late' response includes pathway choice prior to repair. A distinguishing characteristic between these divisions is that the Immediate-Early proteins do not form large easily discernible foci, while most Early and Late response proteins do show such focal recruitment. The nature of these foci is the topic of much debate; they have been described as liquid-liquid phase-separated condensates, droplets, biomolecular condensates, or membrane-less organelles (Fijen and Rothenberg, 2021). Precisely how these Immediate-Early, Early, and Late pre-repair responses tie into actual DSB repair remains unclear. Here, we present a discussion of the roles of PARPs, KU70/80, MRN, and fast-kinetic cNHEJ in the 'Immediate-Early' response; how chromatin alteration and ATM regulate the 'Early' response; the critical role of 53BP1 and BRCA1, as well

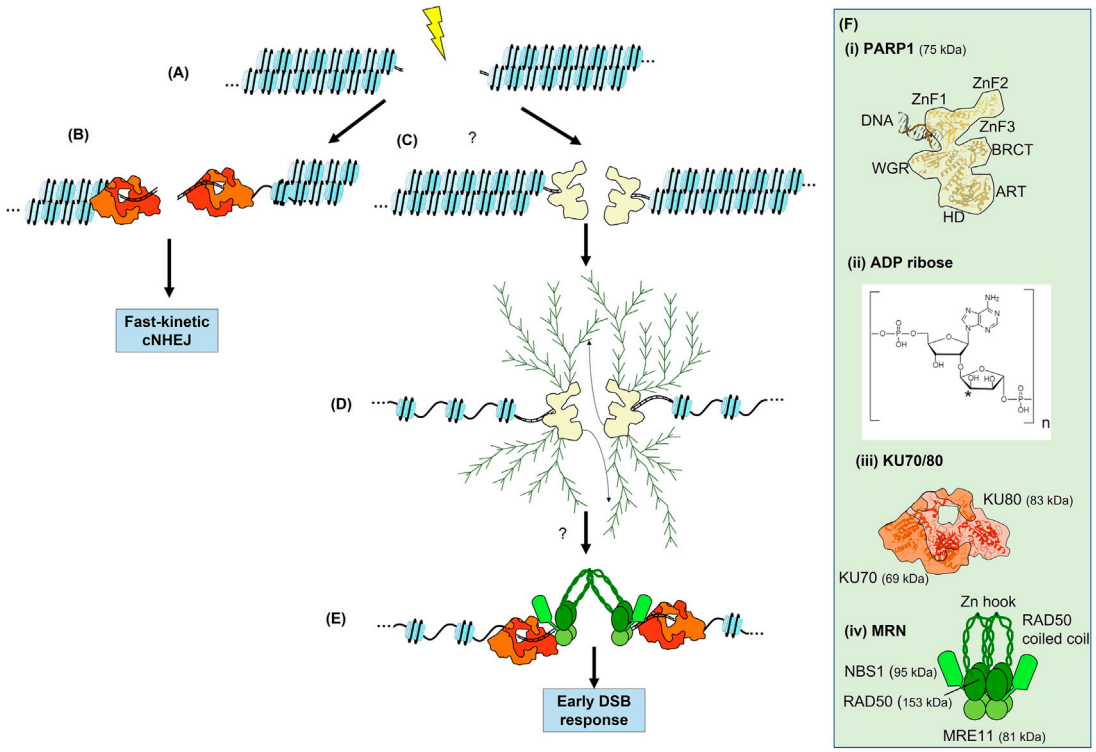

FIGURE 2 | The Immediate-Early response to a DSB. **(A)** Generation of a DSB within a chromatin fibre. **(B)** Recruitment of KU70/80 to some breaks. If KU70/80 is recruited and the break is easily ligatable, fast-kinetic cNHEJ can occur. **(C)** The recruitment of poly(ADP-ribose) polymerases (PARP) to some DSBs. **(D)** Expansion of linear and branched chains of polyADP ribose (PAR) on PARP results in chromatin decondensation. PARylation of other proteins occurs but is not shown. PARylation is a highly dynamic process involving reversal of PARylation by poly(ADP-ribose) glycohydrolase (PARG, not shown). Many DNA repair proteins are recruited to both PARP and PAR chains. The involvement of PARP in the DSB response is not fully elucidated. **(E)** Recruitment of both KU70/80 and MRN are promoted by PARP and PARylation at some DSBs. Initial recruitment of these complexes to the vicinity of the DSB is via interaction with PAR, subsequently they directly bind DSB ends and contribute to synapsis. KU70/80 appears to be recruited with faster kinetics than MRN and is required for fast-kinetic cNHEJ during the Immediate-Early response as seen in **(B)**. Note that both KU70/80 and MRN are required for slow-kinetic cNHEJ but KU70/80 must be evicted prior to MRN-dependent HR. **(F)** Structures of PARP1, ADP-ribose, KU70/80 and MRN. Where known, structure is superimposed within overall architecture as illustrated. **(i)** PARP1, the N-terminal zinc fingers, ART, HD and WGR domains of PARP are indicated (see text for details), as well as the interaction with a DSB (Langelier and Pascal, 2013; Beek et al., 2021). The interaction with HPF1 is not shown. **(ii)** ADP-ribose, linear chains are polymerised via the phosphate groups, whereas the asterisk indicates the position of at which branched chains are attached (Drenichev and Mikhailov, 2016). **(iii)** KU70/80 heterodimer shown in shades of red (Walker et al., 2001). **(iv)** MRN complex in shades of green with the coiled-coils of RAD50 extending outwards (Williams et al., 2009; Casari et al., 2019). In **(D)** these coiled-coils interact via their zinc hooks.

as ongoing roles for KU70/80 and MRN in the 'Late' response; and briefly consider downstream DSB repair.

THE ROLE OF PARP IN THE IMMEDIATE-EARLY DOUBLE STRAND BREAK RESPONSE

The role of PARPs and PARylation in the DSB response is still under debate (Yang et al., 2004; Wang et al., 2006; Patel et al., 2011; Langelier et al., 2012; Caldecott, 2014b; Fouquerel and Sobol, 2014; Strickfaden et al., 2016; Pascal, 2018; Yang et al., 2018; Caron et al., 2019; Murata et al., 2019). While the early literature focussed upon the role of PARPs in SSB repair, it has become more widely implicated in other branches of the DDR. This is largely due to the structure of some DSBs, in which two SSBs on opposites strands of DNA occur near enough that the two ends can separate (**Figure 2A**). There is emerging data showing that PARP1, PARP2, and PARP3 also function at DSBs. This includes structural data showing that PARP1 binds to DSBs (Langelier et al., 2012), as well evidence that PARP1 and KU70/80 compete for DSBs (**Figure 2B**, Wang et al., 2006; Yang et al., 2018), that PARP1 negatively regulates resection (Caron et al., 2019), that defective cNHEJ contributes to the sensitivity to PARP inhibitors (Patel et al., 2011), that PARP3 accelerates cNHEJ (Rulten et al., 2011), and, finally, evidence that PARP1 and KU70/80 can form a complex together (Galande and Kohwi-Shigematsu, 1999). However, it is likely that the linkages between PARPs and DSB responses can be confounded by fast-kinetic cNHEJ functioning in competition with PARP responses during the Immediate-Early response, whereas slow-kinetic cNHEJ that occurs after the Late response appears to be promoted by PARP and PARylation (see discussion on fast- and slow-kinetic cNHEJ in the next section). Important additional considerations are

evidence that PAR-dependent regulation of chromatin remodelling enzymes is required to propagate the DSB signals (Strickfaden et al., 2016); that PARP and PARylation can directly recruit specific DSB repair proteins (discussed below); and, lastly, that the consumption of NAD^+ by PARPs and production of ATP by PARG leads to metabolic shifts that promote specific repair outcomes (Fouquerel and Sobol, 2014; Murata et al., 2019).

PARPs have been extensively studied in the context of SSB repair and BER (Chambon et al., 1963; Benjamin and Gill, 1980; Caldecott, 2014b). There are seventeen members of the PARP family in humans; most of these primarily add a mono(ADP-ribose) to their target proteins (Beek et al., 2021). The mono(ADP-ribose), termed MAR, is most often added to the side (R) carboxyl groups of glutamate and aspartate via an ester bond, but can also be added to the R groups of cysteine and lysine (Wei and Yu, 2016). PARP1 and PARP2 have largely overlapping functions in the DNA damage response, although PARP1 is most prevalent, accounting for 80%–90% of the PARylation in response to strand breaks (Caron et al., 2019). PARP3, which adds mono(ADP-ribose) groups, has also recently been identified as a regulator in the DSB response (Beck et al., 2014). Thus, although PARP1 is the major player, PARP1, PARP2, and PARP3 are collectively responsible for the emerging roles of PARP in the DSB response through their auto-PARylation and PARylation of downstream targets (**Figures 2C,D** and Wei and Yu, 2016). Although the structure of the individual domains of PARP1 have been solved, its complete structure remains elusive (**Figure 2F**). Three zinc-finger (ZnF) domains compose the N-terminal, the first two of which are homologous and recognize DNA (Langelier and Pascal, 2013). Next, the BRCA1 C-terminal (BRCT) domain mediates PAR-dependent protein-protein interactions. The WGR domain is named after its conserved amino acid sequence, and is also involved in DNA binding (Beek et al., 2021). Finally, the catalytic domain, which binds NAD^+ and catalyses addition of ADP-ribose, is comprised of a helical subdomain (HD) and an ADP-ribosyl transferases (ART) subdomain. Interestingly, PARP2 and PARP3 lack the N-terminal ZnF1-3 and BRCT domain present in PARP1 but are still able to bind to DNA via the retained WGR domain.

PARP1/2 are nuclear and depend upon an accessory factor, histone PARylation factor 1 (HFP1), for their activity (Krüger et al., 2020; Suskiewicz et al., 2020). Due to the conformation of the domains of PARP1/2 around the broken DNA end, PARP1/2 preferentially adds PAR to itself via auto-modifications. However, when HPF1 is bound near the PARP1/2 active site, it provides a new catalytic amino acid, N285, that allows PARP1/2 to target serine residues rather than aspartate and glutamate residue targets, contributing to PARP1/2's activity of initiating and elongating the PAR chains. Interestingly, although HPF1 is expressed at relatively low, it appears to be only needed at a ratio of 1:50 to switch the activity of the highly abundant nuclear protein PARP1 (Langelier et al., 2021).

PARP1 is the earliest known protein that senses DNA strand breaks, and binds rapidly to free DNA ends through its DNA-binding domain (DBD) (Ali et al., 2012). It accumulates at lesions in as little as half a second post photoinduced irradiation, and peaks around 5 s (Haince et al., 2008). While the rapid localisation of PARP1 to single-strand breaks has been well characterised, its precise mechanism of localisation to DSBs remains unknown (Liu et al., 2017). In SSB repair, PARP1 and its associated PARylation events recruit X-ray repair cross-complementing protein 1 (XRCC1) to SSB sites, with XRCC1 functioning as a scaffold for the subsequent binding of SSB repair proteins (Masson et al., 1998; Breslin et al., 2015; Hanzlikova et al., 2017; Adamowicz et al., 2021). It is currently unclear how the cell might differentiate between isolated SSBs and those that occur in very close proximity but on opposite strand (i.e., DSBs) after the initial PARylation. One hypothesis is via a possible 'PAR code', an emerging model in which the length and branched nature of the PAR chain controls specific protein recruitment, and thus repair pathway choice (Aberle et al., 2020). A second hypothesis is that due to the unique structure of PARP1, which allows it to be allosterically regulated, the type of DNA break itself could determine the type of PAR chain, which in turn could regulate specific DDR protein recruitment (Pascal, 2018). However, it is currently unknown if the structure of PAR chains differs between the SSB and DSB responses (Leung, 2020).

An observation favouring the involvement of PARPs in the DSB response is that many DSB response proteins bind PAR through their BRCT and forkhead-associated (FHA) domains (Leung and Glover, 2011; Li et al., 2013). PAR-dependent recruitment of DSB repair proteins supports a model in which PARPs and PARylation are required for DSB repair, rather than being merely a remnant of failed attempts to repair DSBs using the SSB repair machinery. Importantly, the KU70/80 and MRN complexes can bind to PAR and have been reported to be dependent upon PARP1/2 activity for their recruitment to DSBs (**Figure 2E** and Haince et al., 2008; Beck et al., 2014; Caron et al., 2019). In addition, PAR interacts with ATM (Aguilar-Quesada et al., 2007), DNA-PKcs (Spagnolo et al., 2012), and BRCA2 (Bryant et al., 2005). PARPs further promote the recruitment of CHD2 (a chromatin remodeller) and BRCA1 (Pascal, 2018). Furthermore, many proteins within the DSB response are targets for PARylation, including RPA (Maltseva et al., 2018), BRCA1 (Li and Yu, 2013), and BARD1 (Li and Yu, 2013). Interestingly, KU70/80 is also PARylated by PARP3 (Beck et al., 2014). The persistence of PARPs and PARylation throughout the Immediate-Early, Early, and Late DSB responses is consistent with a model in which the activity of PARP enzymes is required throughout the DSB response. However, the complex dynamics of PARP1, PARP2, and PARP3 binding to DSBs and the resulting PARylation remain to be fully elucidated and functionally defined.

The rapid PARylation that occurs in the vicinity of a strand break leads to local decondensation of the chromatin, believed to provide space for subsequent protein recruitment (Strickfaden et al., 2016; Pascal, 2018). Of such recruitments, three involve the chromatin remodelling enzymes: amplification in liver cancer 1 (ALC1), and chromodomain helicase DNA binding proteins CHD2 and CHD7 (Luijsterburg et al., 2016; Rother et al., 2020; Verma et al., 2021). CHD7 acetylates histone H4, leading to further chromatin decondensation, facilitating recruitment of histone deacetylase 1 and 2 (HDAC1/2). The ensuing deacetylation of histones leads to recondensation of the

chromatin. Together, this expansion and contraction of chromatin comprises a dynamic process sometimes termed 'chromatin breathing' (Lans et al., 2012). Chromatin breathing offers a more dynamic and nuanced view of the role of chromatin state in the DSB response, rather than a simpler model in which condensed or open chromatin favours either cNHEJ or HR, respectively. In addition, PARP-dependent expansion of the damaged chromatin has recently been shown to recruit the zinc-finger protein ZNF384, which binds DNA ends *in vitro* and is recruited to DSBs *in vivo* via its C2H2 motif. ZNF384 then functions as an adaptor of KU70/80, which promotes the assembly of KU70/80 at DSBs for repair by cNHEJ (Singh et al., 2021).

The metabolic state of the cell is also important to the Immediate-Early DSB response, as PARylation and repair consumes energy. PARylation is a NAD^+-dependent reaction and depends heavily on cellular metabolism. The reverse reaction, dePARylation, by PARG, recycles some of that ATP. PARG has not yet been as extensively studied as PARP1 in the DDR, but some recent studies indicate that PARG binds to nudix hydrolase 5 (NUDT5) and, as PARG hydrolyses the PAR chains, NUDT5 is able to convert the ADP-ribose into ATP, providing energy for downstream processes (Wright et al., 2016). Perhaps the roles of PARG in the Immediate-Early DSB response could be as diverse as those of PARP itself (Feng and Koh, 2013), and future work will be needed to fully decipher its role in DSB repair (Feng and Koh, 2013).

While PARP and KU70/80 have been reported to form a complex (Galande and Kohwi-Shigematsu, 1999), they have also been reported to be mutually exclusive at some lesions (Wang et al., 2006). The latter study supports competition between PARP and KU70/80 at DSBs, but also describes an 'alternate' NHEJ pathway, that is sensitive to PARP inhibitors, involving the core cNHEJ factors as well as Artemis. We interpret this 'alternate' pathway to be what has now been termed slow-kinetic cNHEJ. Another report is consistent with both PARP and KU70/80 being recruited to breaks earlier than other DSBs sensors (Yang et al., 2018). Surprisingly, they report that only KU70/80 binds to DSBs in G1, while in S/G2 both KU70/80 and PARP compete for binding with PARP regulating KU70/80 removal. On the other hand, PAR-dependent recruitment of KU70/80 to DSBs and, this time, retention has been reported in *Dictyostelium discoideum* (Couto et al., 2011). Importantly, this study provides evidence for evolutionarily conservation of PARP function in some cNHEJ repair. Yet another study shows that PARP1 has a role in resection, and that loss of PARP1 results in hyper-resection as well as loss of KU70/80, 53BP1, and RIF1 consistent with PARP having functions upstream of slow-kinetic cNHEJ and HR (Caron et al., 2019). Collectively, these data implicate PARPs in DSB repair, although inconsistencies remain to be resolved. Perhaps some of the contradictory results can be rationalised by the division of cNHEJ into its fast- and slow-kinetics subpathways, PARP-independent and -dependent, respectively (see below).

In summary, SSBs and at least some DSBs appear to require PARPs and the associated PARylation for their repair. Contradictory data on the role of PARPs in DSB responses is a source of confusion in the field, but despite this, PARP and PARylation likely constitutes the initiation of the Immediate-Early response to some DSBs. The ensuing chromatin relaxation and PAR-dependent recruitment of chromatin remodellers and other factors can lead the recruitment and activation of further downstream DSB response proteins.

THE ROLE OF KU70/80 AND MRN IN THE IMMEDIATE-EARLY DOUBLE STRAND BREAK RESPONSE

KU70/80 and MRN recruitment are also part of the Immediate-Early response (**Figures 2B,E**). MRN and KU70/80 are frequently considered as DSB sensing proteins. However, if we consider a 'sensor' to be the initial detection of DSBs, this can be misleading as both MRN and sometimes KU70/80 are loaded subsequent to initial PARylation, and their recruitment can be dependent upon PARP activity (Caron et al., 2019; Ingram et al., 2019). However, if a 'sensor' is more broadly defined as a protein that binds directly to DNA lesions (Jackson, 2002), then PARP, MRN, and KU70/80 can all be counted as DSB sensors. In addition to the complex competition and recruitment interactions between KU70/80 and PARP, KU70/80 and MRN also share what has been termed 'entwined' loading, meaning they are not loaded in defined sequential order or competitively, but rather with more complex dynamics that include multiple points of crosstalk (see below and Rupnik et al., 2008; Shibata et al., 2018; Ingram et al., 2019). In addition, the common model where KU70/80 solely promotes NHEJ by recruiting DNA-PK, and MRN promotes HR by recruiting ATM, is clearly an oversimplification, as both complexes can be loaded to the same DSB (Britton et al., 2013; Ingram et al., 2019; Qi et al., 2021).

The KU70/80 heterodimer is composed of two subunits, 69 and 83 kDa, respectively, forming an open ring around DNA ends (**Figure 2F** and Walker et al., 2001; Jackson, 2002). The major portion of the KU70/80 complex cradles the DSB, effectively covering one surface of the DNA helix but leaving the other surface more open to allow recruitment of further end joining proteins. Once bound, KU70/80 not only facilitates synapsis but also protects DNA ends from resection, thereby promoting cNHEJ. Emerging data supports a division of cNHEJ into two distinct biphasic pathways, termed fast-kinetic and slow-kinetic cNHEJ (**Figures 1B**, **2B,E**, and Jakob et al., 2011; Biehs et al., 2017; Chang et al., 2017; Löbrich and Jeggo, 2017; Shibata et al., 2018; Frit et al., 2019; Setiaputra and Durocher, 2019; Shibata and Jeggo, 2020a; Shibata and Jeggo, 2020b; Qi et al., 2021). The fast-kinetic cNHEJ is also termed 53BP1-, Artemis-, or resection-independent cNHEJ, with Artemis clearly function downstream of ATM (Riballo et al., 2004; Woodbine et al., 2011). It relies upon the essential core cNHEJ factors KU70/80, DNA-PKcs, XRCC4, XLF, and LIG4, which do not form detectable foci during the Immediate-Early response. Fast-kinetic cNHEJ repair likely repairs low complexity breaks that are easily ligatable, and is estimated to repair around 70%–80% of DSBs resulting from X-ray irradiation throughout the cell cycle. KU70/80 appears to be recruited within a second of PARP1, while initial recruitment

of MRN is typically in the range of tens of seconds later (Mari et al., 2006; Haince et al., 2008; Liu et al., 2017; Yang et al., 2018). Their rapidity of recruitment and high nuclear abundance likely makes the kinetics of protein recruitment difficult to study during the Immediate-Early response, as visible foci do not form. The Immediate-Early loading of KU70/80 directs easily repairable DSB towards highly efficient fast-kinetic cNHEJ, likely coinciding with recruitment of PARPs to some breaks, and prior to subsequent loading of MRN. However, it remains unclear how fast-kinetic cNHEJ ties into the nature of KU70/80 and MRN loading, specifically when both complexes are loaded onto the same DSB (Britton et al., 2013; Ingram et al., 2019).

MRN consists of a hetero-hexameric complex consisting of two molecules each of MRE11, RAD50, and NBS1, although there is some discrepancy over whether one or two monomers of NBS1 are associated (**Figure 2F** and Paull, 2018; Syed and Tainer, 2018; Tisi et al., 2020). The MRN complex changes conformation upon ATP binding, enabling MRE11 to span both sides of the DSB, with the coiled-coil domain of RAD50 bridging the gap between the DNA ends. MRE11 is a short-range exonuclease that chews back DNA in a 3′ to 5′ direction, revealing short ssDNA tracts. It also has endonuclease activity important for bypassing blocked DSB ends. Except for fast-kinetic cNHEJ, which does not require resection or processing, tracts of ssDNA are required for all remaining DSB repair pathways; hence MRN is not likely to be critical for fast-kinetic cNHEJ. Consistent with a later function for MRN, the most important role of NBS1 appears to be subsequent binding to ATM during the Early response (Wu et al., 2012; Tisi et al., 2020). Additionally, recruitment of multiple MRN molecules to DSBs has been shown *in vitro*, and proposed to contribute to synapsis in a process that has been termed 'molecular velcro' (De Jager et al., 2001; Rupnik et al., 2009). MRN may initially be recruited to the immediate vicinity of DSBs via an interaction with PAR chains, although its initial recruitment could also be due to its 'facilitated diffusion' capabilities, in which MRN can localise to DNA via RAD50-dependent scanning of DNA for broken DNA ends, which are then recognised by MRE11 (Myler et al., 2017).

The recruitment of KU70/80 within seconds of PARP1 suggests a causal relationship, and an interaction between PARP1 and KU70/80 has been reported, although the detailed mechanism of by which KU70/80 and PARP1 crosstalk with each other remains unclear (Galande and Kohwi-Shigematsu, 1999; Isabelle et al., 2010; Liu et al., 2017; Caron et al., 2019). A possible point of insight is that KU70/80 has been reported to be a PARylation target of PARP3, albeit PARP3 plays a more minor role than PARP1 in the DDR (Beck et al., 2014). New data also supports that PARP-dependent chromatin decondensation facilitates KU70/80 loading via ZNF384 binding (Singh et al., 2021). The Immediate-Early recruitment of MRN complex, which is slightly later than KU70/80, is likely to be at least partially explained by the ability of NBS1 to recognise PARylation (Haince et al., 2008). Whether MRN and KU70/80 load onto the same break, the relative order of this loading and whether they both persist throughout the DSB response is the subject of debate (Hartlerode et al., 2015; Ingram et al., 2019; Paull, 2021), although *in silico* modelling has supported a so-called 'entwined pathway' in which there are multiple point of crosstalk between KU70/80 and MRN loading as opposed to competitive or sequential loading (Ingram et al., 2019).

In summary, if the break is easily ligatable, KU70/80-dependent repair fast-kinetic cNHEJ occurs (**Figure 2B**). KU70/80 and MRN complexes can be recruited during the Immediate-Early response, which is initiated mainly by PARP1 and the subsequent PARylation events and chromatin decondensation (**Figures 2C–E**). These proteins are damage 'sensing' proteins in the sense that they bind directly to the DNA damage. It is possible that there are other proteins that fit this definition, such as the recently reported SIRT6 (Onn et al., 2020). Although MRN and KU70/80 can both be loaded together at a single DSB, only KU70/80 is needed for fast-kinetic cNHEJ. However, MRN's end processing activities and the recruitment of ATM are required for both slow-kinetic cNHEJ and HR, and KU70/80 is likely retained at breaks reapired by slow-kinetic cNHEJ.

THE EARLY RESPONSE TO DOUBLE STRAND BREAKS IS CHROMATIN-BASED

In addition to events at the DSB, parallel chromatin-based responses occur both proximally and distally to the DSBs. Separately from the Immediate-Early DSB sensing events discussed earlier, the chromatin-based Early response to DSBs revolves around the trimethylation of histone H3 K9 (H3K9me3) (**Figure 3**). Regulation of this histone modification by proteins such as heterochromatin protein 1 (HP1, also termed chromobox protein homolog 1) and Tat-interactive protein 60 kDa [TIP60, also termed lysine (K) acetyl transferase, KAT5] centres on ATM activation. TIP60 is the acetyltransferase component of the multicomponent NuA4 complex, which acetylates lysines in multiple targets, including ATM and histone H4, and is important for transcription and DNA repair, as well as contributing to histone exchange (Lee and Workman, 2007; Price and D'Andrea, 2013; Jacquet et al., 2016).

Interestingly, the state of chromatin condensation plays an important role in activating and maintaining the DSB responses leading to DSB repair. Condensed chromatin is regulated by the binding of HP1 (Ayoub et al., 2008; Becker et al., 2016; Kumar and Kono, 2020). HP1β is the most abundant isoform of HP1, while the HP1α and γ variants play lesser roles in chromatin condensation (Kumar and Kono, 2020). HP1β binds to the H3K9me3 heterochromatin marker to maintain the condensed chromatin state. However, the H3K9me3 mark is also present in euchromatin prior to damage, where the level of H3K9 methylation is maintained by a combination of methylases and demethylases (**Figure 3A**). Methyltransferases include suppressor of variegation 3–9 homolog 1 and 2 (SUV39H1 and SUV39H2), and SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) (**Figure 3D** and Monaghan et al., 2019). The demethylases include a family of proteins called lysine (K) demethylases 4 (KDM4A, also termed JMJD2A) that act as demethylases of H3K9me2/3 (Mallette et al., 2012).

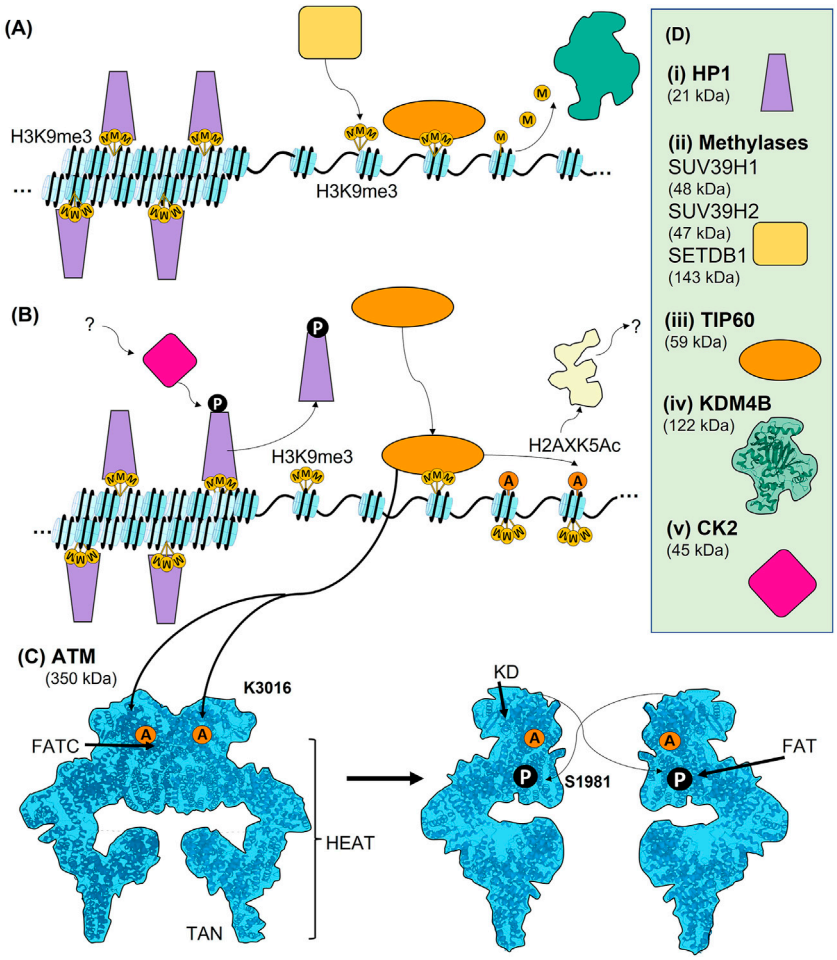

FIGURE 3 | Early chromatin events leading to the activation of ATM. **(A)** The compaction of chromatin prior to DNA damage involves trimethylation of histone H9 at lysine 9 (H3K9me3). In heterochromatin, HP1 binds to H3K9me3 and contributes to chromatin condensation. Although a largely heterochromatic mark, H3K9me3 is also present in euchromatin, where it contributes to transcriptional regulation, and is therefore tightly regulated. It can be methylated by SUV39H1, SUV39H2, and SETDB1, and demethylated by KDM4B. When free of other binding proteins H3K9me3 can bound by TIP60 in its role as a regulator of transcription. **(B)** The regulation of H3K9me3 upon DNA damage and the activation of TIP60. In heterochromatin, CK2-dependent phosphorylation of HP1 (T51) causes its release from H3K9me3, leading to chromatin decondensation. Free H3K9me3 can then be bound by TIP60. When TIP60 is bound it can acetylate lysine 5 of histone H2AX (H2AXK5Ac), leading to further chromatin decondensation. H2AXK5Ac can also contribute to PARP-dependent PARylation around DSBs. **(C)** Activation of ATM occurs via TIP60-dependent acetylation of K3016 within the FATC domain of ATM. ATM is present as a largely inactive dimer prior to damage, and this acetylation causes it to monomerise. Autophosphorylation of S1981 within the FAT domain of ATM also likely contributes to activation of ATM. In addition to the FATC and FAT domain, ATM also is comprised of a kinase domain (KD) and HEAT repeats. Both the FAT and TAN domain are specialised HEAT repeats. **(D)** Where known, structure is superimposed within overall architecture as illustrated. (i) Schematic of HP1. (ii) General schematic of H3K9 methylases, including SUV39H1, SUV39H2, and SETDB1. (iii) TIP60 schematic. (iv) CK2 schematic.

Interestingly, KDM4B recruitment is promoted by PARP (Khurana et al., 2014), suggesting crosstalk with the Immediate-Early response. Importantly, the level of histone H3K9 methylation is in constant flux dependent upon the cell cycle and the specifics of hetero- or euchromatic packaging (Sulkowski et al., 2020).

In heterochromatin after damage, HP1β is phosphorylated (on T51) by casein kinase 2 (CK2), causing its displacement from H3K9me3 (**Figure 3B** and Ayoub et al., 2008). CK2, which is functionally highly pleiotropic in cellular signalling, also phosphorylates multiple other targets in the DDR, although its precise regulation and functions in the DDR are unclear. For example, it is not known how CK2 is activated upon DNA damage to phosphorylate HP1β on residue T51 (Ayoub et al., 2008). Although primarily a mark of heterochromatin, H3K9me3 also functions within euchromatin as a regulator of transcription. DSBs within euchromatin result in a rapid spike of H3K9me3 in the chromatin flanking the DSB, although precisely how this is regulated is not well understood. It is possible that because KDM4B constantly removes H3K9 methylation, its inhibition at DSBs would allow for a quick and local increase in H3K9 methylation (Sulkowski et al., 2020). Another possibility causing

this rapid increase in H3K9me3 upon DNA damage is a pathway that involves ufmylation, a ubiquitin-like protein, of histone H4 at K31 (H4K31Ufm) by ULF1, which is recruited by the MRN complex. H4K31Ufm is read by serine/threonine-protein kinase 38 (STK38) and somehow facilitates recruitment of SUV39H1 to breaks, which then locally trimethylates H3K9 (Qin et al., 2019; Qin et al., 2020). However, this ufmylation-dependent pathway is thought to be more likely an MRN-dependent positive feedback loop and does not account for the initial spike of H3K9me3, but rather its spreading and subsequent activation of ATM (Qin et al., 2019; Qin et al., 2020). Upon HP1β displacement, the histone acetyltransferase TIP60 can bind, via its chromodomain, to H3K9me3 (Sun et al., 2009). It is interesting that although HP1β must be removed, it has also been shown to be recruited to DSBs via an unclear mechanism that involves its chromoshadow domain, suggesting an additional function in the DSB response (Luijsterburg et al., 2009). TIP60 can also bind to H3K36me3, and together, these two chromatin marks act as allosteric regulators of TIP60 acetyltransferase activity (**Figure 3B** and Bakkenist and Kastan, 2015).

Once the H3K9me3 histone mark is revealed within heterochromatin or generated within euchromatin, TIP60 binds H3K9me3 and acetylates K3016 of ATM (**Figure 3C** and Sun et al., 2010; Bakkenist and Kastan, 2015). In fact, ATM and TIP60 can form a stable complex through the FATC domain of ATM, and this interaction is likely what brings TIP60 into proximity with K3016, allowing the acetylation. The TIP60-ATM interaction appears constitutive, although TIP60's histone acetylation activity and the kinase activity of ATM are indeed damage-dependent (Sun et al., 2005). Acetylation of ATM, which is present as an inactive dimer in the nucleus prior to damage, causes ATM to monomerise and autophosphorylate on residue S1981. It is not currently known if this phosphorylation event is *in cis* or *in trans*, or if this phosphorylation is necessary for activation of ATM or just a marker of active ATM (Bakkenist and Kastan, 2003; Zong et al., 2015; Burger et al., 2019). In fact, ATM has been reported to have several other sites of autophosphorylation, which likely play roles in DSB repair yet to be elucidated (Kozlov et al., 2006; Kozlov et al., 2011). At DSBs, ATM phosphorylates many substrates, resulting in complex signal transduction involving numerous distinct PTMs (Matsuoka et al., 2007; Mu et al., 2007; Bensimon et al., 2010; Dou et al., 2011; Brown and Jackson, 2015; Yu et al., 2020). Thus, the TIP60 acetylation-dependent monomerisation of ATM and its likely phosphorylation-dependent activation leads to an extensive leads to an extensive signal transduction network in the Early DSB response (see below).

It is intriguing to note that PARP1/PARylation could aid in recruiting TIP60 and ATM to sites of damage, as ATM binds PARP1 in a PAR-dependent manner (Aguilar-Quesada et al., 2007; Chaudhuri and Nussenzweig, 2017). ATM has also been shown to be activated by treatments that do not directly cause DNA damage but induced global decondensation of chromatin in the absence of any detectable DNA damage (Bakkenist and Kastan, 2003). Thus, in addition to MRN-dependent ATM recruitment, PAR-dependent chromatin decondensation that occurs at DSBs could also contribute to ATM activation and subsequent recruitment. The activation of ATM creates a positive feedback loop that is dependent upon chromatin decondensation and driven by the binding of TIP60 to H3K9me3, which is either revealed by release of HP1β or promoted by damage-induced formation of H3K9me3 proximal to the DSB. TIP60 also acetylates H2AX on lysine 5 (H2AXK5Ac) in the chromatin proximal to a DSB, promoting PARP1-dependent PARylation, which in turn could contribute to the dynamic chromatin decompaction believed to facilitate DNA metabolic activities at DSBs (**Figure 3B** and Ikura et al., 2016). In fact, this study showed that PARP1 was part of the TIP60 complex, which is a potential link between the Immediate-Early PARP response and the chromatin-based Early response to DSBs. PARP1, and no doubt PARG, may have functions throughout the DSB response, although, apart from its Immediate-Early functions, the multiple potential roles of PARP1 during the Early and Late responses remain to be defined.

There are other forms of chromatin reorganisation that take place in response to DSBs, such as removal or sliding of nucleosomes, as well as histone exchange (Price and D'Andrea, 2013; Pessina and Lowndes, 2014; Dhar et al., 2017). Pathway choice depends on chromatin state, as for resection to occur, the DNA must be accessible to the resection machinery. Note, however, that similarly to other DNA metabolic transactions such as transcription and replication, nucleosomes may not have to be physically removed for resection to occur. There are many histone modifications that promote or impede resection (Clouaire and Legube, 2019). In general, the balance between such histone modifications affects the binding of factors required for either limited or more extensive resection, which in turn impacts upon pathway choice and fidelity of repair. Specifically within active transcription units, H3K36me3-dependent recruitment of Lens epithelium-derived growth factor p75 splice variant (LEDGF) promotes HR by damage-induced recruitment of CtIP and subsequently the other proteins required for extensive resection (Daugaard et al., 2012; Aymard et al., 2014).

Additionally, ATM-dependent phosphorylation of the RNF20-RNF40 heterodimer, an E3 ubiquitin ligase, results in monoubiquitination of H2B (H2BK120ub1) and the consequent decondensation of the chromatin around DSBs (Moyal et al., 2011). In undamaged cells this monoubiquitination of H2B is normally associated with transcription elongation, but upon damage contributes to the further relaxation of the chromatin flanking DSBs to facilitate recruitment of both NHEJ and HR proteins. Similarly, the ATM-dependent phosphorylation of KAP-1 on S824 leads to decondensation of heterochromatin (Goodarzi et al., 2011). Interestingly, DSBs within heterochromatin relocate to the periphery of the heterochromatic clusters where they can be more easily repaired (Jakob et al., 2011; Hausmann et al., 2018; Clouaire and Legube, 2019).

Together, the highly complex regulation of a multitude of histone modifications in the chromatin flanking DSBs contributes to the activation of ATM and its downstream targets, as well as contributing significantly to downstream

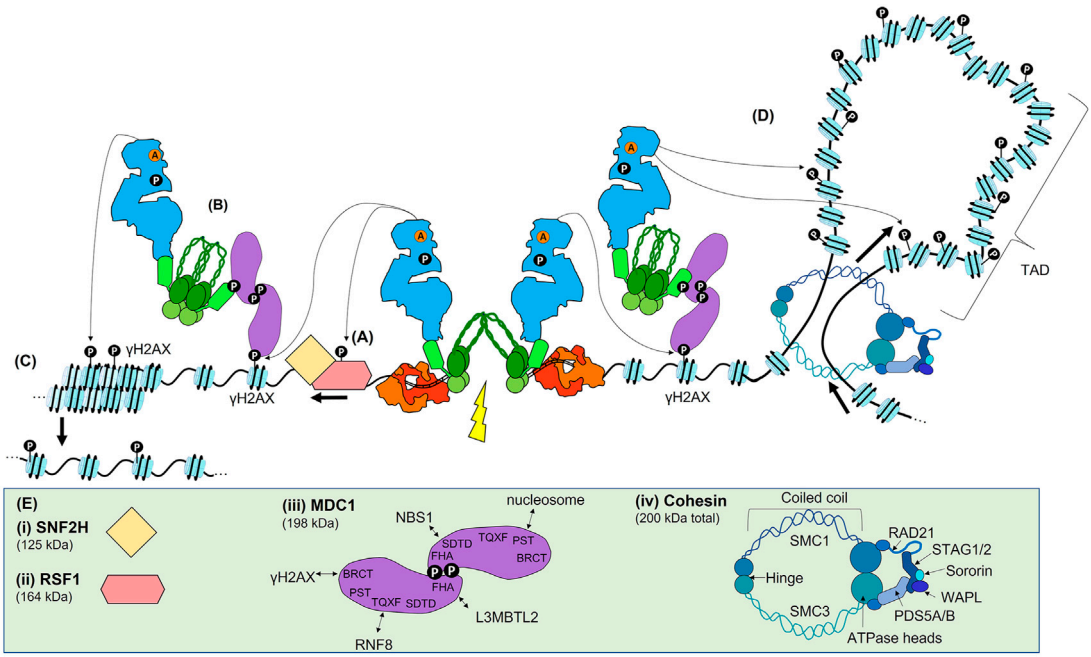

FIGURE 4 | ATM signalling in the Early DSB response. **(A)** Once active, ATM is recruited to sites of DSBs by an interaction with NBS1, where it phosphorylates many target proteins. One of the earliest targets is RSF1, which leads to nucleosome sliding to reveal the DNA surrounding the break. **(B)** ATM phosphorylates H2AX (γH2AX), which allows the scaffold MDC1 to bind via its BRCT domain. As MDC1 is constitutively bound to MRN, the recruitment of MDC1 recruits further MRN and ATM. **(C)** ATM propagates γH2AX via continued MCD1, MRN, and ATM recruitment, leading to chromatin relaxation. **(D)** In addition to this method of γH2AX propagation, γH2AX may also be spread via proposed 'loop extrusion' mechanism. In this model, the DSB machinery blocks one direction of the normal loop extrusion that leads to the formation of TADs. As nucleosomes are extruded, ATM phosphorylates H2AX within a given TAD. **(E)** Schematics or structures are shown to the extent of current data. **(i)** and **(ii)** schematic of RSF1 and SNF2H. **(iii)** MDC1 contains many SQ/TQ sites that are phosphorylated by ATM and are required for protein binding. The FHA domain allows for formation of head-to-head dimers of MDC1, and also contributes to L3BMTL2 binding. The SDTD domain interacts with NBS1. The TQXT domain interacts with RNF8. The BRCT domain interacts with γH2AX. **(iv)** Cohesin is made up of the indicated domains.

pathway choice. While initiated during the Early response, the dynamics of chromatin modification are fluid, continuously adjusting to the specific circumstances of each DSB throughout the entire DSB response.

THE ROLE OF ATM IN THE EARLY DOUBLE STRAND BREAK RESPONSE

Once ATM is activated by TIP60-dependent acetylation, the cell continues with the Early DSB response. The signal transduction pathway initiated by active ATM results in the ubiquitination of histone H2A variants on K13/15 [termed H2A(X)K13/15ub]. The known order of recruitment to chromatin in the vicinity of DSBs is ATM, MDC1, MRN, RNF8, L3MBTL2, and RNF168 (**Figures 4**, **5** and Salguero et al., 2019). These Early DSB response proteins are notable for their easily visible focal recruitment into micron scale condensates that form around DSBs. A large contribution to the versatility, efficiency, and integrated 'decision' making of the DSB repair response is no doubt due to the locally high concentration, ensured by their liquid-liquid phase separation properties, of the many proteins found within foci (Fijen and Rothenberg, 2021).

Once activated (**Figure 3**), the ATM monomer initiates a phosphorylation cascade involving many transducers and effector proteins including those with roles yet to be defined or those yet to be identified (some of the key proteins are illustrated in **Figures 4**, **5**). Perhaps the first ATM-dependent chromatin event is the phosphorylation-dependent recruitment of the remodelling and spacing factor 1 (RSF1) which is required for reorganisation of the nucleosome(s) immediately proximal to the DSB that is essential for both slow-kinetic cNHEJ and HR (**Figure 4A** and Helfricht et al., 2013; Min et al., 2014; Pessina and Lowndes, 2014). Another important phosphorylation target of ATM is the MRN complex (Lavin et al., 2015; Syed and Tainer, 2018). MRN bound to broken DNA (**Figure 2E**) recruits active ATM monomers via an interaction with NBS1, which possibly involves prior K63-linked ubiquitination of NBS1 (**Figure 4A** and Wu et al., 2012). A proportion of MRN proximal to DSBs during the Immediate-Early response via MRE11-dependent DSB-specific binding DNA, as well as a proportion likely recruited via interaction with PAR (see earlier). However, the dramatic focal accumulation of ATM and MRN in the vicinity of DSBs is chromatin-mediated, rather than directly DNA- or PAR-mediated. Once recruited, ATM phosphorylates H2AX, a variant of histone H2A often found in euchromatin, at residue

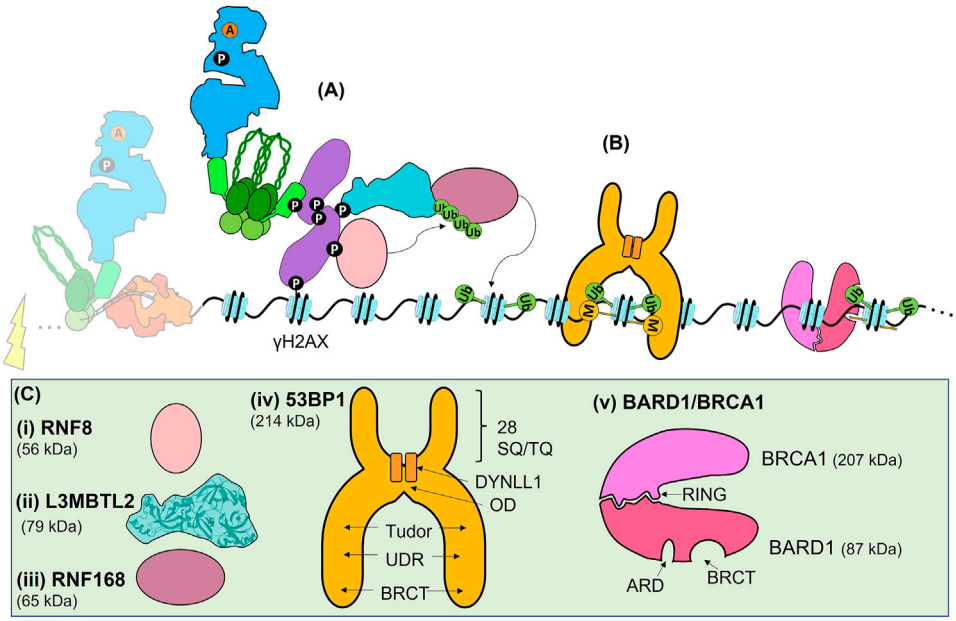

FIGURE 5 | The Early ATM-dependent DSB response results in the ubiquitination of histone H2A(X) (H2A(X)13/15ub) needed for Late protein recruitment. **(A)** DSB signal transduction. The ATM-dependent phosphorylation of MDC1 provides a docking site for the E3 ubiquitin ligase RNF8, and the ATM-dependent phosphorylation of S335 of L3MBTL2 allows it to bind to the FHA domain of MDC1, bringing it in close contact with RNF8. RNF8 polyubiquitinates L3BMTL2 on K659 via K48-linkage, providing a platform for RN168 to bind. RNF168 then monoubiquinates H2A(X)K13/15. **(B)** Together, H2A(X)K15ub and the replication-dependent methylation state of H4K20 recruits either 53BP1 or BARD1, which is in complex with BRCA1. **(C)** Schematics or structures of RNF8, L3BMTL2, RNF168, 53BP1, and BARD1/BRCA1 are shown where known, or informed by known domains where the full structure is not solved. **(i)** RNF8 is an E3 ubiquitin ligase, that interacts with the E2 ubiquitin ligase UBC8 or UBC13 (not shown). **(ii)** L3MBTL2 structure. **(iii)** RNF168 E3 ubiquitin ligase interacts with the UBE2N or UBC13 E2 ubiquitin ligase (not shown). **(iv)** 53BP1 schematic. The UDR domain binds to H4K20me2 and its tandem Tudor domain binds to H2A(X)K15ub. Dimerization of 53BP1 is promoted by the OD and DYNLL1. 28 SQ/TQ sites in the N terminal can be phosphorylated for downstream protein recruitment. **(v)** The ARD domain of BARD1 binds the H4K20me0 mark, while its BRCT domain binds H2A(X)K15ub. BARD1 is in complex with BRCA1. Note that for clarity the DSB and Early ATM signalling proteins are faded out.

S139. This PTM, widely known as γH2AX, provides a docking site for MDC1 via its BRCT domain, with MDC1 then acting as a scaffold protein for further protein recruitment, including further ATM and MRN, throughout the remainder of the DSB response (**Figure 3B** and Jungmichel et al., 2012). In addition to this widely appreciated mechanism of ATM accumulation at DSBs, an additional regulator has been suggested: Pellino1, yet another E3 ubiquitin ligase, that is recruited to the DSB, phosphorylated by ATM, and then binds to γH2AX to further promote the accumulation of ATM and MRN, and subsequently, of MDC1 (Ha et al., 2019).

Prior to damage, a proportion of the MDC1 scaffold is already bound to NBS1 via the SDTD domain of NBS1, requiring CK2-dependent phosphorylation of the SDTD motif in MDC1 (Goldberg et al., 2003; Chapman and Jackson, 2008; Spycher et al., 2008). Once more, the role of CK2 in the DDR is enigmatic, as whether CK2 is regulated to phosphorylate the MDC1's SDTD domain is unclear. Phosphorylation of the N-terminus of MDC1 regulates its dimerization, which in turn appears to be required for an effective DSB response (**Figure 4E** and Luo et al., 2011; Liu et al., 2012). Regardless, the initially recruited MDC1 then recruits more MDC1-bound MRN complexes (**Figure 4B** and Melander et al., 2008; Spycher et al., 2008; Salguero et al., 2019), while additional active ATM monomers are recruited through their interaction with NBS1. The accumulating ATM then propagates γH2AX, spreading across megabases of chromatin domains on either side of the DSB (**Figure 4C**). Normally, H2AX is phosphorylated by ATM activity, but can also be phosphorylated by DNA-PKcs and ATR (Stiff et al., 2004; Wang et al., 2005; Caron et al., 2015), and increasing levels of γH2AX results in further chromatin decondensation and amplification of the DSB repair signal.

Recent data suggests another potential mechanism, loop extrusion, which could facilitate γH2AX propagation (**Figure 4D** and Arnould et al., 2021). In this ATM-dependent mechanism, γH2AX is specifically propagated throughout an entire topologically associated domain (TAD). TADs are structured chromatin domains actively maintained by cohesin and CCCTC-binding factor (CTCF) and formed by loop extrusion, in which chromatin is pushed through the cohesin molecules until opposing CTCF are encountered (**Figure 4E** and Rajarajan et al., 2016; Marchal et al., 2019). The authors propose that a DSB blocks extrusion, leading to unidirectional loop extrusion with the DSB repair machinery anchored one side of the extrusion process, allowing ATM to phosphorylate H2AX as the nucleosomes are extruded (Arnould et al., 2021) (**Figure 3D**). While γH2AX spreading within TADs may be facilitated by loop-extrusion, it is likely to be additive with phosphorylation via the previously described the MDC1/MRN/ATM positive feedback loop.

The phosphorylation of the TQXF motif of MDC1 by ATM provides a binding site for RNF8, an E3 ubiquitin ligase (**Figures 4E**, **5A** and Nowsheen et al., 2018; Salguero et al., 2019). RNF8 interacts with several E2 enzymes, including the ubiquitin charged proteins UBCH8 and UBC13, leading to catalysis of either K48- or K63-linked ubiquitin chains, respectively (Lok et al., 2012). Ubiquitination is best known for marking proteins for degradation by the proteasome via K48-linked chains, but the K63-linkage plays has an important role as a signalling mark in the DSB response, as well as other pathways (e.g., protein kinase activation, and receptor endocytosis) (Lok et al., 2012). There has been some discussion on the major target of RNF8 in the DSB response. It was initially reported that RNF8 ubiquitinates histone H1 (Thorslund et al., 2015). However, more recent data established that RNF8 targets a protein termed Lethal(3) malignant brain tumour-like protein 2 (L3MBTL2) (Nowsheen et al., 2018). L3MBTL2 contains malignant brain tumour (MBT) repeats, which often function as 'chromatin readers' able to bind to histone modifications, and is one of at least three MBT-containing proteins active in the DSB response (Bonasio et al., 2010). Like RNF8, L3MBTL2 is also recruited to the MDC1 scaffold, this time by an ATM-dependent phosphorylation of L3MBTL2 (S335) which interacts with the MDC1 FHA domain (**Figures 4E**, **5A**). The proximity of RNF8 and L3MBTL2, both bound to MDC1, facilitates polyubiquitination of L3BMTL2 (K659, via K63 linkages) by RNF8. This polyubiquitination serves as a platform for the binding of RNF168, another E3 ubiquitin ligase. The key role of RNF168 is mono-ubiquitination of H2A isoforms, including H2AX, on residues K13 and K15 [H2A(X)K13/15ub] (Mattiroli et al., 2012). Although RN168 can ubiquitinate both K13 and K15 residues, the role of K13ub in the DSB response is not understood; however, the damage-inducible ubiquitination of K15 is required for both 53BP1 and BRCA1 recruitment (**Figure 5B** and Mattiroli et al., 2012).

In addition to their transduction of the ATM damage signal, RNF8 and RNF168 also have other regulatory roles in the Early DSB response (Lok et al., 2012; Bartocci and Denchi, 2013). For example, the monoubiquitin on H2A(X)K13/15 can be extended by RNF8; while this polyubiquitination has unclear effects on 53BP1 and BARD1 binding, it is required for recruitment of RAP80 in a complex with BRCA1 (Hu et al., 2011). Other roles for RNF8 and RNF168 in the DSB response include ubiquitination-dependent regulation of L3MBTL1, KDM4A (JMJD2A), and 53BP1. However, these roles have not been fully elucidated and involve K48-linkages more typically involved in proteolysis. In addition, RNF8-dependent ubiquitination of NBS1 may aid the stabilization of MRN at DSBs (Lu et al., 2012). Furthermore, a poorly characterised scaffold protein, WRAP53β, has been reported to contribute to RNF8 recruitment through an unknown mechanism involving phosphorylation by ATM (at S64) and co-localisation with MDC1 (Henriksson et al., 2014; Rassoolzadeh et al., 2015; Coucoravas et al., 2017).

It is likely that there are many other undiscovered regulators of ATM recruitment and early phosphorylation events. A further example is the transcription factor SP1, which is phosphorylated by ATM and co-localises with γH2AX and members of the MRN complex, although its mechanism of interaction and regulatory impact have not yet been reported (Beishline et al., 2012). Finally, ufmylation of MRE11 on K282 has been reported to promote ATM activation, although the mechanistic details remain to be characterised (Wang et al., 2019). In fact, it is likely that many more details of how ATM regulates the response to DSBs remain to be reported and dissected and will add still further complexity to an already complex pathway. To date, the 'major players' required to transduce the Early DSB response include ATM, MRN, MDC1, RNF8, L3MBTL2, and RNF168, while multiple additional proteins are required to fine tune this signal transduction pathway.

THE ROLE OF 53BP1 AND BRCA1 IN THE LATE DOUBLE STRAND BREAK RESPONSE

Emerging data has demonstrated that two histone modifications are critical for pathway choice between NHEJ and HR (Fradet-Turcotte et al., 2013; Pellegrino et al., 2017; Nakamura et al., 2019; Becker et al., 2020; Dai et al., 2021; Hu et al., 2021; Morris, 2021). Ubiquitination of H2A isoforms [H2A(X)K13/15ub] together with the methylation state of histone H4 lysine 20 (either H4K20me0 or H4K20me2) recruit the critical readers of these bivalent chromatin marks, 53BP1 and BARD1, which is in complex with BRCA1 (**Figure 5B**). The control of these two PTMs is highly regulated; H2A(X)K13/15 is initially mono-ubiquitinated by the E3 ligase RNF168 in the chromatin flanking DNA damage, while the methylation of H4K20 is widespread throughout the genome. The recruitment of 53BP1 and BARD1-BRCA1 to these histone modifications occurs during the Late stage of the DSB response, and constitutes some of the last steps prior to pathway choice. Here we will briefly discuss the known mechanisms underlying the choice between slow-kinetic cNHEJ and HR (**Figure 6** and reviewed in Panier and Boulton, 2013; Ceccaldi et al., 2016; Krenning et al., 2019; Scully et al., 2019; Mirman and de Lange, 2020; Ronato et al., 2020).

Previously we discussed the regulation of H2A ubiquitination [H2A(X)K13/15ub], which appears to be the critical damage-dependent regulatory event of the Early response. Di-methylation of H4 (H4K20me2) is largely constitutive and widely distributed throughout the genome. Importantly, for BARD1-BRCA1 recruitment, immediately post DNA replication, newly incorporated nucleosomes are transiently unmethylated (H4K20me0), although the existing nucleosomes retain methylation (H4K20me2) (Botuyan et al., 2006; Saredi et al., 2016; Nakamura et al., 2019; Becker et al., 2020). H4K20me2 is normally methylated by three methyltransferases, where SET8 (also termed SETD8, Pr-SET7, and KMT5A) provides the initial monomethylation, then SUV4-20H1 and its homologue SUV4-20H2 add the second and even a third methyl group (Jørgensen et al., 2013). Demethylation can occur via two RAD23 homologues, hHR23A and hHR23B (Cao et al., 2020). In addition to the post replication control of H4K20 methylation, H4K20me2 can be masked by either KDM4A (JMJD2A) or L3MBTL1 prior to damage (Acs et al., 2011; Butler et al.,

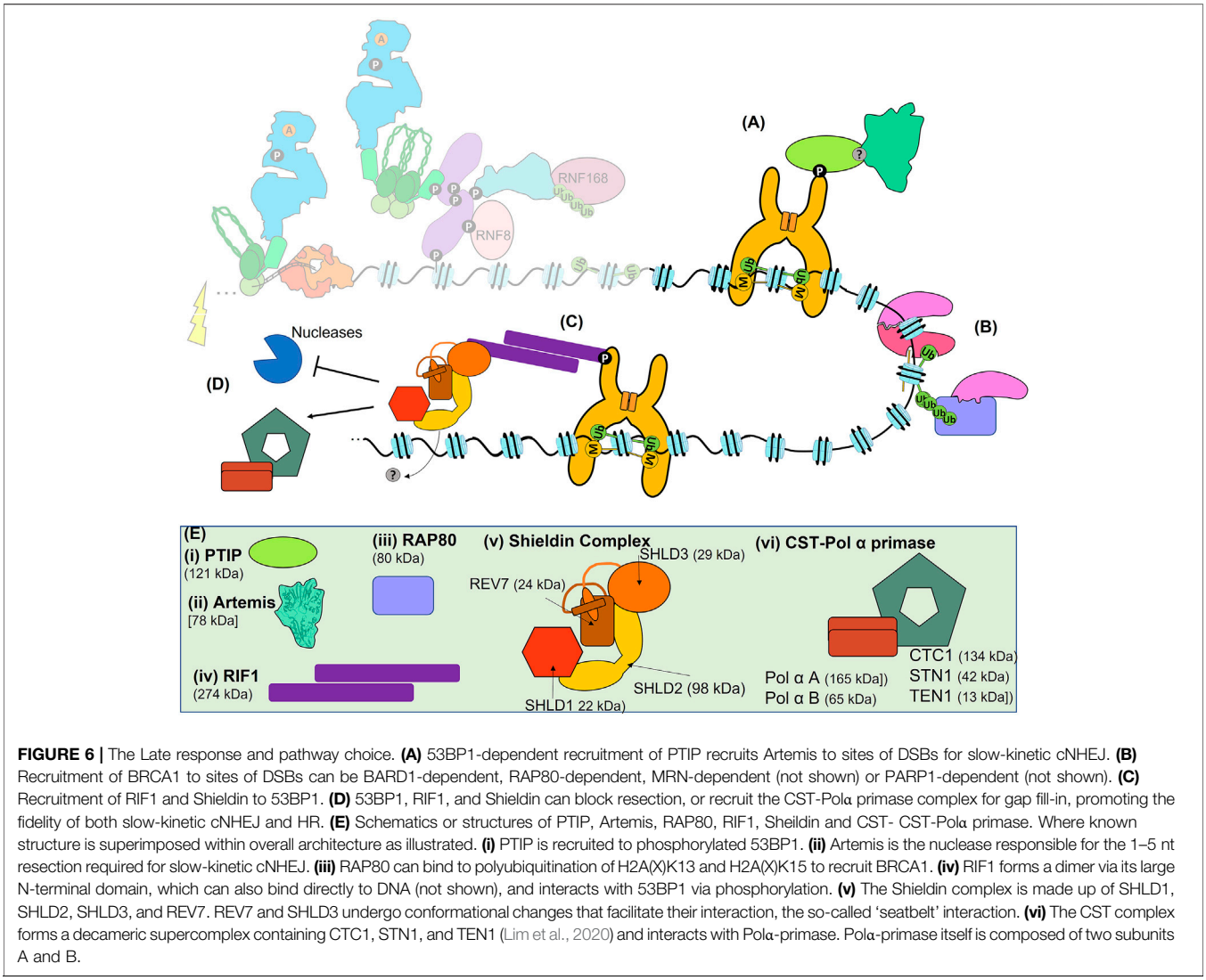

FIGURE 6 | The Late response and pathway choice. **(A)** 53BP1-dependent recruitment of PTIP recruits Artemis to sites of DSBs for slow-kinetic cNHEJ. **(B)** Recruitment of BRCA1 to sites of DSBs can be BARD1-dependent, RAP80-dependent, MRN-dependent (not shown) or PARP1-dependent (not shown). **(C)** Recruitment of RIF1 and Shieldin to 53BP1. **(D)** 53BP1, RIF1, and Shieldin can block resection, or recruit the CST-Polα primase complex for gap fill-in, promoting the fidelity of both slow-kinetic cNHEJ and HR. **(E)** Schematics or structures of PTIP, Artemis, RAP80, RIF1, Sheildin and CST- CST-Polα primase. Where known structure is superimposed within overall architecture as illustrated. **(i)** PTIP is recruited to phosphorylated 53BP1. **(ii)** Artemis is the nuclease responsible for the 1–5 nt resection required for slow-kinetic cNHEJ. **(iii)** RAP80 can bind to polyubiquitination of H2A(X)K13 and H2A(X)K15 to recruit BRCA1. **(iv)** RIF1 forms a dimer via its large N-terminal domain, which can also bind directly to DNA (not shown), and interacts with 53BP1 via phosphorylation. **(v)** The Shieldin complex is made up of SHLD1, SHLD2, SHLD3, and REV7. REV7 and SHLD3 undergo conformational changes that facilitate their interaction, the so-called 'seatbelt' interaction. **(vi)** The CST complex forms a decameric supercomplex containing CTC1, STN1, and TEN1 (Lim et al., 2020) and interacts with Polα-primase. Polα-primase itself is composed of two subunits A and B.

2012; Mallette et al., 2012). Upon DNA damage, the concerted action of RNF8 and RNF168 ubiquitinate KDM4A via K48-linkage that targets it for proteasomal degradation, revealing the H4K20me2 mark for 53BP1 binding. Unmasking L3MBTL1 to reveal H4K20me2 is achieved somewhat differently. RNF8 and RNF168 are required to recruit the AAA-ATPases valosin-containing protein (VCP) and nuclear protein localization protein 4 (NPL4) to DSBs in order to remove L3MBTL1 freeing the H4K20me2 mark for 53BP1 (Jacquet et al., 2016).

In the context of a DSB, and the associated H2A ubiquitination [H2A(X)K15ub] of the flanking chromatin, the greatest binding affinity of 53BP1 and BARD1 is to H4K20me2 and H4K20me0, respectively (Pellegrino et al., 2017; Nakamura et al., 2019; Becker et al., 2020; Dai et al., 2021). Although H4K20me2 is abundant, it is also known to be damage-inducible via the histone methyltransferase MMSET (also known as NSD2 or WHSC1), which is recruited in a γH2AX- and MDC1-dependent manner (Pei et al., 2011). This could be particularly important for regions of the genome relatively depleted in the H4K20me2 modification. More typically, the H4K20me2 is only absent on newly synthesised chromatin. Thus, the brief availability of H4K20me0 in newly replicated chromatin facilitates recruitment of BARD1, immediately after replication fork passage, which directs repair towards HR as BARD1 forms a heterodimer with BRCA1 via the RING domain of BRCA1 (**Figure 5C**). BARD1 binds to H4K20me0 through its ankyrin repeat domain (ARD) domain, while its BRCT domain binds H2A(X)15ub (Nakamura et al., 2019). The affinity of BARD1 for H2A(X)K15ub is higher than that of 53BP1 (Dai et al., 2021). Once H4K20 becomes methylated, the window for repair via HR closes and repair is once more directed towards the slow-kinetic cNHEJ pathway. Although this is not the only method of BRCA1 recruitment, as BRCA1 forms many complexes, including BRCA1-A (Abraxas & RAP80 containing), BRCA1-B (BACH1 containing), BRCA1-C (CtIP and MRN containing) and the BRCA1/PALB2 complex, that are separately recruited (**Figure 6B** and reviewed in Her et al., 2016). As previously

noted, it seems that BRCA1-A complex can be recruited to sites of DSBs by interaction between RAP80 and polyubiquitination of either H2A(X)K13 or H2A(X)K15, which is extended from monoubiquitination of either residue by RNF8 (Mattiroli et al., 2012). The post replication window during which newly incorporated histone H4 remains unmodified at lysine 20 methylation suggests a mechanism of how the cell successfully deals with one-ended DSBs that can occur at replication forks. One-ended DSBs cannot be accurately repaired by slow-kinetic cNHEJ (joining to another one-ended DSB elsewhere in the genome would result in a chromosomal rearrangement) and are instead repaired via break induced replication (BIR), a homology-dependent mechanism requiring the sister chromatid (Anand et al., 2013). While there are multiple mechanisms by which BRCA1 is recruited to two-ended DSBs, precisely how BRCA1 outcompetes 53BP1 to favour HR at those breaks preferentially repaired by this pathway remains to be fully elucidated.

The structure of 53BP1 allows it to bind to the bivalent damage-induced H2A(X)15ub and the largely constitutive H4K20me2 (see above and Fradet-Turcotte et al., 2013; Mirman and de Lange, 2020; Ronato et al., 2020). The C-terminus of 53BP1 consists of a tandem Tudor domain with a closely associated ubiquitin-dependent recruitment (UDR) motif, followed by a tandem BRCT domain which separately binds p53 (**Figure 5C**). The Tudor domain of 53BP1 specifically binds H4K20me2, while the UDR motif binds monoubiquitinated H2A(X)K15ub, but not H2A(X)K13ub (Botuyan et al., 2006; Panier et al., 2012; Uckelmann and Sixma, 2017). Effective binding of 53BP1 to these marks also requires its constitutive dimerization, achieved via its oligomerisation domain (OD), and facilitated by its interaction with DYNLL1 (also LC8). Interestingly, DYNLL1 interaction with MRE11 disrupts its nuclease activity, suggesting another mechanism by which 53BP1 inhibits resection (He et al., 2018). The large N-terminus of 53BP1 is unstructured and contains 28 S/TQ sites that can be phosphorylated by ATM and form a platform for recruitment of multiple factors such as RIF1, which in turn leads to the recruitment of Shieldin (**Figure 6C** and Findlay et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018).

The Shieldin complex, consisting of REV7 (MAD2L2), SHLD1, SHLD2, and SHLD3 is recruited to DSBs to block resection (**Figure 6E**). Shieldin can also recruit Polα-primase via its accessory factor CTC1-STN1-TEN1 (CST) to achieve the correct balance between resection and fill-in DNA synthesis (**Figure 6D** and Mirman et al., 2018). This may allow slow-kinetic cNHEJ to occur with higher fidelity and indicates an active role for 53BP1 in efficient slow-kinetic cNHEJ. A further active role for 53BP1 in slow-kinetic cNHEJ is suggested by its recruitment of PTIP, which in turn has functions in localising Artemis to DSBs that must be processed prior to repair (**Figure 6A** and Callen et al., 2013; Wang et al., 2014). The nuclease activity of Artemis is then required to process the DNA end (Riballo et al., 2004). Intriguingly, in addition to slow-kinetic cNHEJ, Artemis has also been shown to promote HR by removing lesions or secondary structures that inhibit repair by either pathway (Beucher et al., 2009). Regardless of these active roles in slow-kinetic cNHEJ, 53BP1-dependent recruitment of RIF1 and Shieldin inhibits HR, as well as the more mutagenic Alt-EJ, BIR, and SSA mechanisms of DSB repair.

53BP1 is clearly important for slow-kinetic cNHEJ and regulation of HR. These roles are supported by its recruitment into chromatin spanning megabases of DNA either side of a DSB, as well as its focal recruitment into large, micron sized, phase-separated condensates (Clouaire et al., 2018; Kilic et al., 2019). 53BP1 foci have been recently resolved into substructures termed 'nanodomains', which appear to correlate with TADs (Ochs et al., 2019; Caron and Polo, 2020). By mechanisms involving RIF1 and Shieldin, that have not been fully deciphered, these nanodomains reorganise into circular structures (termed 'microdomains'). Each microdomain is composed of about five 53BP1 nanodomains/TADs, only one of which contains the DSB. The relationship between spherical foci and circular microdomains is unclear, but the arrangement of nanodomains might protect the integrity of chromatin in those TADs close to the TAD harbouring the DSB. Interestingly, pro-resection factors localises to the centre of the microdomain, likely to segregate such proteins away from the DNA until such time as they are needed, while anti-resection factors congregate within individual nanodomains.

Regulation of 53BP1 is even more complex than its histone modification-dependent recruitment. 53BP1 is recruited to DSBs that are ultimately repaired by either slow-kinetic cNHEJ or HR. As discussed, its role in slow-kinetic cNHEJ is not just limited to inhibition of resection but it may also have active roles in slow-kinetic cNHEJ (e.g., recruitment of Artemis, CST-Polα-primase), while in HR 53BP1 promotes fidelity by preventing excessive resection (Wang et al., 2014; Ochs et al., 2016; Löbrich and Jeggo, 2017; Mirman et al., 2018; Zhao et al., 2020; Kelich et al., 2021). Given such important roles, it is not surprising that 53BP1 recruitment is tightly regulated by multiple additional mechanisms: 1) the Tudor interacting repair regulator (TIRR) can bind the Tudor domain of 53BP1 to block its H4K20me2 binding (Drané et al., 2017; Dai et al., 2018; Wang et al., 2018); 2) acetylation within the 53BP1 UDR domain (K1626/K1628) by CREB-binding protein (CBP) disrupts 53BP1 binding to the nucleosome (Guo et al., 2018); 3) RNF169, an RNF168 paralogue, appears to be able to antagonise 53BP1, as well as RAP80-BRCA1, accumulation at DSBs through a mechanism that remains enigmatic (Chen et al., 2012; Panier et al., 2012; Poulsen et al., 2012; An et al., 2018); 4) phosphorylation of the damage dependent H2AK15 ubiquitin tag (UbT12p) itself also inhibits binding of 53BP1, but not HR factors (Walser et al., 2020); intriguingly, and in contrast to these negative regulatory mechanisms, 53BP1 binding can be positively regulated by the kinesin, KIF18B, in a mechanism requiring a direct interaction with the 53BP1 Tudor domain and the motor activity of KIF18B (Luessing et al., 2021).

The regulation of 53BP1 and BRCA1 recruitment to DSBs defines the Late response to DSBs that occur prior to repair by specific pathways, and is clearly complex and not yet fully understood. Emerging data demonstrates that both 53BP1 and BARD1-BRCA1 can bind to related bivalent histone marks, providing a DNA damage histone code. Both factors can bind

to the chromatin flanking the same DSB. Details of how BRCA1 outcompetes 53BP1 at some DSBs are emerging. In particular, at one-ended DSBs produced at replication forks specific recruitment of BARD1-BRCA1 drives repair towards HR. Precisely how BRCA1 outcompetes 53BP1 at two-ended DSBs destined for repair via HR is as yet unknown. Furthermore, improved resolution of how 53BP1 and BRCA1 are physically segregated within three-dimensional space proximal to DSBs could provide important insight into pathway choice.

THE ROLE OF KU70/80 AND MRN AND IN THE LATE RESPONSE

Despite being two of the fastest-recruited proteins in the DSB response, KU70/80 and MRN have additional roles in repair choice that occur during the Early and Late response. Earlier we mentioned that proteins in the Immediate-Early damage response do not form visible foci, such as the ionising radiation induced foci (IRIF) that occur during the Early and Late response. While MRN foci have been well characterised, KU70/80 has also been shown to form detectable foci-formation at later timepoints (Britton et al., 2013). Focal recruitment indicates accumulation of sufficiently large amounts of protein that then become easy to detect by immunofluorescence. Unlike MRN foci, which can be visualised through conventional immunofluorescence, visualisation of KU70/80 foci requires pre-treatment with RNAseA and pre-extraction buffer (Britton et al., 2013). This is likely because, in addition to binding DNA ends, KU70/80 is also believed to bind RNA, also found in foci (Fijen and Rothenberg, 2021). Thus, revealing DSB-dependent foci during the 'Late' response appears to require removal of RNA to facilitate antibody access to KU70/80 (Britton et al., 2013; Sharma et al., 2021). However, neither KU70/80 nor MRN foci have been demonstrated within the Immediate-Early response, which takes place within the seconds immediately after DSBs formation and during which fast-kinetic cNHEJ occurs. Other than its DNA end binding activity, and possibly also a reported interaction between PARP1 and KU70/80, the mechanism of KU70/80 recruitment, particularly into foci, is less defined than for MRN, which is primarily ATM- and MDC1-dependent as previously discussed. Although, as a further complication, there is evidence that binding of human single-stranded DNA binding protein 1 (hSSB1) to resected DNA facilitates enhanced recruitment of MRN and increased MRE11 endonuclease activity (Richard et al., 2008; Richard et al., 2011a; Richard et al., 2011b).

Importantly, it appears that while KU70/80 must be retained at DSBs for slow-kinetic cNHEJ, for HR KU70/80 has to be evicted during the Late response. KU70/80 eviction is achieved by a combination of nucleolytic and proteolytic activities. Interestingly, the major nuclease implicated in KU70/80 eviction is MRE11, indicating crosstalk between these two end binding complexes, while CtIP also plays a role (Langerak et al., 2011; Chanut et al., 2016; Myler et al., 2017). Proteolytic eviction of KU70/80 is regulated by RNF8 and RNF138, yet another E3 ubiquitin ligase, which can tag KU70/80 for degradation using K48-linked polyubiquitin (Feng and Chen, 2012; Ismail et al., 2015). Ubiquitination of KU70/80 appears to be promoted by yet another post translational modification, neddylation (Brown et al., 2015).

It is possible that MRN could also contribute to the proximal 'melting' of the broken DNA ends to facilitate the loading of RNA polymerases. The resulting non-coding RNAs (ncRNAs) have been reported to be processed by the RNAses DROSHA and DICER to become the so-called DNA damage response or damage-inducible RNAs (DDRNA or diRNA), reported to regulate the DSB response (Francia et al., 2012; Wei et al., 2012). Another recently reported role for RNA in the DSB response is to hybridise to the 3′ overhanging strand after resection thereby protecting it from nucleases. RNA Pol III has been reported to synthesise the RNA that forms these transient RNA-DNA hybrids, and impacts upon high fidelity repair by both slow-kinetic cNHEJ and HR (Liu et al., 2021). Indeed, beyond the scope of this review, there is emerging evidence that RNA plays many important roles in the DSB response (Chowdhury et al., 2013; Barroso et al., 2019; Crossley et al., 2019; Bader et al., 2020; Ketley and Gullerova, 2020; Guiducci and Stojic, 2021; Jimeno et al., 2021; Marnef and Legube, 2021; Palancade and Rothstein, 2021). Perhaps the ability of KU70/80 to bind RNA could be important with respect to the emerging roles for RNA in the responses to DSBs.

DOWNSTREAM DOUBLE STRAND BREAK REPAIR PATHWAYS

After the Late DSB response, largely constituting a delicate balance between 53BP1 and BRCA1 recruitment, the remaining DSBs can be repaired by either slow-kinetic cNHEJ or HR (**Figures 1E,I**). However, these two high-fidelity repair pathways are often counted among five distinct pathways. These are: i) slow-kinetic cNHEJ (note that fast-kinetic cNHEJ is an Immediate-Early response, see **Figure 2**); ii) alternative end joining (Alt-EJ; often referred to as microhomology-mediated EJ, or MMEJ); iii) break induced replication (BIR); iv) single strand annealing (SSA), and v) gene conversion (GC), which can result from two distinct HR mechanisms, either synthesis dependent strand annealing (SDSA, also called short tract GC) or double Holliday Junctions (dHJ, also called long tract GC) mediated recombination (**Figures 1E–I** and Mehta and Haber, 2014; Chang et al., 2017; Malkova, 2018; Krenning et al., 2019). How the cellular DSB repair machineries funnel DSBs into the possible repair outcomes is not yet fully understood, but influencing factors include cell cycle stage, chromatin context (especially with respect to transcriptional status), the type and extent of the breaks, and the amount of resected ssDNA (Ronato et al., 2020). The historical perspective that cNHEJ and HR are resection independent or dependent, respectively, has been revised by the realisation that in addition to Alt-EJ, some slow-kinetic cNHEJ also relies upon resection (Shibata and Jeggo, 2019).

Slow-kinetic cNHEJ accounts for repair of about 20% of IR induced DSBs, and has been termed ATM, 53BP1-, or Artemis-dependent cNHEJ and, as it requires some limited (1–5 nt)

resection, is additionally termed resection-dependent cNHEJ (**Figure 1E** and Chang et al., 2017; Jeggo and Löbrich, 2017). This pathway also depends upon other indirect factors impacting upon pathway choice, including RIF1, Shieldin, and CST-Polα primase. Whereas the core cNHEJ proteins are required by both fast- and slow-kinetic cNHEJ, 53BP1, RIF1, and Shieldin are anti-resection factors and CST- Polα primase balances resection with *de novo* DNA synthesis, likely improving fidelity (Mirman et al., 2018). However, fast- and slow-kinetic cNHEJ differ in their ability to repair simple versus complex DSBs, have different recruitment pathways, and are used to different extents throughout the cell cycle (Setiaputra and Durocher, 2019; Shibata and Jeggo, 2020a; Shibata and Jeggo, 2020b; Qi et al., 2021). Intriguingly, emerging data suggests that slow-kinetic cNHEJ can avoid mutagenic deletions by using RNA molecules as homology templates for retrieving sequence information that can be lost during resection (Storici et al., 2007; Chakraborty et al., 2016; Meers et al., 2016; Mazina et al., 2017).

Alt-EJ encompasses vestigial NHEJ repair pathways that do not require KU70/80, XRCC4, or LIG4 (**Figure 1F** and Iliakis et al., 2015; Wyatt et al., 2016; Dutta et al., 2017; Hanscom and McVey, 2020; Ramsden et al., 2021). These repair subpathways occur after Artemis and CtIP-dependent slow-kinetic cNHEJ fails to repair the DSB, and when there is insufficient homology (less than 25 nt) for HR. Interestingly, Alt-EJ pathways can still occur in cells with functioning cNHEJ and HR, albeit at a frequency of just 0.5%–1% (Hanscom and McVey, 2020). Given the many descriptors (a-EJ, alterative NHEJ, backup NHEJ, MMEJ (microhomology-mediated end joining), TMEJ [polymerase theta (Pol θ)-Mediated End joining], Synthesis-dependent MMEJ, etc.) and the obvious confusion generated, Alt-EJ subpathways may best be considered as being either Pol θ–dependent or independent. During, MMEJ resection reveals microhomologies allowing annealing, followed by removal of the 3′ non-homologous tails, gap filling, and ligation. It is interesting to note that PARP1 plays a role in Alt-EJ subpathways such as MMEJ (Mansour et al., 2010; Dutta et al., 2017). TMEJ, on the other hand, still uses microhomologies, but also relies on Pol θ in order to prime the synthesis of up to 25 nt of nascent DNA (Hanscom and McVey, 2020). More recently, TMEJ has been shown to be cell cycle regulated, repairing one-ended DSBs that arise in S-phase in early mitosis (Llorens-Agost et al., 2021). In this pathway, RAD52 and BRCA2 delay TMEJ until early mitosis, by which time one-ended DSBs have been converted to two-ended DSBs. A recent study also demonstrated that the polymerase activity of Pol θ is not the only function required for TMEJ; amazingly, its DNA polymerisation domain can also function nucleolytically for 3′ end trimming (Zahn et al., 2021). When we consider NHEJ as a whole network of pathways, it is important to remember that fast-kinetic cNHEJ occurs upstream within the Immediate-Early response, while all other subdivisions, including slow-kinetic cNHEJ, MMEJ, TMEJ, and any other Alt-EJ pathways, are all resection-dependent repair pathways.

In mitotic cells HR has three main subdivisions: GC, BIR, and SSA (Jackson, 2002; Mehta and Haber, 2014; Chang et al., 2017; Krenning et al., 2019; Pham et al., 2021). GC is the highest-fidelity repair; in yeast requiring just 20–80 nt of *in trans* homology, and resecting 2–6 kb, while in mammalian cells the minimal *in trans* homology is unclear, but resection can occur for up to 3.5 kb (Ronato et al., 2020). In mammals, GC is dependent upon the nuclease activity of MRN and other proteins such as CtIP, BLM, EXO1, RPA1, BRCA1, PALB2, BRCA2, XRCC3, RAD51, and RAD54 (**Figure 1I**). This repair pathway requires end resection, ssDNA protection, search for homology, strand invasion, and resolution of the resulting Holliday Junction (Jackson, 2002; Li and Heyer, 2008). GC has two subdivisions: SDSA and dHJ mediated recombination, which are also referred to as short tract GC (STGC) and long tract GC (LTGC), respectively (Elbakry and Löbrich, 2021). In SDSA, an unstable displacement loop (D-loop) is formed as an intermediate composed of a double stranded DNA double helix invaded by the broken DNA end, leading to short-tract DNA synthesis. The second end of the break is then annealed to this newly synthesised DNA, resulting in repair that is cross-over independent. This is the most common form of DSB repair, as it minimises the chance of mutations to DNA near the DSB (Pham et al., 2021). On the other hand, dHJ resolution begins with the invasion of the broken strand to form a stable D-loop, followed by long-tract DNA synthesis. The second end of the DSB is eventually captured, leading to the formation of joint molecules. The resolution of these joint molecules results in cross over and non-cross over events with equal frequencies (Elbakry and Löbrich, 2021).

It is interesting to note that the involvement of BRCA1 and BRCA2 in HR is an area of intensive research stimulated by the roles of these DSB repair factors in heritable *BRCA* defective breast and ovarian cancers. Furthermore, *BRCA* defective cancer cells are sensitive to PARP inhibition (Antolin et al., 2020; Jannetti et al., 2020; Rose et al., 2020), and this synthetic lethality suggests that PARP and BRCA1/2 function in different pathways. The mechanism by which PARP inhibitors function remains to be fully deciphered and is subject to much debate, but it has been proposed to be due to defective SSB repair, which results in one-ended DSBs during S phase that require HR for their repair (Helleday, 2011; Murai et al., 2012; Horton et al., 2014). However, it is likely that Artemis-dependent, or slow-kinetic cNHEJ, also contributes to PARP inhibition-dependent lethality in HR-defective cells (Patel et al., 2011; De Lorenzo et al., 2013), consistent with PARP performing some roles in multiple DSB repair pathways.

BIR is a sub-pathway of HR which uses the invading strand for long-range DNA synthesis without the engagement of a second DSB end (Elbakry and Löbrich, 2021). It therefore repairs one-ended DSBs arising from fork collapse and provides an alternative mechanism for telomere maintenance when telomerase is lost (**Figure 1G** and Malkova, 2018). In budding yeast, BIR requires approximately 72 nt of homology and can resect up to 1 kb (Ronato et al., 2020). This recombination-based method of conservative DNA replication copies from a template DNA until the end of the DNA template. The invasion of the single DNA end and subsequent replication during BIR relies on RPA, Rad52, Rad51, and to some extent, Rad54, Rad55, and Rad59 (Anand et al., 2013; Malkova, 2018). Although it is not currently known what restrains BIR at two ended breaks and

promotes GC, the proteins Rad52, Rad58, Mph1, and MRX have been implicated in yeast studies (Pham et al., 2021).

SSA is not dependent on a sister chromatid for homology and results in deletions (**Figure 1H**). Resection reveals *in cis* homologous repeat sequences which then anneal together with the resulting 3′ flap structures being removed (**Figure 1H** and Onaka et al., 2020). Studies in budding yeast have shown that SSA relies on 63–89 bp homology, while the end is resected until homology occurs (Ronato et al., 2020). In yeast or mammalian cells, mutagenic SSA occurs when GC is unavailable, for example, when RAD51 or RAD54 are depleted, the cell switches to the RAD52-dependent SSA repair (Ochs et al., 2016; Onaka et al., 2020).

When considering the DDR, there are other pathways that tie into these described repair pathways that have not been discussed in this review, for example, DSBs arising at a replication fork. The kinase ATR can be activated in response to resected DSBs, but is most often activated in response to the elevated levels of ssDNA couated with RPA, that occurs at stalled replication forks. Such structures can be converted into DSBs by nucleolytic attack or fork collapse (Burger et al., 2019). Alternately, ATR can be activated if repair is unsuccessful, as it is involved in checkpoint signalling and cell fate. Additionally, ICL repair is a critical pathway that repairs one of the most complex DNA lesions (Scully et al., 2019; Panday et al., 2021; Semlow and Walter, 2021). Because ICL repair generates a transient DSB as an intermediate, that is protected within the context of ICL repair, it should be included in the discussion of DSB repair pathways. It depends on FA core proteins, as well as downstream repair proteins involved in both HR and cNHEJ. The repair at an ICL consists of an unhooking step, *trans* lesion synthesis, excision repair, strand invasion, and resolution. During S-phase, there are complex repair requirements at single or converging forks, while replication-independent ICL repair can also outside of S-phase (Semlow and Walter, 2021). Processing of the DSB after the unhooking step depends upon HR proteins for repair via strand invasion and resolution. It should be noted that the DSB produced during ICL repair is protected within the context of this repair pathway. It is therefore not likely to be sensed as a classic DSB that activates the Immediate-Early and Early response.

It is important to note that cNHEJ, both fast- and slow-kinetic, as well as HR appear to be the default pathways in healthy wild-type mammalian cells and they are not usually error prone as they have evolved to operate with high fidelity (Ceccaldi et al., 2016). The physiological relevance of the alternate repair pathways Alt-EJ, SSA, and BIR under normal conditions remains an open question. These mutagenic pathways occur in the absence of certain cNHEJ and HR factors or upon non-physiological levels of replication stress, for example in cancerous cells. Under such cellular conditions, elevated levels of error-prone DSB repair may therefore reflect the enzymatic capabilities of the remaining proteins (Khanna and Jackson, 2001; Iliakis et al., 2019).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Here, we have presented an integrated view of the pre-repair DSB response at its three main stages, Immediate-Early, Early, and Late. Although there is no clear consensus on a precise DSB sensor in the field, the Immediate-Early response consists of the initial DSB sensing and signalling that occurs within seconds of DSB formation. While PARPs have well defined roles in SSB repair, there is emerging data implicating some roles for PARP1, PARP2, and PARP3 upstream of DSB repair. Furthermore, the complex recruitment and interplay between PARP, KU70/80, and MRN contributes to downstream pathway choice. In addition, we support emerging evidence for fast-kinetic cNHEJ responsible for the rapid repair of most DSBs during the Immediate-Early response. The remaining breaks require processing before repair. The activation of ATM and the associated chromatin dynamics constitutes the Early response. This culminates in damage-dependent ubiquitination events permissive for recruitment of Late response proteins, such as the 53BP1 and BRCA1 scaffold proteins.

Every step of the pre-repair responses, Immediate-Early, Early, and Late, appears to be important for pathway choice. This requires complex integration of multiple factors to achieve the optimal outcome, which in turn will be specific to the context of each DNA lesion. These factors include the complexity of the DSB itself, chromatin context, cell cycle phase, and availability of the specific repair factors required to achieve the highest fidelity possible. Critical molecular events include the PARP-dependent PARylation response, the recruitment of KU70/80 and/or MRN, dynamic chromatin decondensation and condensation, the activation of ATM, and damage-dependent histone modifications defining a histone code for DSB repair. While there is crosstalk between the Immediate-Early, Early, and Late responses, according to our current understanding it is not until after 53BP1 and BRCA1 recruitment that a cell commits to a specific DSB repair pathway. However, much remains to be discovered about how these responses crosstalk, overlap, and compete.

A key emerging question is apparently simple, yet of deep complexity: for those breaks that are not immediately ligated, at what stage is a DSB committed to a specific repair pathway? Instead of pathway choice occurring downstream of 53BP1 and BRCA1, could it not be more useful to consider pathway choice as a continuous process? It is likely that regulation and crosstalk between the pre-repair pathways allows integration of the many factors required for normal maintenance of genome stability. A related question is whether, if repair fails, can the repair machinery backtrack and attempt to repair the lesion using an alternative high-fidelity approach, before resorting to a more error-prone mechanism. Additionally, the interplay between the PARP-dependent Immediate-Early response and the ATM- and chromatin-dependent Early response has not been fully deciphered. Also, despite its pleiotropic roles throughout the DSB response, how CK2 is activated to specifically regulate so many steps remains enigmatic. It is important to consider that highly error prone mechanisms are unlikely to be physiologically relevant under normal conditions, and are likely to be rare events in normally growing unstressed wild-type cells. Under suboptimal conditions, such as the loss of specific DSB factors that occurs during cancer, or where

elevated and non-physiological levels of damage are induce by exogenous agents, repair outcomes become skewed towards mutation. Under such conditions, and if apoptosis is not triggered, repair is likely to proceed using whatever machinery is available. Full understanding of the DSB response remains a challenge for the future. No doubt, these challenges will be met and will expand our evolving understanding of how Immediate-Early, Early, and Late DSB responses are coordinated and integrated to achieve the optimal downstream repair outcomes.

AUTHOR CONTRIBUTIONS

SRK and NFL discussed concepts, planned, and wrote the paper. SRK drafted the Figures.

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METHODS
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Protocol: A Multiplexed Reporter Assay to Study Effects of Chromatin Context on DNA Double-Strand Break Repair

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DNA double-strand breaks (DSBs) can be repaired through various pathways. Understanding how these pathways are regulated is of great interest for cancer research and optimization of gene editing. The local chromatin environment can affect the balance between repair pathways, but this is still poorly understood. Here we provide a detailed protocol for DSB-TRIP, a technique that utilizes the specific DNA scars left by DSB repair pathways to study pathway usage throughout the genome. DSB-TRIP randomly integrates a repair reporter into many genomic locations, followed by the induction of DSBs in the reporter. Multiplexed sequencing of the resulting scars at all integration sites then reveals the balance between several repair pathways, which can be linked to the local chromatin state of the integration sites. Here we present a step-by-step protocol to perform DSB-TRIP in K562 cells and to analyse the data by a dedicated computational pipeline. We discuss strengths and limitations of the technique, as well as potential additional applications to study DNA repair.

Keywords: DNA repair, reporter, chromatin, protocol, non-homologous end-joining, microhomology-mediated end-joining, single-strand template repair, CRISPR

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1 INTRODUCTION

The double-strand break (DSB) repair machinery consists of multiple pathways, including non-homologous end-joining (NHEJ), homologous recombination (HR) and microhomology-mediated end-joining (MMEJ) (McVey and Lee, 2008; Iliakis et al., 2015; Chang et al., 2017; Scully et al., 2019). Furthermore, a pathway named single-strand templated repair (SSTR) has been identified that can be utilized for templated CRISPR editing (Lin et al., 2014; Richardson et al., 2016). Many factors can influence which pathway is used to repair a specific lesion (reviewed in Brandsma and Gent, 2012; Ceccaldi et al., 2016; Scully et al., 2019). Several studies have demonstrated that the local chromatin state is one of the factors that influences which pathway is preferentially used to repair a DSB. These studies used methods ranging from using single imprinted endogenous loci (Kallimasioti-Pazi et al., 2018), single transgenic loci (Lemaitre et al., 2014), hundreds of endogenous loci (Iacovoni et al., 2010; Massip et al., 2010; Aymard et al., 2014) to thousands of integrated reporters as presented here (Gisler et al., 2019; Pokusaeva et al., 2021; Schep et al., 2021). All these studies rely on endonucleases creating a DSB at a defined locus in the genome, targeting either a definite sequence (e.g., restriction

enzymes) (Iacovoni et al., 2010) or a user-defined one (Kallimasioti-Pazi et al., 2018; van Overbeek et al., 2016; Chakrabarti et al., 2019) [e.g., with CRISPR/Cas9 (Jinek et al., 2012; Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013)]. While there are other methods to generate DSBs across the genome, such as ionizing radiation, non-ionizing radiation or chemically induced DSBs (reviewed in Vitor et al., 2020), they are less suitable to link chromatin state to DNA repair pathway usage.

The advantages of using a single endogenous or transgenic site are that these loci can be easily imaged and perturbed in a controlled manner [e.g., nuclear lamina targeting (Lemaitre et al., 2014)], which allows for precise dissection of their repair kinetics [e.g., imprinted loci (Kallimasioti-Pazi et al., 2018)]. However, this approach does not provide the diversity of sites that multiplexed assays offer. Working with single loci can also be much more labour intensive to collect sufficient data to dissect the effects of the large variety of chromatin states on DNA repair.

Other techniques use restriction enzymes cutting multiple endogenous loci. The I-PpoI endonuclease targets the ~300 copies of the 28S ribosomal RNA gene plus 15 other unique sites. It was mainly used to understand the interplay between DNA repair and transcription, as well as histone distribution (Berkovich et al., 2007; Goldstein et al., 2013; Kim et al., 2016). The Legube lab developed the DSB inducible *via* AsiSI (DIvA) cell line expressing the AsiSI restriction enzyme (Iacovoni et al., 2010; Massip et al., 2010; Aymard et al., 2014). The enzyme is fused to a ligand-inducible domain for controlled nuclear localization and can reliably create ~150 endogenous breaks in U2OS cells in an inducible manner. This method can accurately measure differences in repair pathway choice between transcribed and non-transcribed regions. Unfortunately, in this system, the cutting efficiency is in general very low in heterochromatin (Aymard et al., 2014). DNA repair can therefore not be accurately measured throughout this major chromatin type. Other limitations with these restriction enzymes are that the pool of target sites is fixed and that the varying sequence surrounding the target site might still affect the repair pathway balance.

Here we provide a detailed protocol of DSB-TRIP, a technology to measure the relative activity of multiple DSB repair pathways in many genomic locations with different chromatin states (Schep et al., 2021). DSB-TRIP is an adaptation of TRIP (Thousands of Reporters Integrated in Parallel), which was initially designed to measure the impact of chromatin context on gene regulation (Akhtar et al., 2013; Akhtar et al., 2014). The multiplexed nature of DSB-TRIP enables the probing of DSB repair hundreds or thousands of genomic locations, providing the statistical power needed to link differences in DSB repair pathway usage to a variety of chromatin features. We found that DSB-TRIP can detect DSB repair events across all chromatin states, including all known types of heterochromatin. Moreover, the design of DSB-TRIP effectively rules out confounding effects of surrounding DNA sequences.

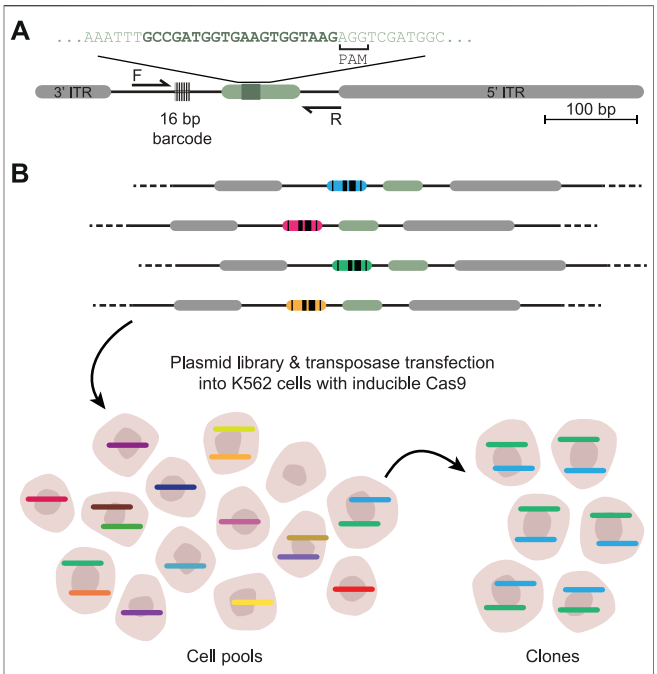

FIGURE 1 | Scheme of DSB-TRIP. **(A)** Scheme of the barcoded DSB reporter. In grey the PiggyBac inverted terminal repeats, in light green the LBR endogenous sequence with the 20 bp gRNA sequence in dark green. Primers F and R are used to sequence the indels and the barcode. **(B)** Representation of the barcoded library and an illustration of the TRIP cell pools and clones with the different barcodes represented by different colours. Adapted from (Schep et al., 2021).

1.1 Concept

DSB-TRIP works by random integration of a specially designed DSB repair pathway reporter into hundreds or thousands of genomic locations in a pool of cells, by means of a transposon vector. The reporter is short [~650 base pairs (bp)] and devoid of transcriptionally active sequences that could change the local chromatin environment (**Figure 1A**). Each copy of the reporter is marked by a random barcode, which allows for decoding of individual reporters and linking them to their genomic location. First, the genomic locations of the reporter integrations in the pool of cells are mapped. It is also possible to generate clonal cell lines that carry up to dozens of reporters. Next, a DSB is introduced inside each reporter by means of Cas9. Repair of the resulting DSBs results in specific "scars" [insertions and deletions (indels)] that can be used to identify the repair pathways that were active at a DSB (Allen et al., 2018; Chakrabarti et al., 2019; Chen et al., 2019; Shen et al., 2018; van Overbeek et al., 2016; Brinkman et al., 2018). Our current reporter can detect NHEJ, MMEJ and SSTR. After DSB induction and DNA repair, the genomic DNA is extracted and the reporter "scar" and the flanking barcode are jointly amplified by PCR and subjected to high throughput sequencing. A computational pipeline counts the scars for each individual barcoded reporter, infers from these counts the relative activity of each pathway, and links the results to the genomic location. Overlay

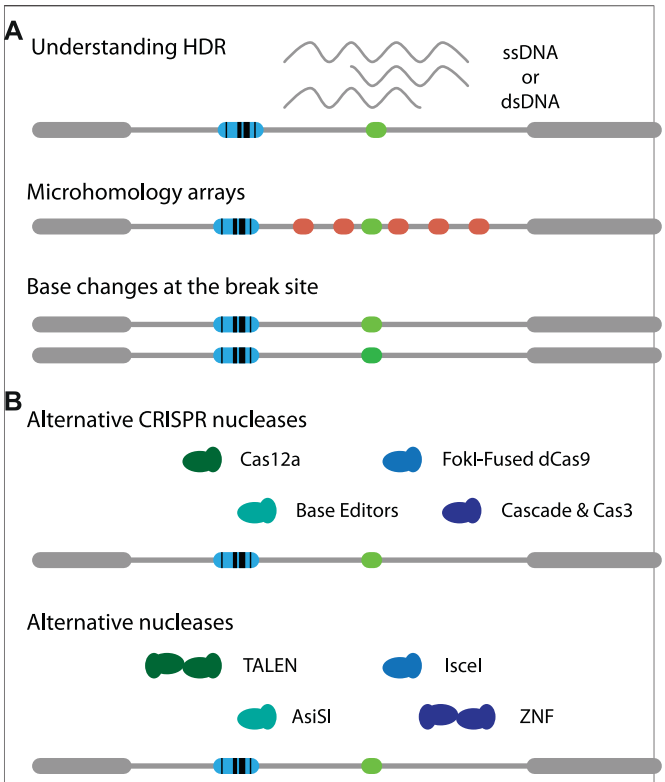

FIGURE 2 | Adaptations of DSB-TRIP. **(A)** Potential variations of the reporter itself. One can study the effects of chromatin on (1, top) MMEJ by creating tiled microhomologies (red), (2, middle) on Cas9 mutagenesis with small mismatches (middle) or (3, bottom) on HDR based transgenesis. **(B)** Illustration on variations on CRISPR nucleases (top) and other nucleases (bottom). Target cut site in light green, IPR barcode in blue.

with epigenomic mapping data then uncovers any correlations between pathway usage and local chromatin features.

A key feature of DSB-TRIP is that an identical reporter sequence (except for the short barcodes) is integrated into many different chromatin environments. Hence, differences in pathway usage between integration sites can be attributed to differences in the chromatin context. Thus, insights are obtained in the impact of the chromatin environment on DSB repair.

1.2 The Protocol in Brief

The protocol consists of two main components: the wet-lab experiments, and the computational analysis of the resulting sequencing data.

The wet-lab part starts with the design of the DSB repair reporter, which is a DNA sequence that, when repaired after a DSB, produces a specific indel pattern that can be associated with either NHEJ or MMEJ. We then describe how to make reporter plasmid libraries, how to transfect them into K562 cells and how to generate cell pools and clones carrying multiple integrations (**Figure 1B**). Next, two sets of data are generated: mapped reporter integrations and indel scores per reporter. First, the genomic location of the integrated pathway reporters (IPRs) in these clones are mapped by inverse PCR (iPCR), as explained in detail in (Akhtar et al., 2014). Second, the DSBs are induced by Cas9 induction, cells are cultured up to 72 h to allow the DSBs to be repaired, after which genomic DNA is collected to create sequencing libraries.

The sequencing data of the mapping and the indels are then processed with a computational pipeline that produces tables of the mapping per barcode and indels per barcode for further analysis.

The most basic implementation of the data analysis pipeline uses two types of sequencing data: iPCR reads used to link barcodes to the integration locations in the genome (mapping) and indel PCR sequences to assess repair outcomes. The standard output for the iPCR is a table containing the barcode, its genomic location (chromosome, start, end, and orientation), reads mapped, mapping quality and mapped sequence. The output for the indel PCR is a table containing four columns: the barcode sequence, the mutation class (e.g., wild-type, insertion, deletion or "unclear"), the size of the detected indel in bp and the number of occurrences. Two main extensions can be implemented for more detailed output. First, the pipeline can be run to count specific repair scars. This can be used to differentiate MMEJ from non-MMEJ scars with the same indel size. This functionality is available in the pipeline but requires a significantly longer runtime. The second option available, is the ability to add a recognition sequence to detect homology directed repair.

1.3 Choice of Cell Line

A prerequisite for DSB-TRIP is a cell line in which a DSB can be induced in a specific and reproducible way. We recommend establishing a clonal founder cell line with a stably integrated, inducible Cas9. We use K562 cells expressing Cas9 from the human PGK promoter and fused to a destabilization domain (DD) at its N terminus. This DD causes active degradation of the protein unless it is stabilized by addition of the small molecule Shield-I (Banaszynski et al., 2006; Brinkman et al., 2018). This allows for tight control of Cas9 activity. We note that in our cell line (clone K562#17) full induction of DD-Cas9 takes ~16 h, which should be taken into account in the experimental design. Possibly, Cas9 can be introduced into cells by lentiviral transduction, or by transient transfection with an expression vector or Cas9 ribonucleoprotein. However, this may not yield homogeneous expression in 100% of the cells, which may compromise the data quality.

Instead of K562 cells, other cells may be used. However, there are some practical restrictions. First, the cell line must tolerate DNA transfections and transposon integrations; this is a requirement for the reporter insertions as well as for sgRNA transfections. Second, the cells must tolerate some levels of DNA damage, as some individual cells in the pool might carry more than 20 IPRs in their genome, and thus may potentially need to cope with as many simultaneous DSBs. Third, epigenome mapping data must be available for the cell line. This is essential for linking of the DSB-TRIP results to the chromatin state of the IPRs.

1.4 Designing the Reporter

One should consider the following elements when designing the reporter. First, a reporter of small size (≤1 kb) and devoid of any

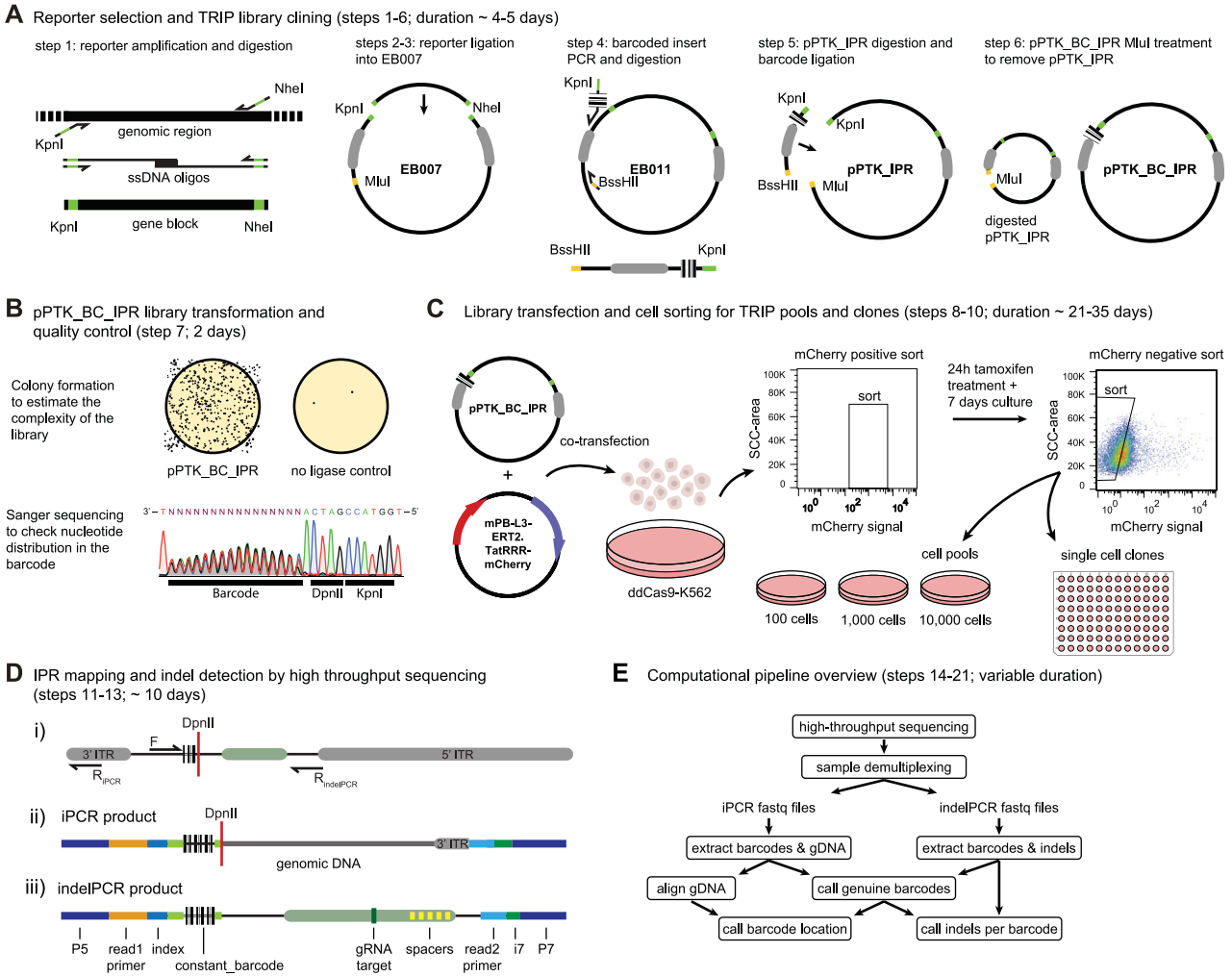

FIGURE 3 | Step by step DSB-TRIP. **(A)** DSB-TRIP cloning steps. DNA sequences are in black, important restriction enzyme sites in green and orange and the PiggyBac ITRs are in grey. **(B)** Library quality control. Top: assessing the complexity of the library by counting the colonies. Bottom: A Sanger sequence track from step 7, spanning the barcode and the DpnII and KpnI restriction sites in the plasmid library, The sequence is flipped to match figure orientation. All the bases are equally present in the 16 bp barcode. **(C)** Library transfection and FACS sorting strategy to generate pools and clones. **(D)** i) Scheme of the IPR with the main primers used for generating the mapping (F & R_{iPCR}) and the indel (F & $R_{indelPCR}$) sequencing libraries. DpnII restriction site is indicated with a red line. ii) Scheme of a raw iPCR library PCR product, created from the circularisation of the fragment by ligation of a genomic DpnII site with the DpnII site from above. From left to right: P5 Illumina adapter, read1 primer site, 16 bp IPR barcode, DpnII restriction site in red, genomic DNA dark gray, 3′ITR in light gray, i7 Illumina index, P7 Illumina adapter. iii) Scheme of a raw indelPCR library PCR product, indicating the Illumina adapter and primer locations (P5, read1 primer, read2 primer, i7 and P7) as well as constant sequences related to the config file. From left to right: P5 Illumina adapter, read1 Illumina primer, index, constant_barcode (light green) with 16 bp IPR barcode embedded in between, LBR sequence containing the Cas9 target site (dark green) and multiple spacers in yellow (spacer_list), read2 Illumina primer, i7 Illumina index, P7 Illumina adapter. **(E)** Diagram of the analysis pipeline.

active transcription will have less impact on the chromatin state at the integration site. Second, the reporter sequence should produce signature "scars" after repair of the DSB that are characteristic of the repair pathway(s) of interest. We used a short sequence derived from the *LBR* gene (Schep et al., 2021), but it may be replaced by other sequences depending on the pathway of interest. Third, when designing the reporter sequence it is important to have the correct restriction sites at each end of the sequence (**Section 1**) as well as the *IndelPCR1_rv* primer binding site at the NheI end to be able to follow the library preparations steps (**Section 13**).

1.5 Limitations of DSB-TRIP

Despite the power of DSB-TRIP, it has some limitations. First, DSB-TRIP is based on the detection of signature indels that are produced by the respective pathways. However, HR does not usually generate indels, and hence this pathway cannot be measured with DSB-TRIP. The reporter that we present here is also sensitive to large deletions or extensive resection that might remove primer binding sites or the reporter barcode. In part this can be overcome by using Tn5 transposon-based library preparation as we presented in (Schep et al., 2021) or with UdiTaS (Giannoukos et al., 2018) which requires only one

specific primer site. Second, while Cas9 has been the most prominent tool for gene editing, the DSBs that it creates may not be representative for DSBs that occur naturally. For example, the cutting and repair rates that have been observed with Cas9 appear to be extremely slow (Brinkman et al., 2018). This should be kept in mind while interpreting the data. Due to the slow rates of cutting and repair (a typical DSB-TRIP experiment takes 3 days), it is difficult to link observed pathway activities to cell cycle stages. Finally, in the cell pools the number of integrations per cell can vary substantially, which may lead to different DNA damage loads that in turn may affect the DNA repair kinetics or pathway balances.

1.6 Additional Applications of DSB-TRIP

While the generation of TRIP cell pools and clones can be time consuming there are many ways to use these TRIP cell pools and clones. The cell pools offer many different genomic loci, but note these experiments also require many cells to have sufficient coverage per IPR. For more multiplexed applications, such as small molecule or CRISPR screens, we recommend the use of clones carrying multiple IPRs. DBS-TRIP is compatible with experiments in 96- or even 384-well plates. This format is perfect for screens and other automated applications. For instance, a multiplexed, automated time series analysis in 96-well format was presented in Schep et al. (2021).

The reporter assay can be modified in many ways. For example, the DSB target site may modified with different microhomologies to study MMEJ in more detail. It is also possible to add variations in the target sequence to study how chromatin might affect slight changes in affinity of Cas9. This may provide a better understanding of off-target cutting by Cas9 and the ensuing repair (**Figure 2A**, middle). Using the exact same reporter as presented here it is feasible to study SSTR in the context of chromatin, by co-transfecting a single-stranded oligonucleotide that carries a small insertion (Schep et al., 2021). It may also be possible to provide different types of templates, for instance a double-stranded DNA template (Wienert et al., 2020). Furthermore, the effects of adding mismatches and varying the homology arm lengths may be investigated (**Figure 2A**, bottom) (Richardson et al., 2018; Hussmann et al., 2021). Potentially, time-series measurements on DSB-TRIP cell pools or clones combined with mathematical modelling (Brinkman et al., 2018) may reveal how rate constants of individual steps of the cut-and-repair process are affected by the local chromatin state.

It will be interesting to apply DSB-TRIP with other CRISPR endonucleases such as Cas12a (Zetsche et al., 2015) or rare-cutter restriction enzymes such as I-SceI (Niu et al., 2008) or AsiSI (Iacovoni et al., 2010) (**Figure 2A**, bottom). I-SceI was successfully applied to study Single Strand Annealing in a TRIP assay (Pokusaeva et al., 2021). Another option are transcription activator-like effector nucleases (TALENs) (Boch et al., 2009; Gaj et al., 2013; Moscou and Bogdanove, 2009), especially since it was found that these nucleases are more efficient in heterochromatin, compared to Cas9 (Jain et al., 2021). Furthermore, mismatch repair may be studied using base editors (**Figure 2A**, top) [reviewed in (Anzalone et al., 2020)]. For each of these endonucleases it is essential that they are expressed in a tightly controlled inducible manner, otherwise the DSBs are already generated and repaired while the cell pools with IPRs are being established.

Finally, it will be interesting to study chromatin context effects on DNA repair in cells with particular genetic alterations, such as mutations in specific repair proteins or defects in chromatin organisation, such as Hutchinson-Gilford Progeria that is the result of specific mutations in the Lamin A gene (De Sandre-Giovannoli et al., 2002; Eriksson et al., 2003). With its flexibility, multiplexing ability and detailed readout, DSB adds a versatile tool to study DNA repair, gene editing and the interplay between repair pathways and chromatin.

2 STEP BY STEP METHODS

2.1 Experimental Procedure

2.1.1 DSB-TRIP Library Cloning

1. Preparation of the reporter insert (**Figure 3A**)—30 min

NOTE: Can be done in parallel with **Section 2**.

1.1. Digest 100 ng—1 µg of the insert (amplified by PCR, from oligos, or as a geneblock—i.e., gBlocks™ from IDT) with the following mix:

Component	Amount (µl)	Final concentration
10× CutSmart buffer	5	1×
0.1–1 µg insert	a	
NheI-HF (20 U/µl)	1	0.4 U/µl
KpnI-HF (20 U/µl)	1	0.4 U/µl
Nuclease-free water	43—a	
Total	50	

1.2. Incubate 10 min at 37°C

1.3. Purify the digested product with a PCR purification kit, following the manufacturer's instructions. Kits such as PCR Isolate II PCR and Gel Kit (Bioline) or CleanPCR beads (cleanNA) can be used for any PCR purification steps in this protocol.

1.4. Measure the insert concentration by nanodrop.

2. Preparation of the TRIP vector (**Figure 3A**)—30 min

2.1. Digest 100 ng of the plasmid EB007 as described above.

2.2. Dephosphorylate the ends using either Quick CIP (NEB #M0525), Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371) or Antarctic Phosphatase (AP) (NEB #M0289) and heat inactivate the enzymes, using the supplied protocol.

3. Ligation and Transformation of the reporter into the TRIP vector (**Figure 3A**)—1.5 days

NOTE: This can be done in parallel with the barcoded insert preparation—**Section 4**).

3.1. Mix 50 ng of the insert with 1 µl of digested EB007, 1 µl of T4 DNA ligase (Roche Cat#: 10799009001) and 2 µl T4 DNA Ligase Buffer in 20 µl final volume.

Component	Amount (µl)	Final concentration
10× T4 DNA ligase buffer	2	1×
50 ng reporter insert from step 1.4	a	
Digested EB007 from step 2.2	1	0.3 U/µl
T4 DNA Ligase (5 U/µl)	1	0.3 U/µl
Nuclease-free water	16−a	
Total	20	

3.2. Incubate 10 min at RT (room temperature).
3.3. Transform 2 µl of the ligation reaction into JM109 competent cells together with a no-ligation control.
3.4. Pick 10 colonies and grow them in 2 ml LB with 100 µg/ml ampicillin for 8 h, purify the plasmids using PureLink™ HQ Mini Plasmid DNA Purification Kit (Thermo—or similar) and quantify using a nanodrop.
3.5. Verify the correct plasmid sequence with Sanger sequencing using primer *barcode-sanger-rv*.

barcode-sanger-rv | TAC0005 | CGCCAGGGTTTTCCCAGTCACAAG.

3.6. Expand the selected mini culture in 100 ml LB with 100 µg/ml ampicillin and purify pPTK_IPR with PureLink™ HiPure Plasmid Midiprep Kit (or similar).
3.7. Measure pPTK_IPR concentration by nanodrop.

4. Preparing the barcoded insert (**Figure 3A**)—3 h
4.1. Prepare the following PCR mix to make the barcoded insert. Split the total volume in five 100 µl reactions.

Component	Amount (µl)	Final concentration
5× Phusion high-fidelity buffer	100	1×
5 ng EB011	a	10 pg/µl
barcoding-primer-fw (100 µM)	2	0.4 µM
barcoding-primer-rv (100 µM)	2	0.4 µM
dNTP mix (10 mM)	10	0.2 mM
Nuclease-free water	376−a	
Phusion DNA polymerase (2 U/µl)	10	0.04 U/µl
Total	500	

NOTE: Do not use EB007 here as it has a point mutation in the 3′ITR of the PiggyBac transposon.

Cycle number	Denature	Anneal	Extend
1	95°C 1 min		
2–26 (25 cycles)	95°C 30 s	58°C 30 s	72°C 30 s
27			72°C 1 min

4.2. Amplify using the following PCR program:
4.3. Pool the PCR tubes and run 5 µl on gel, a single band of 200 bp should appear.
4.4. Purify the PCR product with a PCR purification kit, elute in 100 µl nuclease-free water and quantify by nanodrop. Aim for a yield of ~ 6–7 µg.
4.5. Digest the barcoded PCR product with KpnI and BssHII.

Component	Amount (µl)	Final concentration
10x CutSmart buffer	20	1x
pPTK_IPR from step 3.7	A	
BssHII (5 U/µl)	12	0.3 U/µl
KpnI-HF (20 U/µl)	3	0.3 U/µl
Nuclease-free water	165−a	
Total	200	

4.6. Incubate at 37°C for 2 h.
4.7. Purify with a PCR purification kit and elute in 30 µl nuclease-free water and quantify by nanodrop. Aim for a yield of ~4–5 µg.

5. Digestion of the pPTK_IPR for the barcode ligation (**Figure 3A**)—4 h
5.1. Digest the pPTK_IPR (step 3) with KpnI-HF and MluI-HF in the following reaction:

Component	Amount (µl)	Final concentration
10× CutSmart buffer	20	1×
pPTK_IPR from step 3.7	a	
MluI-HF (20 U/µl)	3	0.3 U/µl
KpnI-HF (20 U/µl)	3	0.3 U/µl
Nuclease-free water	174−a	
Total	200	

5.2. Incubate at 37°C for 2 h.
5.3. Purify with a PCR purification kit and elute in 88 µl nuclease-free water.
5.4. Dephosporylate and heat inactivate all of the digested pPTK_IPR using either Quick CIP (NEB #M0525), Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371) or Antarctic Phosphatase (AP) (NEB #M0289) using the supplied protocol.
5.5. Purify the vector with a PCR purification kit, elute in 30 µl nuclease-free water and quantify by nanodrop. Aim for yield of ~1.5–2 µg.

6. Ligation of the pPTK_IPR with the barcoded insert (pPTK_BC_IPR) (**Figure 3A**)—1.5 days
6.1. Prepare the ligation mix on ice as well as a control reaction without insert. The control should be processed in parallel with the real TRIP library until step 7.8.

Component	Amount (µl)	Final concentration
Nuclease-free water	16−a−b	
10× T4 Ligase buffer	2	1×
Purified KpnI-MluI-digested pPTK_IPR vector	A	~15–25 ng/µl
Purified KpnI-BSSHII digested barcoded insert	B	molar ratio of 1:5 vector: insert
T4 DNA ligase (5 U/µl)	2	0.5 U/µl
Total	20	

6.2. Incubate at 16°C overnight.
6.3. Add 80 µl nuclease-free water and heat-inactivate T4 DNA ligase for 10 min at 65°C.
6.4. Purify with a PCR purification kit and elute in 43 µl.
6.5. Prepare a digestion mix to digest any remaining original non barcoded vector:

Component	Amount (µl)	Final concentration
10× CutSmart buffer	5	1×
Purified pPTK_BC_IPR from step 6.4	43	
MluI (10 U/µl)	2	0.4 U/µl
Total	50	

6.6. Incubate for 1 h at 37°C
6.7. Purify the digestion with a PCR purification kit and elute in 50 µl nuclease-free water. NOTE: Include one extra wash step to make sure all remaining salt is removed. Small traces of salt can hinder the subsequent electroporation into bacteria.

NOTE: In case of issues with electroporation, extra bead purification using magnetic beads such as CleanPCR beads eluted in 10 µl can be used.
NOTE: It is important to elute in nuclease-free water, and not TE or EB as the sample might need to concentrated by SpeedVac in step 6.9 and high salt concentrations can affect the electroporation in step 7.1.

6.8. Measure the concentration of DNA by Qubit or Nanodrop spectrophotometer.

NOTE: The total yield should be about ~250–400 ng of DNA.

6.9. Concentrate the DNA in a SpeedVac to reach a concentration of ~50–200 ng/µl in a minimum of 5 µl.

NOTE: the pPTK_BC_IPR DNA can be stored at −20°C indefinitely.

7. Transformation of bacterial cells and preparation of the DSB-TRIP library (**Figure 3B**)—1.5 days
7.1. Electroporate the pPTK_BC_IPR into CloneCatcher DH5α electrocompetent *E. coli* (Genlantis) using an Electroporation machine (Gene Pulser—Bio-Rad or similar) setup: Capacitance = 25 -Set volts to 2.00 (kV)–400 Ω resistance.

NOTE: It is essential that the *E. coli* are highly electrocompetent to generate a high complexity TRIP library. This would be a good starting point for trouble shooting any issues with getting high enough complexity in the libraries.
NOTE: The time constant should be >4 ms. A lower time constant is most likely the result of an arch discharge (spark) and might result in a lower TRIP complexity.

7.2. Immediately add 2 ml recovery medium (that comes with the electrocompetent cells) to the cells and let the cells recover for 30 min at 37°C on a shaker at 400 rpm.
7.3. To estimate the complexity of the pool, make three dilutions the transfected cells using a small fraction of the cells (20 µl) and plate these in three different LB agar plates containing 100 µg/ml ampicillin. We recommend final dilutions of 1:1,000, 1:10,000 and 1:100,000. Incubate these plates at 37°C O.N. Repeat this with the no insert negative control.

NOTE: This will be used to estimate the complexity of the library, count the number of colonies per plate and multiply that by each dilution factor. This will typically range between 1 and 10×10^6, with at least $2–3 \times 10^5$. The control plate should have 50–100 times less cells, indicating minimal contamination (1–2%) of the initial vector.

7.4. Transfer the remaining cells into a sterile flask with 200 ml of LB medium containing 100 µg/ml of ampicillin, incubate at 37°C O.N. with vigorous shaking.
7.5. Collect the cells from the previous step and pellet them by centrifugation at 5,000 *g* for 15 min at 4°C and keep them on ice.
7.6. Purify the plasmid DNA using a plasmid maxi kit following the manufacturer's instructions. Elute the pPTK_BC_IPR library in 600 µl of nuclease-free water.
7.7. Measure the concentration with a Nanodrop spectrophotometer. Standard yield is about 1 µg/µl. NOTE: This plasmid library can be stored at −20°C indefinitely.
7.8. Verify the correct distribution of nucleotides by Sanger sequencing the plasmid library using primer *indelPCR1-rv*, following the recommendations of the Sanger sequencing service used.

NOTE: It is important to see an equal distribution of the nucleotides at the position of the barcode. If the distribution of the four nucleotides is not equal this might mean that the barcode library is biased and might not be as complex as expected (**Figure 3B**).

2.1.2 Creating DSB-TRIP Cell Pools and Clones

8. Transfection of K562-DD-Cas9 cells (**Figure 3C**)—2 h
8.1. Culture a fresh batch of K562-DD-Cas9 in complete RPMI, expand the cells to reach at least 20 million cells for the transfection.
8.2. Plate 9 million cells per plate in 2 10-cm dishes. One plate will be used for the actual library, the second will serve as a mock transfection.

NOTE: For the mock transfection replace the mPB-L3-ERT2.TatRRR-mCherry plasmid with nuclease-free water.

8.3. Prepare the lipofection DNA dilution in OptiMEM as follows:

Component	Amount (µl)	Final concentration (ng/µl)
TRIP library (30 µg; from step 7.7)	*X*	20
mPB-L3-ERT2.TatRRR-mCherry plasmid (5 µg)	*Y*	4
Opti-MEM medium	1,500	

8.4. Prepare the lipofectamine dilution in OptiMEM as follows:

Component	Amount (µl)	Final concentration
Lipofectamine 2000	60	4%
Opti-MEM medium	1,500	

8.5. Incubate both dilutions for 10 min at RT.
8.6. Mix the solutions together and flick the tube to mix the lipofection solution. Incubate for 20 min at RT.
8.7. Add the lipofection solution dropwise to the plates. Incubate the cells at 37°C overnight. Refresh the medium the next morning by centrifuging the cells at 300 g for 5 min at RT and resuspending them in fresh complete RPMI medium.
8.8. Asses the transfection efficiency (mCherry signal) after ~18 h under a fluorescence microscope. The proportion of mCherry-positive cells should be at least 10%.

9. Sorting of the transfected cells (**Figure 3C**)—7 days
9.1. After 18–30 h of transfection, prepare the cells for FACS sorting. Transfer the cells into a 15-ml conical tube and centrifuge for 4 min at 300 g at RT.
9.2. For the transfected cells, aspirate the medium and resuspend the cells in 1.5 ml of PBS supplemented with 1% FBS. Place the cells in a FACS tube on ice and head to the sorting machine.
9.3. Right before the sort, pass the cells through a cell-strainer cap on a FACS tube and install the tube in the machine.
9.4. For the sorting strategy follow step 52 [Nature Protocols (NP):52] in (Akhtar et al., 2014).
9.5. Centrifuge the sorted cells for 4 min at 300 g at RT. And resuspend the cells in warm fresh RPMI medium at approximately 10^5 cells per ml containing 0.5 µM 4-hydroxytamoxifen (4-OHT) to activate the transposase.
9.6. Culture the cells for 24 h and then wash the cells by centrifugation at 300 *g* for 4 min at RT and resuspend them in fresh complete RMPI medium to avoid rehopping of the transposon.

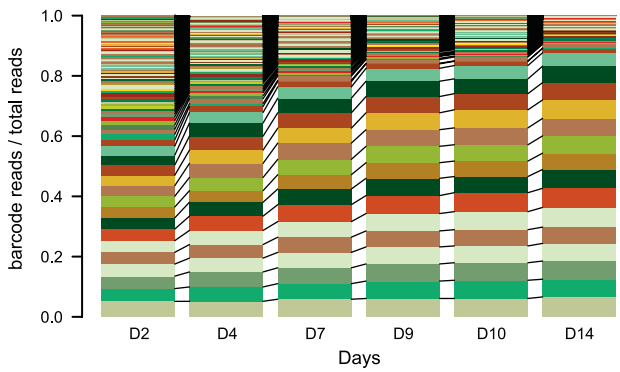

FIGURE 4 | IPR expansion and drift over time. Stacked barplot of the percentage of reads each barcode represents over time (days). Each color represents a different barcode (IPR) that are linked between the plots *N* = 1. Over the time course of the experiment samples were taken from a TRIP cell pool and processed as for scoring indels. The barcodes were retrieved from the indelPCR data, counted and their proportion was plotted per day.

9.7. Continue culturing the cells for ~5–7 days and expand the culture conditions if they become too confluent. Aim for at least 5× the plasmid library complexity (step 7.3) by the end of this period for generating the TRIP pools and clones.

NOTE: This period is required do get rid of the free-floating plasmids in the cells that might cause background rehopping of the transposon.

10. Generate TRIP cell pool and clones. (**Figure 3C**) >7 days
10.1. Passage the cells one final time 24 h at a density of 2–5 × 10^5 cells per ml before the second sort to make TRIP cell pools. This is needed to make conditioned medium for the TRIP cell pools and clones for a higher chance of recovery.

NOTE: We recommend collecting 12 ml conditioned medium per 96-well plate and 25 ml for a full 24 well plate for the cell pools.

10.2. To prepare the cells for the FACS sort to make TRIP cell pool, collect the cells and pellet them by centrifugation at 300 *g* for 4 min at RT. Collect the medium from the cells and filter it through a 20 µm sterile filter. Prepare the collection plates. Distribute 1 ml per well in a 24-well plate for the pools and 100 µl per well in two 96-well plates for the clones. Keep the plates at 37°C until the sort.
10.3. Resuspend the cells in PBS supplemented with 1% FBS and place them on ice until the sort. Right before the sort, pass the cells through a cell-strainer cap on a FACS tube and install the tube in the machine.
10.4. Sort live cells to generate pools with different starting number of cells, this will determine the complexity of the pool. The type of following experiment will determine if higher or lower complexities are required. Therefore, we recommend sorting cells in pairs with different starting number (500–10,000).
10.5. Sort single live cells into the 96-well plates to directly generate clones.

NOTE: in our experience, we obtain ~10–20 fit and healthy clones from one 96-well plate.

10.6. Collect the left-over cells as a backup, culture them for one more day to recover and cryo-store them in complete RPMI medium +10% FBS.
10.7. Expand the TRIP cell pools and clones and prior to cryo-storage collect ~10^6 cells for integration counting and mapping.

NOTE: It can take more than 3 weeks before the clones reach at least 10^6 cells and they might grow at different rates.

10.8. To measure the average number of integrations per cell, we suggest two options:
a) By qPCR: follow the Box 2 in (Akhtar et al., 2014).
b) If clonal lines were generated from the TRIP cell pool, the number of integrations can be estimated by high throughput sequencing of barcodes in each clone.

When clones are randomly selected, these clones should give a fair representation of the number of integrations per cell.

11. Mapping the IPRs (**Figure 3D**, i,ii)—5 days
    11.1. Collect genomic DNA (gDNA), by using a standard genomic DNA extraction kit, from TRIP cell pools and/or clones to obtain about 1 µg of gDNA for the clones and 6 µg of gDNA for the pools.
    11.2. For the mapping of the pools and the clones by inverse PCR, follow steps NP:83–105 from (Akhtar et al., 2014) with the following modifications:
        a) We recommend setting up the DpnII digestion twice (step NP:87) in case of low yield after purification or a failed PCR step.
        b) The ligation purification by precipitation at step NP:89–91 can also be done by bead purification at a beads:sample ratio of 1.5:1 following the manufacturer's instructions.

2.1.3 DSB Induction and Repair

NOTE: The required number of cells will differ depending on the complexity of TRIP integrations in the cell pools. Aim for at least 1,000× of the pool's starting number of cells prior to nucleofection. This aims to correct for cell death after nucleofection and to have sufficient coverage per IPR for the library preparation. E.g., 2×10^6 cells for a cell pool of 2000 starting cells (TRIP-2000 pool).

NOTE: Do not culture the pools for too long. After a lot of culturing, the complexity of the TRIP cell pools can decrease as fast cycling cells will take over most of the reads in the pools within two to 3 weeks (**Figure 4**).

NOTE: Sequencing the whole pool without damage induction might help understanding the total complexity (number of different barcodes) and the barcode distribution. If the barcode distribution is strongly skewed toward more abundant ones, we recommend increasing the starting material during the library prep as well as increasing the sequencing depth. This will have to be optimized for every specific library and experimental setup.

12. sgRNA plasmid transfection, Cas9 activation and cell collection—3 days

For the K562-DD-Cas9 TRIP-2000 pool:

    12.1. Collect all the cells from a high density 10 cm dish in a 15 ml falcon and count the cells using a cell counter.
    12.2. Transfer four million cells into a new 15 ml falcon and centrifuge at 300 g for 4 min at RT. Two million for the treated cells and 2 million for the no-guide transfection control.
    12.3. During this time prepare the transfection buffer: add 8 µl of 100 mM ATP to 100 µl of homemade Amaxa buffer (reagent setup) per sample.
    12.4. Prepare two Eppendorf tubes for the plasmids to transfect. Add 2 µg of sgRNA plasmid to one tube and 2 µg of GFP expressing plasmid to the other tube.

NOTE: The quantity of sgRNA plasmid can be optimized by transfecting different amounts of sgRNA plasmid and checking for indel frequencies by TIDE in the parental cell line or one of the TRIP cell pools while the other clones and pools are growing.

    12.5. Remove the supernatant and resuspend the cells in 200 µl of complete transfection buffer.
    12.6. For each transfection reaction, take 100 µl of cell suspension and mix it with the plasmid in the tube and directly transfer the whole volume into a nucleofection cuvette.
    12.7. Nucleofect the cells with program T-016 on an Amaxa 2D Nucleofector.
    12.8. Immediately add 900 µl of complete RPMI medium to each cuvette. And then, using a small Pasteur pipet, transfer the cells to a new 10 cm dish with fresh complete RPMI.
    12.9. Let the cells recover from the transfection for ~16 h.
    12.10. Add 500 nM Shield-1 to the cells to activate Cas9.
    12.11. Mix by swirling the plate and place the plate in the incubator.
    12.12. Collect 2×10^6 cells in a 15 ml falcon at the desired time points, typically 72 h for the endpoint of the cutting and repair reaction.
    12.13. Centrifuge the cells at 300 g for 4 min at RT and remove the supernatant.
    12.14. Extract gDNA by using a genomic DNA extraction kit following the manufacturer's instructions.

2.1.4 High Throughput Sequencing Libraries

13. Library preparation for the indel detection (**Figure 3D**, iii)—1 day

NOTE: It is advised to check for total indel frequency by *TIDE* prior to library preparation (Brinkman et al., 2014; Brinkman and van Steensel, 2019).

    13.1. Prepare the indelPCR1 mix as follows:

Component	Amount (µl)	Final concentration
gDNA—200–500 ng	*x*	4–10 ng/µl
MyTaq™ Red mix 2×	25	1×
indelPCR1-fw-indexed 10 µM	0.5	100 nM
indelPCR1-rv 10 µM	0.5	100 nM
Nuclease-free water	24—*x*	
Total	50	

NOTE: A complex pool will require more starting material than clones or less complex pools. Considering a mainly triploid K562 genome (Zhou et al., 2019), 100 ng of gDNA represents about 10^4 K562 cells. For complex pools aim for at least a 50× coverage of the starting number of cells ($50 \times 2{,}000 = 10^5$ cells; so ~1 µg of gDNA). This should be sufficient to recover most of the IPRs in a reproducible manner as we account for some cell death during the pool expansion (not all 2,000 cells will survive) and during standard culturing. In our case we will run this in triplicates to increase the coverage of our TRIP-2000 pool.

NOTE: The indexed PCR primers allow sample multiplexing for next generation sequencing. The indices from the indelPCR1-fw-

indexed primer count as internal barcodes and will not be automatically demultiplexed by the Illumina sequencer.
NOTE: Important considerations to take when mixing indices. Pick a varied set of indices, if possible, avoid mixing indices with only a couple of mismatches. Avoid indices starting with or containing two guanines (Gs) in a row, especially for NovaSeq and NextSeq machines from Illumina. The Gs are not illuminated in these machines and therefore are prone to mistakes if they are at the start of the read.

13.2. Run the indelPCR1 with the following conditions.

Cycle number	Denature	Anneal	Extend
1	95°C 1 min		
2–4 (3 cycles)	95°C 15 s	58°C 15 s	72°C 10 s
4–13 (10 cycles)	95°C 15 s	68°C 15 s	72°C 10 s
14			72°C 1 min

13.3. Prepare the indelPCR2 mix as follows:

Component	Amount (µl)	Final concentration
indelPCR1 product	5	
MyTaq™ Red mix 2×	25	1×
indelPCR2-fw 10 µM	0.5	100 nM
indelPCR2-rv-indexed or indelPCR2-rv 10 µM	0.5	100 nM
Nuclease-free water	19	
Total	50	

NOTE: Depending on the complexity of the library, indelPCR2-rv can be used with or without an index (i7).

13.4. Run the indelPCR2 with the following conditions

Cycle number	Denature	Anneal	Extend
1	95°C 1 min		
2–4 (3 cycles)	95°C 15 s	58°C 15 s	72°C 10 s
4–13 (10 cycles)	95°C 15 s	70°C 15 s	72°C 10 s
14			72°C 1 min

13.5. Run 10 µl of indelPCR2 on a 1% agarose gel, a band should be visible around 280 bp.
13.6. Pool ~10 µl of the samples based on the band intensity to approximately match the concentrations.
13.7. Purify the library by bead purification following the manufacturer's instructions at a bead:sample ratio of about 0.8:1, to keep only fragments >200 bp.
13.8. Measure the concentration using a Qubit dsDNA HS Assay Kit on a Qubit Fluorometer.
13.9. Sequence the library with the aim to obtain about four million reads per pool, single-end, 150 bp read length. NOTE: For a TRIP cell pool it is recommended to aim for at least a median 2000 reads per IPR per sample. For a trip clone, ~500 reads per IPR per sample is sufficient.

2.2 Bioinformatics Pipeline

2.2.1 Setting up the Environment for the Snakemake Pipeline (Figure 3E)

14. Downloading the scripts—10 min

NOTE: all code below is run in the *linux* shell from the terminal.

14.1. To successfully run the *Snakemake* pipeline we recommend to download the DSB-TRIP *github* repository (https://github.com/vansteensellab/DSB_TRIP_protocol):

```
git clone https://github.com/vansteensellab/DSB_TRIP_protocol
```

15. Setting up the conda environment—variable
15.1. Install the *conda* environment 'DSB_TRIP_env.yml' that is supplied with the repository

```
conda env create -f config/DSB_TRIP_env.yml
```

15.2. Activate the *conda* environment

```
source activate dsb_trip
```

16. Download extra dependencies to lib folder—10 min

```
./download_dependencies.sh
```

17. Generating Bowtie index for reference the genome—2 h
17.1. Download the reference genome of interest (e.g., hg38).
17.2. Build a *Bowtie* index:

```
bowtie2-build </path/to/genome.fa.gz> </path/to/index>
```

18. Configure the pipeline—10 min
18.1. The example configuration file provided in the *GitHub* repository can be adapted to fit the users' specific requirement. This could be a matter of changing some local paths (e.g., the path to the *Bowtie* index).

2.2.2 Running the Pipeline

19. Running the Snakemake pipeline—1–4 h (depending on the amount of data and number of cores used)
    19.1. Run the DSB_TRIP *Snakemake* pipeline with 20 cores:

```
snakemake –s src/dsb_trip.snake \
--configfile <path/to/config.yaml> \
--use-conda -j 20
```

20. Calculate pathway balance and link to IPR location in genome (detailed below in the *Results* section)—1 h
    20.1. Select barcodes with unique location
    20.2. Filter out lowly abundant barcodes
21. Downstream analysis (detailed below in the *Results* section)—8 h
    21.1. Calculate pathway balance measures (e.g., ratio's/proportions)
    21.2. Correlate the pathway balance measure of interest with chromatin information available (e.g., chromatin states/ChIP signal). This last step is outside the scope of this paper.

3 DSB-TRIP PIPELINE

The main functionality of the DSB-TRIP pipeline is to extract the integration site for every barcode from the iPCR data, and the indel data for every barcode from the indel PCR. With the correct configuration it is able to give easily interpretable results.

3.1 Pipeline Configuration

The DSB-TRIP pipeline is written as a *Snakemake* pipeline (Molder et al., 2021). Two files are required for successful execution: 1) a meta-data file with information on the samples, and 2) a pipeline configuration file with pipeline parameters.

1. Meta-data file

A tab-delimited file with information on the samples: sample name, file location, type of PCR (e.g., iPCR, indelPCR), and plasmid guide information.

2. Configuration file

The configuration file determines the DSB-TRIP pipeline execution. Parameters include but are not limited to: description of the read structure, thresholds used in the pipeline, information on the reporter for DNA scar analysis, and alignment parameters.

3.2. Pipeline Process

The DSB-TRIP pipeline consists of several modules which will perform the following four tasks: pre-processing of the sequencing reads, barcode clustering, genomic alignment of the iPCR reads and scar analysis of the indel PCR reads.

1. Barcode retrieval

First, the barcode is extracted from iPCR and the indel reads. This implementation is based on *Cutadapt* (version 1.11) (Martin, 2011) and custom scripts. For both read types, a 16 bps barcode is positioned within a constant 5′ adapter sequence: "GTCACAAGGGCCGGCCACAA{barcode}TGATC". The barcode is extracted using a three-step strategy: 1) matching of the entire sequence, 2) matching of the 5′ sequence, 3) matching of the 3' sequence. This approach ensures accurate retrieval of barcodes shorter or longer than 16 bps (15 or 17 bp barcodes can be present). Only reads with successful barcode retrieval are selected for further processing. This barcode retrieval is performed on read 1 of the iPCR paired-end sequencing reads. An additional constant sequence is required for read 2 of the pair. Finally, the reverse complement of these constant patterns are removed from the 3′ end of the reads if present due to read-through into the backbone.

2. Barcode clustering

The retrieved barcode sequences are classified as "genuine" or "mutated" using the *starcode* clustering algorithm (Zorita et al., 2015). Barcodes are clustered based on Levenshtein distance (<2) and the most abundant barcode in the cluster is considered the "genuine" barcode. Currently this step is used as a filtering step, but alternatively, barcodes can be rescued by assigning all information from the mutated barcodes to the genuine barcode.

3. Reporter alignment

Remaining iPCR sequences are aligned to the human genome with *Bowtie 2* (Langmead and Salzberg, 2012). This is carefully done to deal with potential chimeric reads created by the circular ligation [as shown in (Akhtar et al., 2014) Supplementary Figure 1]. For such reads, two pieces of genomic DNA are ligated at an unknown junction. Briefly, to remove these events, two sequential alignment steps are performed. First, the read pairs are mapped independently and the mates reading directly into the genome from the edge of the transposon are filtered based on potential soft-clip at the 5' end. These reads are either discarded, or if >16 bps soft-clipping occurred, the clipped sequence is extracted and realigned to the genome in a second alignment step. This ensures that in case of chimeric reads, the genomic sequence found next to the integrated reporter is aligned instead of some distant genomic sequence that got inserted at the circular ligation step. Reads that pass the quality thresholds are aggregated based on their barcode sequence. For every barcode, read counts and mapping quality for the two locations with most supporting reads are returned. This allows custom filtering to select trustworthy integration sites.

4. DNA scar analysis

The DNA scar analysis tests for two points: the presence of the wild-type sequence and the size of a possible indel compared to the original sequence. First, reads are matched against known

recognition sequence(s), made up of the wild-type sequence and optionally, any number of expected introduced mutations. Second, the location of specific downstream sequences is compared with their location in the wild-type fragment to calculate an indel size. In our case, these are seven 6 bps sequences that are originally are positioned between 83 and 124 bps from the start of the sequence (**Figure 3D**, iii). This approach allows us to pick up the complete range of deletion sizes that can be captured by the PCR performed and deal with potential read errors or mutations downstream of the break site.

When a recognition sequence is found, the call will be named after the recognition sequence ("wt" for wild-type sequence, or in case of a directed mutation, the name given in the *configuration file*). When no recognition sequence is found, the call depends on the indel size, this call will be "del", "wt_point_mut" or "ins" for indel sizes *<0*, *0*, *>0*, respectively. When no recognition sequence or indel size can be calculated, the call will be "not_clear". The indel size will be calculated and reported even if in the first step a recognition sequence was found. This way, some reads might be given a "wt" call, but an indel size other than 0 (e.g., −1). This can occur due to either a technical artefact or biological mutation outside of the Cas9 targeted mutagenic event of interest.

Two alternatives to this scar analysis are implemented: 1) pairwise alignments that capture the exact DNA scar, 2) direct comparisons of the DNA scars with expected scars as predicted by *inDelphi* or *FORECasT* (https://partslab.sanger.ac.uk/FORECasT) (Allen et al., 2018; Shen et al., 2018). Both of these options will allow more fine separation of repair scars at the cost of significantly longer processing time. This allows for more detailed analysis such as separating MMEJ scars from NHEJ scars that resulted in the same size deletion.

4 RESULTS

4.1 Pipeline Output

The DSB-TRIP pipeline creates various output files and log files with details on pipeline execution. Output is organized following the four steps described previously.

1. Barcode retrieval

The statistics file for every sequencing sample is produced and includes for each read all the intermediate steps in the barcode retrieval analysis. This information is useful to identify and solve read parsing issues.

2. Barcode clustering

The barcode clustering returns a table with three columns containing barcodes: one column with clustered barcodes, the second with the most prominent within the cluster (we call this the "genuine" barcode), and one column with the total number of occurrences of barcodes within the cluster. The two columns containing barcodes are used to filter intermediary files for iPCR and indel reads that only the rows with "genuine" barcodes are left. The total cluster count is ignored by the pipeline, but can be used to assess how well this barcode clustering performed.

3. Reporter alignment

For each iPCR sample two tables are generated (we call these the "mapping tables") containing barcode locations and quality measures, one for each mate of the paired end reads. The P7 read (read 2, file ending in "2.table") is most important since that read contains the part of the sequence starting at the end of the transposon (**Figure 3D, ii**). The P5 read (read 1, file ending in "1.table") is partly made up of the barcode and surrounding transposon sequence, there is still some genomic DNA left in the sequence which should be aligned at a nearby GATC side where DpnII cut the DNA before circularization. The table of this mate in the read pair can be used as quality control.

4. DNA scar analysis

The ".count" files generated by the scar analysis contain a four column table with the total number of reads per combination of barcode, mutation call and indel size produced by step 4 of the pipeline (see above).

4.2 Pipeline Evaluation

Finally, the various pipeline output files can be used to evaluate the DSB-TRIP experiment. We advise several quality controls and downstream filtering steps, as described below:

- The first thing to do is to select the barcodes which give an accurate and unique location. We advise to select all barcodes which are supported by at least 5 iPCR reads with an average mapping quality larger than 10 at the primary location, having at least 95% of the reads located at this locus, with not more than 2.5% of the reads at a secondary location. These ratio's and averages can be calculated from the read counts and sum of mapping quality found in the "mapping table" (file ending in ".2.table", see above). This will remove barcodes with multiple alignment locations either because of technical reasons such as repetitive sequences or because a single barcode is integrated in multiple locations.
- The second step to see if the pipeline was correctly configured and that the data is of good quality is to check whether repair scars were correctly identified. There are three sequence calls which can be used as indicators for bad quality/incorrect pipeline configuration: "not_clear" calls, "wt_point_mut" calls and wild-type ("wt") calls with non-zero indel size. All these calls should be minor, since they indicate situations which are not expected to result from normal repair events. The "not_clear" calls include reads that failed both the recognition sequence search as well as the indel calculation. The "wt" calls are expected to be made for reads of indel size 0, therefore, "wt" calls with non-zero indel size and indel size of 0 without "wt" call ("wt_point_mut") should be rare.
- We recommend to filter out the "not_clear" calls. In previous analysis we had a mean of 17.3% (95% CI:

16.7–17.8%) of "not_clear" calls that were filtered out this way. In this analysis we left in the "wt_point_mut¨ and wild-type calls that didn't have an indel size of 0. A different option is to remove these from further analysis. However, the previous evaluation step should have indicated these events to be of minor impact on the resulting indel ratios.

- After initial filtering, when working with a TRIP cell pool, barcodes can be filtered on abundance. Since barcodes in a pool are found in a limited number of cells, it is important to assess whether a barcode is represented by enough cells to obtain an accurate measurement. One way to filter for abundance is to estimate, for each barcode, the number of cells in which this barcode can be found. In (Schep et al., 2021) this was done by assuming that, for each experiment, there are approximately 100,000 cells (based on the amount of DNA used) and every cell has on average 6 barcodes (based on the clonal selection). For each barcode an approximate cell number can be calculated summing up its count for all mutation calls, dividing over the total count of the experiment and multiplying this by 600,000. In (Schep et al., 2021) only barcodes were considered that were represented in at least 50 cells estimated by this approximation.

4.3 Downstream Analysis

After quality control and filtering, indel ratios can be calculated. In (Schep et al., 2021) this was done by normalizing each replicate over library size and biological replicates were averaged. The frequency of each indel type as proportion of total reads was calculated on that average. Pathway frequency per IPR was calculated as a proportion of the specific mutation over all indels (excluding wild-type sequences).

It is important to consider that wild-type (wt) sequences can be of various origins during the analysis. They can be either uncut or perfectly repaired by HR, NHEJ or another pathway. Additionally, wt sequences could undergo cycles of cutting and repair as long as they are repaired perfectly. However, we previously estimated that perfect repair of this reporter sequence is rare (Brinkman et al., 2018). As many factors can affect the abundance of the wt sequence in the cells, it is crucial to not overinterpret these results and take cell viability, transfection efficiency and various repair pathway activities into account.

The next step is to classify indels in pathways. Schep et al., 2021 used multiple gRNA target sites within the LBR gene, the main one was termed "LBR2" ("LBR1" in Brinkman et al., 2014) (Brinkman et al., 2014; Brinkman et al., 2018; Schep et al., 2021). This target site was used since it had a predictable repair outcome with very prominent outcomes for MMEJ events (7 bp deletions, flanked by 3 bp microhomologies) and NHEJ events (1 bp insertions). 7 bp deletions were classified as MMEJ, and 1 bp insertions were classified as NHEJ. Schep et al., 2021 focused on the balance between these outcomes (Brinkman et al., 2014; Brinkman et al., 2018; Schep et al., 2021). This classification can easily be adapted for different guides, when clear microhomologies and NHEJ outcomes are known. To discover which indels are caused by the NHEJ pathway, in Schep et al., 2021 the M3814 inhibitor was used (Schep et al., 2021). Indels with an indel ratio of at least 0.01 in the DMSO setting and a significant decrease (adjusted p-value < 0.05) in the M3814 inhibitor setting (one-sided Wilcoxon test) were classified as NHEJ. Alternatively, for guides that result in a broader spectrum of multiple mutations, a pipeline setting can be used to detect specific MMEJ events. This alternative pipeline setting performs pairwise alignments with the wild-type sequence was used, resulting in more detailed scar information at the cost of extra computational time. Adapting this strategy for LBR2, other microhomologies can be identified. These include a 14 bp deletion with a 3 bp microhomology and a 22 bp deletion with a 6 bp microhomology.

After mutation counts are separated by MMEJ and NHEJ (and unclassified) events, different calculations can be made for pathway balance and mutational rates. To calculate basic mutational rates a division over total indel can be a simple measure. For comparison in pathway balance between NHEJ and MMEJ, a relative pathway proportion can be calculated using: $\frac{MMEJ}{(MMEJ+NHEJ)}$.

5 MATERIALS AND METHODS

5.1 Sequences

1. DSB-TRIP plasmids (sequences available at https://osf.io/k2fwt/files/)
   a. EB007
   b. EB011 (EB011_pPTK-P.CMV.584-eGFP-trim1-PI04_mutated)
   c. pPTK-BC-IPR (GenBank: MW408732)
   d. EB032_pBKS-sgRNA
   e. EB032_pBKS-sgRNA-LBR2
2. Oligos

Name	Number	Sequence (5′ -> 3′)
barcoding-primer-fw	TAC0003	ACTGATCATGGGTACCGATCA**(N)16**TTGTGGCCGGCCCTTGTGACCTGCA
barcoding-primer-rv	TAC0004	AAAAGCGCGCATACTAGATTAACCCTAGAAAGATAATCATATTG
TIDE_endo_LBR_F	TAC0017	GTAGCCTTTCTGGCCCTAAAAT
TIDE_endo_LBR_R	TAC0018	AAATGGCTGTCTTTCCCAGTAA
indelPCR1-fw-indexed	TAC0007.1–24	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**(N)10**GTCACAAGGGCCGGCCACA
indelPCR1-rv	TAC0012	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
indelPCR2-fw	TAC0009	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
indelPCR2-rv	TAC0011	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
indelPCR2-rv-indexed	TAC0159.1–96	CAAGCAGAAGACGGCATACGAGAT**(N)6**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Sanger_map_IPR_F	TAC0065	ATGCTAGCGTGACTGGAGTT

5.2 Reagents

5.2.1 *In Vitro* Applications

1. Nuclease-free water
2. Agarose MP (Roche Cat#: 11388991001; brand not critical)
3. Ethidium bromide (EtBr; Sigma-Aldrich, Cat#: E8751; brand not critical)
4. GeneRuler 100 bp Plus DNA Ladder (ThermoFisher, Cat#: SM0321)
5. 50× TAE (Lonza, cat. no. 51216; brand not critical)
6. MyTac Red Mix 2× (Bioline—Cat#: BIO-25044; brand not critical)
7. NheI-HF® (New England BioLabs—Cat#: R3131S/L—20 U/μl)
8. KpnI-HF® (New England BioLabs—Cat#: R3142S/L—20 U/μl)
9. BssHII (New England BioLabs—Cat#: R0199S/L—5 U/μl)
10. MluI-HF (New England BioLabs—Cat#: R0198S/L—20 U/μl)
11. Ampicilin (brand not critical)
12. LB-Agar (brand not critical)
13. Culture dishes, 10 cm (brand not critical)
14. Lysogeny broth (LB; brand not critical)
15. Shrimp Alkaline Phosphatase (New England BioLabs—Cat#: M0371S—1 U/μl)
16. T4 DNA ligase (Roche Cat#: 10799009001—5 U/μl)
17. Exonuclease I (New England BioLabs—Cat#: M0293S)
18. dNTP Mix (Bioline—Cat#: BIO-39043; brand not critical)
19. Phusion® HF DNA Polymerase (New England BioLabs—Cat#: M0530L)
20. CleanPCR Beads (CleanNA—Cat#: CPCR-0500)

5.2.2 Tissue Culture

1. ddCas9-K562 cell line (our lab)
2. Tissue culture medium, for K562: RPMI 1640 (GIBCO—Cat#: 21875034)
3. Fetal Bovine Serum (Sigma—Cat#: F7524)
4. Penicillin-Streptomycin (5,000 U/ml) (GIBCO–Cat#: 15070063)
5. PBS
6. T-75 tissue culture flasks
7. 10 cm tissue culture dishes
8. 15 ml falcons
9. 50 ml falcons
10. Lipofectamine 2000 (Invitrogen—Cat#: 11668019)
11. Tamoxifen (resuspended in DMSO at 1 mM; Sigma-Aldrich—Cat#: T5648)
12. Dimethyl sulfoxide (DMSO; Sigma—Cat#: D4540)
13. 50 ml seringe
14. Sterile 0.22 μM filter
15. KH_2PO_4 (e.g., Sigma-Aldrich—Cat# P5655)
16. $NaHCO_3$ (e.g., Sigma-Aldrich—Cat# S5761)
17. $MgCl_2$ (e.g., Sigma-Aldrich—Cat# M8266)
18. Glucose (e.g., Sigma-Aldrich—Cat# G7021)
19. ATP 100 mM (Thermo Scientific—Cat#: R0441)
20. Shield-1 (resuspended in 100% Ethanol at 500 μM; Aeobius—Cat#: AOB1848)

5.2.3 Bacterial Strains

1. CloneCatcher DH5α electrocompetent *E. coli* (Genlantis—Cat# C810111)
2. JM109 competent cells (Promega—Cat#: L2001)

5.2.4 Commercial Kits

1. PCR Isolate II PCR and Gel Kit (Bioline, Cat#: BIO-52060; brand not critical)
2. PureLink™ HQ Mini Plasmid DNA Purification Kit (ThermoFisher, Cat#: K210001; brand not critical)
3. PureLink™ HiPure Plasmid Filter Maxiprep Kit (ThermoFisher, Cat#: K210017; brand not critical)
4. Lipofectamine 2000 (Invitrogen, Cat#: 11668019)

5.2.5 Hardware

1. NanoDrop spectrophotometer (ThermoFisher) or equivalent
2. DNA SpeedVac (New Brunswick Scientific) or equivalent
3. PCR thermocycler (Bio-Rad) or equivalent
4. Microfuge centrifuge (Eppendorf) or equivalent
5. Vortex (VWR) or equivalent
6. Thermomixer (Eppendorf) or equivalent
7. Gel apparatus for electrophoresis (Bio-Rad) or equivalent
8. Gene Pulser™ electroporator with the Capacitance Extender and Pulse Controller modules (Bio-Rad) or equivalent
9. Cell incubator, CO2 (5%) (Thermo Scientific) or equivalent
10. TC20 automated cell counter (Bio-Rad) or equivalent
11. Amaxa 2D Nucleofector (Lonza)
12. MoFlo® Astrios™ Cell Sorter equipped with 561 nm laser (Beckman Coulter) or equivalent

5.2.6 Software

NOTE: All computational software except for *inDelphi* and *SelfTarget* comes with the conda environment as described above. A script is provided on the GitHub to download these additional dependencies.

1. Conda v4.10.3
2. Bowtie2 v2.3.4
3. Samtools v1.5
4. Cutadapt v1.9.1
5. Starcode v1.1
6. inDelphi
7. FORECasT
8. R version 3.6.3 (2020-02-29)

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://osf.io/k2fwt.

Plasmids and cell lines generated in this study are available upon request. Plasmid maps and example data is available at: https://osf.oi/k2fwt/. Code is available at: https://github.com/vansteensellab/DSB_TRIP_protocol.

AUTHOR CONTRIBUTIONS

RS, EB, and BvS conceived and designed the study. RS and EB performed the experiments. CL wrote computational pipeline with input from TvS, RS, and CL worked on figure preparation. BvS performed supervision and project management. RS, CL, and BvS wrote manuscript with contributions from all authors.

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Genomic Reporter Constructs to Monitor Pathway-Specific Repair of DNA Double-Strand Breaks

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Repair of DNA Double-Strand Breaks (DSBs) can be error-free or highly mutagenic, depending on which of multiple mechanistically distinct pathways repairs the break. Hence, DSB-repair pathway choice directly affects genome integrity, and it is therefore of interest to understand the parameters that direct repair towards a specific pathway. This has been intensively studied using genomic reporter constructs, in which repair of a site-specific DSB by the pathway of interest generates a quantifiable phenotype, generally the expression of a fluorescent protein. The current developments in genome editing with targetable nucleases like Cas9 have increased reporter usage and accelerated the generation of novel reporter constructs. Considering these recent advances, this review will discuss and compare the available DSB-repair pathway reporters, provide essential considerations to guide reporter choice, and give an outlook on potential future developments.

Keywords: genomic reporter constructs, double-strand break repair pathway choice, homologous recombination, end-joining, single-strand annealing

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INTRODUCTION

The integrity of our genome is constantly challenged by DNA damaging lesions that arise during normal cell growth and division, and are caused by exposure to environmental mutagens and irradiation (Hoeijmakers, 2009). A particularly toxic lesion is the DNA Double-Strand Break (DSB), which separates a chromosome into two pieces and can thus cause detrimental karyotypic alterations. Detection of a DSB initiates an elaborate signaling response that halts the cell cycle, re-shapes the chromatin environment and recruits repair factors (Smeenk and van Attikum, 2013; Dantuma and van Attikum, 2016). The subsequent repair is performed by one of multiple repair pathways that are mechanistically distinct and range from error-free to highly mutagenic (**Figure 1A**; Scully et al., 2019).

In human cells the majority of DSBs are repaired by classical Non-Homologous End-Joining (c-NHEJ), which requires no or very little (≤4 nucleotides) homology at the DSB-ends to ligate them together (**Figure 1A**; Pannunzio et al., 2018). Repair by c-NHEJ can be either error-free, or introduce small insertions or deletions (InDels) at the break junction due to DSB end-processing by nucleases and polymerases. Alternatively, DSBs can be repaired by Homologous Recombination (HR), which is initiated by extensive nuclease-mediated resection of the DSB-ends to generate 3' single strand overhangs (**Figure 1A**; Jasin and Rothstein, 2013). These overhangs invade homologous double-stranded DNA and prime polymerase-mediated extension. HR can then progress via several sub-pathways, as discussed in detail elsewhere in this special issue (Elbakry and Löbrich, 2021). In the dominant sub-pathway, the extended

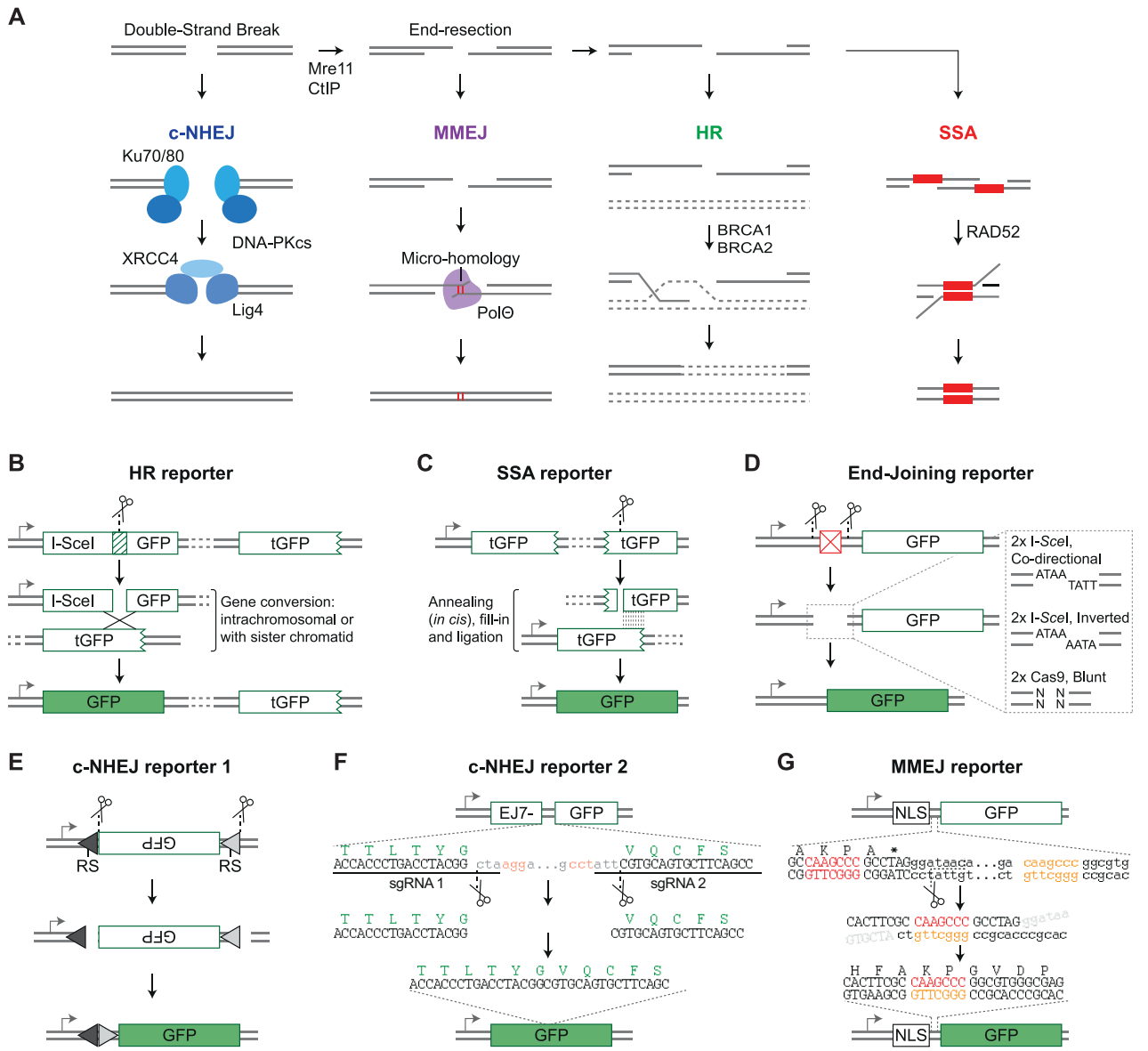

FIGURE 1 | Fluorescent DSB-repair reporter systems. **(A)** Schematic diagram of the four double-strand break repair pathways and their main components. See main text for details. **(B)** DR-GFP (Pierce et al., 1999) is shown as an example HR reporter. Arrow = promoter, I-*Sce*I GFP is a GFP gene disrupted by insertion of an I-*Sce*I target site, tGFP = truncated GFP. **(C)** SA-GFP (Stark et al., 2004) is shown as an example SSA reporter. **(D)** Structure of a generic End-Joining reporter. Expression of GFP is prevented by a sequence element between the gene and the promoter (red box), but can be restored by nuclease-induced excision of this element followed by end-joining mediated repair of the distal DSB ends. The dashed-line box shows a zoom of the DSB ends which can be ligation compatible or non-compatible, depending on the nature of the nuclease and orientation of the target site. **(E)** Structure of pMX-INV (Bredemeyer et al., 2006), which is a VDJ recombination based c-NHEJ reporter specifically used in B-cells. It is cut by RAG nucleases, RS = Recombination Signal. **(F)** Structure of EJ7-GFP (Bhargava et al., 2018), which reports on error-free c-NHEJ. GFP amino acid sequence is depicted in green, sgRNA PAM sequences in red. Dots indicate omitted sequence. **(G)** EJ2-GFP (Bennardo et al., 2008) is shown as an example MMEJ reporter. Designed microhomology sequences are shown in red and orange, dots indicate omitted sequence.

overhang anneals to the opposite DSB-end, and remaining single-stranded (ss) DNA gaps are closed. The genetically identical sister chromatid is the preferred repair template, and therefore HR is considered a high-fidelity repair pathway. However, other homologous sequences, either on nearby chromosomal DNA or on ectopically provided DNA, can also be used as repair template, even if homology is incomplete. In the latter case, HR can lead to sequence alterations at the repaired locus, which is known as gene conversion.

In addition to HR, DSB end-resection can prime repair by either Single-Strand Annealing (SSA) or by Micro-Homology Mediated End-joining (MMEJ), which is also known as alternative End-Joining or Theta-mediated End-joining

(**Figure 1A**; Bhargava et al., 2016; Sallmyr and Tomkinson, 2018; Schimmel et al., 2019). During repair by both pathways, the opposite DSB-ends are joined by annealing of homologous sequence stretches, followed by nuclease-mediated removal of the non-homologous ssDNA ends. MMEJ requires short regions of microhomology (<20 nucleotides) adjacent to the DSB ends, whereas SSA depends on homologous repeats of at least 50 nucleotides long and can occur even if these are separated by distances up to 28 kilobases (Mendez-Dorantes et al., 2018; Kelso et al., 2019). Both pathways are mutagenic, but particularly SSA can result in large deletions.

Which pathway is employed to repair a given DSB is dependent on many factors including sequence context, chromatin environment, and cell cycle stage (Scully et al., 2019). Engagement of the appropriate repair pathway is essential for efficient genome maintenance, whereas an imbalance in pathway choice can have pathological consequences, including cancer development (Knijnenburg et al., 2018). This knowledge has driven studies on the fundamentals of DSB-repair for decades, but recently this research interest has grown exponentially due to the development of genome editing approaches using Cas9 and Cas9-like nucleases (Komor et al., 2017; Knott and Doudna, 2018). Understanding the determinants that direct repair of a Cas9-induced DSB towards a specific pathway is crucial to predict editing outcome, and to identify methods to control this outcome (Yeh et al., 2019). Notably, advances in genome editing techniques have not only spiked DSB-repair research, but also stimulated the development of methods to study it. This includes genomic DSB-repair reporter constructs, which have been essential tools in DSB-repair research by providing an easy and quantitative read-out for repair pathway activity (Gunn and Stark, 2012). Given these new developments, this review will discuss and compare the traditional and more recently published genomic DSB-repair reporter constructs.

TABLE 1 | Overview of fluorescent DSB-repair reporters. HR = Homologous Recombination, SSA = Single-Strand Annealing, c-NHEJ = classical Non-Homologous End-Joining, MMEJ = Microhomology-Mediated End-Joining, fs = frameshift, HITI = Homology Independent Targeted Integration, CD = Co-Directional orientation, Inv = Inverted orientation. "HR (templated)" indicates the requirement of an ectopically delivered repair template to detect repair by HR.

Reporter	Nuclease	# of pathways	Pathway(s) analyzed	Reference
DR-GFP	I-*Sce*I	1	HR	Pierce et al. (1999)
HR-Reporter	I-*Sce*I (2x, Inv)	1	HR	Mao et al. (2007)
pGC	I-*Sce*I	1	HR	Mansour et al. (2008)
SA-GFP	I-*Sce*I	1	SSA	Stark et al. (2004)
RMD-GFP	Cas9 (2x)	1	SSA	Mendez-Dorantes et al. (2018)
NHEJ-C	I-*Sce*I (2x, CD)	1	All distal end-joining	Seluanov et al. (2004)
EJ5-GFP	I-*Sce*I (2x, CD)	1	All distal end-joining	Bennardo et al. (2008)
sGEJ	I-*Sce*I (2x, CD)	1	All distal end-joining	Xie et al. (2009)
pEJ2	I-*Sce*I (2x, CD)	1	All distal end-joining	Mansour et al. (2010)
EJ6-GFP	Cas9 (2x)	1	All distal end-joining	Bhargava et al. (2017)
NHEJ-I	I-*Sce*I (2x, Inv)	1	Mutagenic distal end-joining	Seluanov et al. (2004)
pEJ	I-*Sce*I (2x, Inv)	1	Mutagenic distal end-joining	Mansour et al. (2008)
vGEJ	I-*Sce*I (2x, Inv)	1	Mutagenic distal end-joining	Xie et al. (2009)
EJ-RFP	I-*Sce*I	1	Mutagenic distal end-joining	Bindra et al. (2013)
pMX-INV	RAG (2x)	1	Error-free distal c-NHEJ	Bredemeyer et al. (2006)
EJ7-GFP	Cas9 (2x)	1	c-NHEJ	Bhargava et al. (2018)
EJ2-GFP	I-*Sce*I	1	MMEJ	Bennardo et al. (2008)
EJ7-GFP mHOM[a]	Cas9 (2x)	1	MMEJ	Bhargava et al. (2018)
Traffic Light Reporter (TLR)	I-*Sce*I	2	2 bp fs mutagenic end-joining + HR (templated)	Certo et al. (2011)
GFP to BFP conversion	Cas9	2	Mutagenic end-joining + HR (templated)	Glaser et al. (2016)
DNA repair reporter Arnoult	I-*Sce*I (2x, Inv)	2	Mutagenic distal end-joining + HR (templated)	Arnoult et al. (2017)
FIVER	Cas9 (2x)	2	All distal end-joining + HR/HITI (templated)[b]	Tennant et al. (2020)
CDDR (one cut variant)	Cas9	2	Mutagenic end-joining + HR (templated)	Eki et al. (2020)
HR-NHEJ Reporter	I-*Sce*I (2x, Inv)	2	Mutagenic distal end-joining + HR	Chen et al. (2019)
DSB-Spectrum_V1	Cas9 (2x)	2	Error-free distal c-NHEJ + HR	van de Kooij et al. (2021)
DSB-Spectrum_V2	Cas9	2	Mutagenic end-joining/SSA[c] + HR	van de Kooij et al. (2021)
RFP-SCR	I-*Sce*I	2	Gene Conversion, Short Tract + Long Tract	Chandramouly et al. (2013)
SeeSaw Reporter	I-*Sce*I	2	>39 bp deletions[d] + SSA	Gomez-Cabello et al. (2013)
CAT-R	Cas9 (2x)	2	Mutagenic end-joining + Large deletions[e]	Roidos et al. (2020)
CDDR (two cut variant)	Cas9 (2x)	2	Mutagenic end-joining + Error-free distal c-NHEJ	Eki et al. (2020)
SSA-TLR	I-*Sce*I	3	2 bp fs mutagenic end-joining + SSA + HR (templated)	Kuhar et al. (2016)
DSB-Spectrum_V3	Cas9	3	Mutagenic end-joining + SSA + HR	van de Kooij et al. (2021)

[a]*Several variants of EJ7-GFP were constructed that contain 1–4 nucleotides microhomology.*
[b]*Either HR or HITI can be studied, depending on the provided repair template.*
[c]*Loss of BFP expression can result from mutagenic repair by either end-joining or SSA.*
[d]*The I-SceI target site is located 39 bp behind the GFP sequence, so only repair resulting in deletions >39 bp will disrupt GFP expression.*
[e]*Which repair pathway is responsible for the large deletions that are detected by the CAT-R system has not been determined.*

SINGLE PATHWAY DSB-REPAIR REPORTERS

There are numerous published methods that could be considered reporter assays because they quantitatively detect DSB-repair by a given pathway. For simplicity, this review will be limited to describing the genomic reporter constructs that are designed to detect gain or loss of expression of a marker gene, as a result of defined sequence changes associated with DSB-repair by a specific pathway. The marker gene in the reporter could be a drug resistance cassette, and we will mention a few examples of such reporters. However, the main focus of this review will be on reporters that carry a marker gene that encodes a fluorescent protein (**Table 1**).

Early reporter systems were designed to study HR and the lay-out was based on the *MAT* locus of *Saccharomyces cerevisiae*. This locus is targeted by the HO endonuclease which can result in mating-type switching if the DSB is repaired by HR-mediated gene conversion using one of two homologous *HM* genes (Haber, 2012). To study this gene conversion process further, HR-reporter constructs were cloned that resembled the *MAT* locus, but contained marker genes like LacZ rather than the *MAT* gene (Rudin et al., 1989). This prototypic HR-reporter design was transferred to mammalian cells by the Jasin lab, which modified it to contain the target site for the I-*Sce*I nuclease rather than the HO nuclease, and GFP genes rather than LacZ genes (Rouet et al., 1994; Pierce et al., 1999). The resulting HR-reporter DR-GFP consists of two non-functional GFP gene repeats; the first is disrupted by insertion of an *I-Sce*I target site, while the second lacks a promoter and is C-terminally truncated (**Figure 1B**; Pierce et al., 1999). Expression of GFP, which can be measured by flow cytometry, is therefore dependent on I-*Sce*I-induced gene conversion between the two repeats, and serves as a quantitative read-out for HR. DR-GFP is currently still widely used and inspired the design of many other fluorescent DSB-repair reporter constructs (**Table 1**).

Following DR-GFP, Jasin *et al.* developed SA-GFP, an SSA-reporter that contains a C-terminally truncated GFP gene, and a second N-terminally truncated GFP gene with an I-*Sce*I site (**Figure 1C**; Stark et al., 2004). There is substantial sequence overlap between the truncated GFP genes, and annealing of these homologous sequences during SSA-repair of the I-*Sce*I induced DSB will generate an intact GFP gene. A limited number of alternative fluorescent HR and SSA reporters has been published (**Table 1**), which generally follow the same design principles as DR-GFP and SA-GFP, respectively.

In contrast to HR and SSA, there is an abundance of reporters to study repair by end-joining pathways (**Table 1**). The majority of end-joining reporters are conceptually similar and contain an intact GFP gene that is not expressed due to an upstream inhibitory sequence element, like an out-of-frame start codon or a second gene with a stop codon (**Figure 1D**). DSBs are generated at two nuclease target sites flanking this inhibitory sequence, and fusion of the distal DSB-ends by end-joining repair will excise the inhibitory element and permit GFP expression. Some end-joining reporters generate ligation-compatible distal DSB-ends (see **Figure 1D**), and will therefore measure the collective frequency of error-free c-NHEJ, mutagenic c-NHEJ, and MMEJ. Other end-joining reporters are limited to the detection of mutagenic end-joining as their distal DSB-ends have non-compatible overhangs (**Figure 1D**; **Table 1**). In both reporter types, however, the contribution of each individual end-joining pathway to DSB-repair cannot be distinguished based on GFP expression, although it can be revealed by sequence analysis of the repair junction (Bennardo et al., 2008).

In contrast to these generic end-joining reporters, the pMX-INV reporter specifically measures c-NHEJ (Bredemeyer et al., 2006). It is based on the process of VDJ recombination that occurs in antigen-receptor genes during lymphocyte maturation (Bassing et al., 2002). The pMX-INV reporter is introduced in mouse progenitor B-cells that express viral Abl kinase and are arrested in G1 by Alb kinase inhibitors. This induces expression of the RAG nucleases that cleave adjacent to recombination signals in the reporter to excise an antisense GFP gene (**Figure 1E**). Subsequently, the GFP gene is inverted and re-ligated during a VDJ recombination-like reaction, which puts it behind the LTR promoter and results in GFP expression. As VDJ recombination is strictly dependent on c-NHEJ repair of the RAG-induced DSBs (Helmink and Sleckman, 2012), GFP expression from pMX-INV is a direct measure for c-NHEJ activity. More recently the EJ7-GFP reporter was developed, which also specifically quantifies repair by c-NHEJ, but resembles the other end-joining reporters rather than pMX-INV, and, unlike pMX-INV, can be used in any genetically modifiable cell-line. It contains a GFP gene with an intragenic 46 basepair (bp) spacer sequence that can be removed by targeting Cas9 precisely to the edges, and which results in an intact GFP sequence if the distal ends are joined by error-free c-NHEJ (**Figure 1F**; Bhargava et al., 2018).

Finally, the Stark lab published two reporters that were designed to monitor MMEJ. The EJ2-GFP reporter contains two regions with 8 nucleotides microhomology flanking an I-*Sce*I site. Repair of the I-*Sce*I-induced DSB by MMEJ will remove a stop codon and put GFP in frame with an upstream NLS-tag sequence (**Figure 1G**; Bennardo et al., 2008). Notably, sequence analysis of repair junctions from GFP positive cells revealed that 10% of the repair products contained a deletion without microhomology flanking the break, indicating that microhomology-independent DSB-repair of EJ2-GFP can also result in GFP expression. Furthermore, several EJ7-GFP variants were generated containing microhomology (Bhargava et al., 2018). One variant, with 4 nucleotides microhomology located inward from the DSB-edge, is a bona fide MMEJ reporter, as GFP expression was strongly dependent on the end-resection factor CtIP, and the MMEJ-factor Polϴ (Bhargava et al., 2018).

MULTI-PATHWAY DSB-REPAIR REPORTERS

HR, SSA and end-joining are connected in a DSB-repair signaling network, such that loss of a pathway is compensated for by enhanced engagement of one or more of the remaining pathways (Scully et al., 2019). To study these inter-pathway dynamics,

reporter systems have been developed that can quantify repair by more than one pathway (**Table 1**). Many multi-pathway reporters combine elements from published single-pathway reporter systems, and contain two or more genes encoding different fluorescent proteins to distinguish between the repair pathways. Rather than providing a detailed explanation of each individual multi-pathway reporter, we will discuss a few to highlight the major design concepts, and refer the reader to the references listed in **Table 1** for details.

The majority of multi-pathway reporters were developed to simultaneously monitor end-joining and HR (**Table 1**). This was often achieved by combining a genomic end-joining reporter with an ectopic repair template. For example, the Traffic Light Reporter (TLR) contains a GFP gene disrupted by an internal I-*Sce*I site, followed by an mCherry gene with a reading frame shifted 2 bp compared to that of GFP (Certo et al., 2011). Formation of any 2 bp frameshift-causing mutation at the I-*Sce*I target site, predominantly generated by mutagenic c-NHEJ, will result in mCherry expression. HR can be analyzed by GFP expression, which is caused by gene conversion between the I-*Sce*I site-containing GFP gene and a truncated GFP gene present on an ectopically provided repair template.

In several other multi-pathway reporters gene conversion causes a color-switch, i.e., a change from expression of one fluorescent protein to another. A particularly practical and widely used HR-dependent color switch is GFP to BFP conversion, or the other way around (Glaser et al., 2016). The BFP and GFP genes are completely homologous with exception of two amino acids that determine their fluorescent properties, and can therefore function as reciprocal repair templates without the need for additional homologous sequences. BFP to GFP conversion is also used as read-out for HR in the DSB-Spectrum reporters (van de Kooij et al., 2021). Conveniently, no ectopic repair template is required in these multi-pathway reporter systems because the truncated GFP repair template is located on the same construct, downstream of the BFP gene that is targeted by Cas9.

Several dual pathway reporters monitor sub-pathways rather than any of the four major DSB-repair pathways. A recent example of such a reporter is the CDDR (two cut variant), which contains a functional mCherry gene inserted into a split, and thus non-functional, GFP gene (Eki et al., 2020). Cas9 is targeted to sites flanking the mCherry gene such that distal error-free c-NHEJ reconstitutes the GFP gene, and deletes the mCherry gene. This can be distinguished from mutagenic end-joining, which results in mCherry loss without GFP expression.

Finally, whereas there are many dual-pathway reporters, only two systems can monitor three pathways simultaneously. SSA-TLR is a variant of the TLR that is flanked by truncated iRFP genes, which can be joined by SSA to form an intact gene (Kuhar et al., 2016). This system thus reports on 2 bp frameshift inducing c-NHEJ, HR with an ectopic repair template, and SSA. The more recently developed DSB-Spectrum_V3 consists of an intact BFP gene that is targeted by Cas9, and can be disrupted by end-joining mediated mutagenesis. Loss of BFP expression is thus a measure for mutagenic end-joining in general, but primarily for mutagenic c-NHEJ, as indicated by sequence analysis (van de Kooij et al., 2021). Furthermore, the reporter contains a truncated GFP gene that can be used to convert BFP to GFP as a measurement for HR (van de Kooij et al., 2021). These homologous elements can also anneal during DSB-repair by SSA, resulting in the removal of a functional mCherry gene that separates the two. As such, a single reporter can be used to simultaneously measure three DSB-repair pathways.

CONSIDERATIONS FOR USING DSB-REPAIR REPORTERS

There is substantial variation within the reporter repertoire, and one reporter might be more suited to specific research needs than the other. A first consideration is whether to use single-pathway or multi-pathway reporter systems. An advantage of single-pathway systems is their simplicity and, at least for many of them, their extensive validation by widespread use for many years. However, with similar research efforts, multi-pathway reporters generate a more comprehensive view of DSB-repair pathway activity. When studying DBS-repair factors, for instance, such reporters can immediately reveal whether a factor functions in one or multiple pathways, the latter of which can also indicate at which node in the DSB-repair network it acts.

A second consideration is to use either reporters that require ectopic HR repair templates, or repeat-containing reporters that carry the template embedded within the construct. Glaser *et al.* used a single-stranded oligo as template in their GFP to BFP conversion reporter (Glaser et al., 2016), which has been demonstrated to mediate gene conversion by a mechanism that diverges from canonical HR (Bothmer et al., 2017; Richardson et al., 2018). The other templated reporters use double-stranded repair templates, which might be copied in an HR-dependent process. However, unlike the sister chromatid, these ectopic repair templates are present in high copy number, exist throughout the cell cycle, and lack proximity to the broken chromosome. In repeat-containing reporters, the template is on the sister chromatid in S/G2. However, it is also present in G1 and could theoretically be used as a donor during intrachromosomal gene conversion, in contrast to HR at endogenous loci. Although these non-physiological HR-events cannot be completely excluded, early studies on DR-GFP-like reporters indicated them to be rare (Johnson and Jasin, 2000). Moreover, more recently it was shown that all detected gene conversion events with a novel repeat-containing reporter occurred in S-phase (Chen et al., 2019). Therefore, repeat-containing HR-reporters more accurately reflect HR-repair at endogenous genomic loci and may be preferred when studying fundamentals of DSB-repair, whereas templated reporters can be useful when studying genome editing approaches.

A third consideration is that some reporters, in particular end-joining reporters, might not be very specific but actually measure the collective frequency of repair by multiple pathways, as explained above. More specific detection of repair by a single pathway is in most cases preferable. However, it should be taken into account that too high specificity can come at the cost of low frequency. This is, for example, the case in the RFP-SCR reporter, which can distinguish between gene conversion sub-pathways. The

frequency of cells undergoing long-tract gene conversion is consistently less than 0.1%, requiring analysis of large cell populations for reliable quantification (Chandramouly et al., 2013).

Fourth, reporters are designed to detect DSB-repair either by loss or by induction of marker gene expression. The latter confers specificity because the expression is strictly dependent on a defined change in the reporter sequence (**Figure 1**). This could, however, result in an underestimation of pathway usage because not all repair by the assayed pathway necessarily generates that defined sequence change. For example, mCherry expression in the TLR is dependent on generation of 2 bp frameshifts, and it therefore measures only a fraction of mutagenic c-NHEJ (Certo et al., 2011; Kuhar et al., 2016). Loss of marker gene expression, on the other hand, is theoretically less specific because it could be caused by other mutagenic repair pathways than the one measured. Nevertheless, these reporters are more inclusive because they lack the requirement for a unique mutagenic event, and their specificity can easily be validated by sequencing and genetic interrogation.

Finally, even though reporter constructs have proven extremely useful they do have limitations. First, the DSB is generated in a specific sequence context, which can impact repair pathway employment (Shen et al., 2018; Allen et al., 2019). Second, the DSB in reporter constructs is generated by efficient nucleases that keep cutting as long as they are expressed and the target site is intact. As a consequence, the phenotype analyzed might be the result of re-iterative rounds of error-free repair followed by one mutagenic repair event. This might result in an overestimation of the frequency of mutagenic repair. Moreover, the persistence of a constantly regenerated DSB might in specific cases affect pathway choice (Bennardo et al., 2009). Third, the nucleases will most likely cut both sister chromatids, which disables HR and makes the cell more reliant on alternative pathways like SSA or MMEJ. This should be taken into account when using, for example, SSA-reporters. It is, however, not an issue when using HR reporters, because the provided template lacks the nuclease target site. Altogether, these limitations should be considered for correct interpretation of reporter assays. However, they do not prevent the generation of insightful data, as proven by a large body of published DSB-repair literature in which results obtained with reporter assays were validated using orthogonal techniques.

FUTURE DEVELOPMENTS OF DSB-REPAIR REPORTERS

The advent of targetable nucleases like Cas9 has spurred the construction of novel reporter systems (**Table 1**). It has enhanced the flexibility in reporter design because it negates the requirement for a specific I-*Sce*I nuclease target sequence. Moreover, it has also expanded the nuclease repertoire with a variety of blunt-cutting enzymes, the staggered cutting Cas12a/Cpf1, and Cas9 nickase variants that can be targeted to either strand (Komor et al., 2017). Therefore, different DSB-ends can be generated, which has been shown to affect pathway choice (Bothmer et al., 2017; Schimmel et al., 2017). Given these enhanced design possibilities, a four-pathway reporter system measuring the frequency of all major DSB-repair pathways is within reach.

In addition to the development of new reporters, genome editing tools will facilitate targeted integration of reporter constructs. This will allow for comparison of repair pathway usage between genomic loci, for example, in hetero- and euchromatic regions, which is an active area of investigation (Caron et al., 2021; Schep et al., 2021). Interestingly, Cas9-based tools have been developed to modify the chromatin at target loci (Goell and Hilton, 2021). These could be used in combination with reporters to study the effect of specific chromatin marks on DSB-repair pathway choice. Importantly, ongoing research efforts are aimed at developing methods to rapidly activate and de-activate Cas9 (Liu et al., 2020; Marino et al., 2020), so that complete temporal control over Cas9 activity will be possible in the near future, thus eliminating the current problem of re-iterative cutting of reporters. Finally, reporters are ideal tools for pooled high-throughput screening, because repair phenotypes can easily be selected by FACS. Reporter screens have been done using siRNAs, but these screens have been hampered by the strong tendency of siRNAs to silence Rad51 expression as an off-target effect (Adamson et al., 2012; Howard et al., 2015). CRISPR-based screens are generally less affected by off-target editing, and the first insightful CRISPRi reporter screens have already been published (Richardson et al., 2018; Wienert et al., 2020).

In conclusion, DSB-repair reporters have evolved from designated constructs to study HR, to complex multicolor tools that can measure repair by two or three pathways in one assay. This evolution is expected to continue, driven by Cas9-technology, ensuring that reporters will remain an essential element in the DSB-repair toolkit, as they have been for multiple decades.

AUTHOR CONTRIBUTIONS

Both authors have made an equal contribution to this work and approved it for publication.

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REVIEW
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Meiotic Genes and DNA Double Strand Break Repair in Cancer

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Tumor cells show widespread genetic alterations that change the expression of genes driving tumor progression, including genes that maintain genomic integrity. In recent years, it has become clear that tumors frequently reactivate genes whose expression is typically restricted to germ cells. As germ cells have specialized pathways to facilitate the exchange of genetic information between homologous chromosomes, their aberrant regulation influences how cancer cells repair DNA double strand breaks (DSB). This drives genomic instability and affects the response of tumor cells to anticancer therapies. Since meiotic genes are usually transcriptionally repressed in somatic cells of healthy tissues, targeting aberrantly expressed meiotic genes may provide a unique opportunity to specifically kill cancer cells whilst sparing the non-transformed somatic cells. In this review, we highlight meiotic genes that have been reported to affect DSB repair in cancers derived from somatic cells. A better understanding of their mechanistic role in the context of homology-directed DNA repair in somatic cancers may provide useful insights to find novel vulnerabilities that can be targeted.

Keywords: meiosis, mitosis, meiotic genes, genomic instability, DNA repair, homologous recombination

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INTRODUCTION

In the 19th century, the hypothesis was put forward that cancers arise from embryonic remnants that remain in adult organs (Durante, 1874; Cohnheim, 1875). When the microenvironment changes and provides the necessary blood supply, these remnants grow in an uncontrolled fashion. This theory was based on the observation of pathologists that the microscopic morphology of some cancers (*e.g.* Teratoma and Wilm's tumor) highly resembles that of embryonic tissues. Later on, Theodor Boveri (1862–1915) concluded from his observations that embryonic characteristics of cancer cells are rather side effects of the abnormal distribution of chromosomes and that remnant embryonic tissues only explain rare cases (Boveri, 2008). This fostered the concept that cancer cells can arise from well-differentiated cells and can de-differentiate. Today we know that cancer is caused by various genetic alterations that affect both germ cells and somatic cells. Intriguingly, many somatic cancer cells seem to benefit from the expression of genes that are typically present in germ cells and contribute to meiotic cell division. As several of these affect processing of DNA double strand breaks in the context of homologous recombination, some cancers may benefit from the double-strand break (DSB) repair mediated by aberrantly expressed meiotic genes. If particular cancers are dependent on their expression when exposed to DNA damage, they may provide interesting drug targets. Whereas normal somatic cells do not depend on the expression of meiotic genes for DSB repair, tumor cells that do depend on them in the context of DNA damage may die when their function is blocked. Such a therapeutic approach would still harm germ cells, but since many cancers arise in people beyond

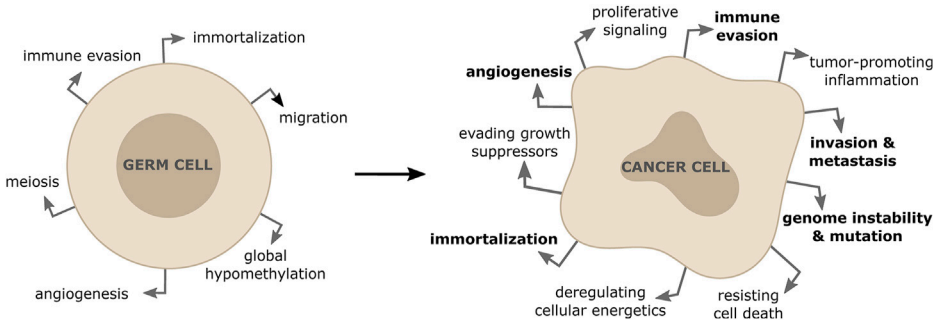

FIGURE 1 | Activation of meiotic genes in somatic cells contribute to properties of tumor formation and progression. The re-expression of meiotic genes in somatic cancer cells is responsible for driving some of the hallmarks of cancers (shown in bold).

the wish to have children, the loss of germ cells may be tolerated. In this review, we briefly highlight mitotic and meiotic cell division with a focus on DSB-related meiotic genes that have been found to be aberrantly expressed in cancer.

The primary goal of each cell division for non-cancerous somatic cells is to ensure that daughter cells are genetically identical to their parent cells (Nurse, 2000). Errors happening during cell division result in various forms of genome alterations in the daughter cells and include mutations of specific genes, amplifications, deletions or rearrangements (including gain or loss) of entire chromosomes (Levine and Holland, 2018). Cells use a number of mechanisms to prevent these alterations, including error-free repair of sporadic DNA damage, high fidelity DNA replication during S-phase, precise chromosome segregation during mitosis and a coordinated cell cycle progression (Shen, 2011). Inherited or acquired defects in DNA repair, DNA replication, chromosome segregation or cell cycle control lead to an increased mutation frequency. Accumulation of these genomic alterations is generally referred to as genome instability, which predisposes cells to malignant transformation (Negrini et al., 2010). In most cases, significant genome alterations result in a non-viable cell, but in rare events it might confer a selective growth advantage, leading to cancer initiation and progression. It has been clear for a long time that such genomic changes involve genes encoding tumor suppressors, proto-oncogene or genes that function to maintain genomic integrity (Negrini et al., 2010). Moreover, there is emerging evidence that an inappropriate activation of meiotic genes in somatic cells results in both initiation and maintenance of the malignant phenotype in a range of cancer types (Feichtinger and McFarlane, 2019). The aberrant expression of meiotic genes in cancer cells has been shown to contribute to various hallmarks of cancer by altering centromeric polarity control, motility, chromosome dynamics and DNA repair (Hanahan and Weinberg, 2011; McFarlane and Wakeman, 2017) (**Figure 1**). In particular, alterations of how cancer cells repair DNA breaks due to unscheduled expression of meiotic genes, has been shown to drive genomic instability and to affect tumor cells' response to anticancer therapies (Nielsen and Gjerstorff, 2016; Mantere et al., 2017; Trussart et al., 2018).

These observations have raised a significant interest towards the study of meiotic genes in somatic cancers, as they could be used as cancer-specific predictive biomarkers of therapy response. Moreover, in the era of immunotherapy aberrantly expressed germ cell proteins are prime targets for cancer vaccination and adoptive T-cell transfer with chimeric T-cell receptors. For example, male germ cells lack HLA-class I molecules and cannot present antigens to T cells to induce immunotolerance (Janitz et al., 1994). When expressed in somatic cancers, cancer/testis antigens therefore represent promising targets for cancer immunotherapy (Gjerstorff et al., 2015).

MITOSIS AND MEIOSIS

Eukaryotic cells can undergo two different types of cell divisions. On the one hand, with the goal of maintaining a functional organism, somatic cells undergo mitosis and thereby create two genetically identical daughter cells (Nurse, 2000). On the other hand, germline cells undergo a different type of cell division, known as meiosis, to produce haploid gametes, which have only one copy of each chromosome. Both processes are tightly regulated by a number of coordinated pathways to ensure the correct segregation of genetic material. The molecular mechanisms of mitosis and meiosis are well described in other reviews (Nurse, 2000; Marston and Amon, 2004; Duro and Marston, 2015; Ohkura, 2015; Bolcun-Filas and Handel, 2018); we therefore provide only a succinct overview of both processes here.

Mitosis

In brief, cells undergo four different phases during the cell cycle: the two main phases, S- and M-phase (mitosis), are separated by two gap phases called G1 (before S-phase) and G2 (after S-phase). To create two identical daughter cells from a parental cell, chromosomes are duplicated during S-phase to form sister chromatids, which will be separated to each daughter cell in the M-phase of the cell cycle. G1 and G2 are important to provide cells time to control the correct replication and chromosomal

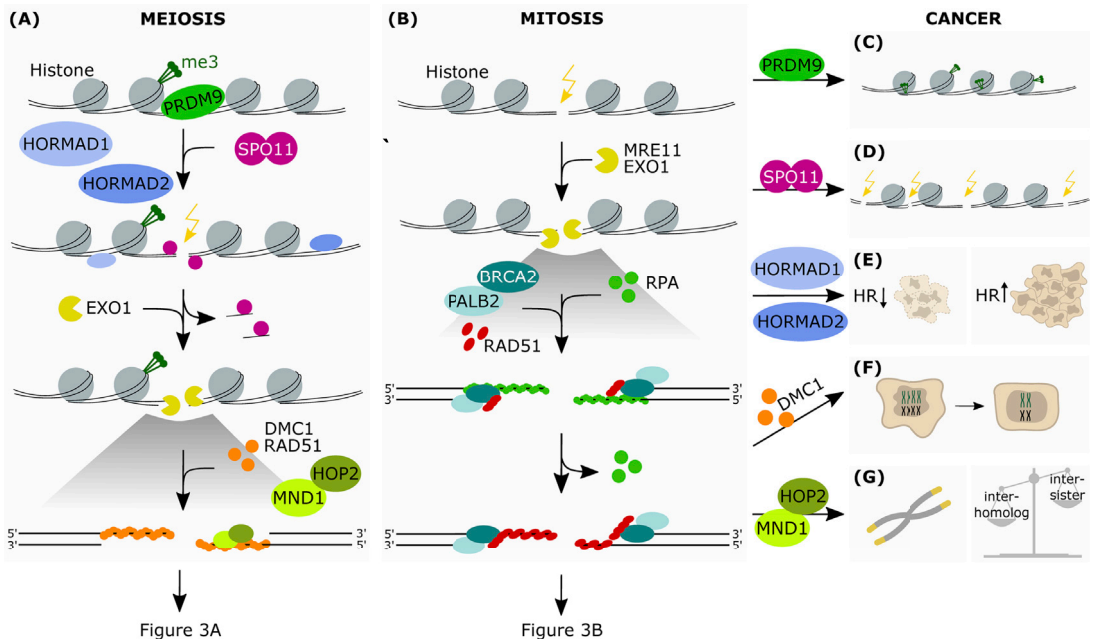

FIGURE 2 | The aberrant expression of meiotic genes in somatic cancers affects HR-dependent-DNA repair. Simplified schematic representation of the first steps of HR in meiosis **(A)** and mitosis **(B) (A)** PRDM9 is the protein responsible for the epigenetic marking of the hotspots for DSB introduction. This allows the binding of SPO11, which is favoured by HORMAD1 and HORMAD2. SPO11 introduces strand breaks at the marked hotspots. This is followed by end resection at the break sites by exonucleolytic activity. The subsequent binding of the RAD51 and DMC1 recombinases onto ssDNA allows the formation of a nucleoprotein filament, which in turn recruits downstream factors promoting interaction between homologous chromosomes. The protein heterodimer HOP2-MND1 acts in concert during this process favoring homology search and therefore resolution of the DSB **(B)** Upon recognition of the break site, the nucleases MRE11 and EXO1 resect the DNA generating ssDNA which are stabilized by RPA. This allows the formation of the RAD51-ssDNA filaments, in cooperation with BRCA2 and PALB2, which search for the homologous DNA template by invading the sister chromatid **(C)** In some cancer cells PRDM9 may also mark DNA regions that are favorable to the formation of chromosomic lesions **(D)** Due to its meiotic function in generating DNA strand breaks, aberrantly expressed SPO11 may then promote crossover events in somatic cancer cells, as well as translocations, insertions and deletions **(E)** Due to its ability in modulating HR-mediated DNA damage repair, increased expression of HORMAD in somatic cancers has been shown to promote or disrupt HR-mediated repair, depending on the genetic background **(F)** Expression of DMC1 promotes meiosis-like reductional segregation of homologues in polyploid cells, restoring the proliferative state of somatic cancer cells **(G)** HOP2-MND1 may function in cancer cells to promote an alternative lengthening of telomeres (ALT) in the absence of telomerase reactivation. Furthermore, as HOP2-MND1 favor recombination between homologous chromatids in meiotic cells, their reactivation in somatic cancer cells could disrupt the recombination bias between sister chromatids that is typical of mitotic cells.

segregation (Nurse, 2000; Williams and Stoeber, 2012). The transition from one phase to another is tightly regulated by cyclin-dependent kinases (CDKs), which phosphorylate downstream factors allowing cells to initiate DNA replication or chromosomal segregation to the daughter cells (Barnum and O'Connell, 2014).

Meiosis

Meiosis is cell division for the generation of gametes in sexual reproduction. The key feature of this process is the reduction of the DNA content with the final goal of generating gametes with a haploid set of DNA. This process involves two cycles of cell division: meiosis I and meiosis II. In meiosis I, homologous chromosomes are replicated and subsequently segregated, generating diploid daughter cells. Meiosis I is followed by another round of chromosome-segregation (Meiosis II), which does not include another phase of DNA replication and gives rise to four haploid gametes. Gametes originating from the same cell are genetically different from each other, not only due to the independent segregation of maternal and paternal DNA but also due to another mechanism exclusive to meiosis I: before segregation of the homologous chromosome pairs in meiosis I, chromosomes undergo a programmed recombination of the genetic material, also known as homologous recombination (HR), which involves the formation of several DSBs. The repair of these lesions is associated with non-crossover or crossover events, which in the latter case leads to the exchange of genetic information and thus to an increase in inter-individual diversity (Ohkura, 2015; Bolcun-Filas and Handel, 2018).

In contrast to the programmed generation and repair of DSBs during meiotic cell division, DNA lesions occur randomly in somatic cells, and need to be repaired in an error-free manner to minimize the risk of DNA alterations. To this purpose, somatic cells also use HR, which repairs DSBs with high fidelity. HR in somatic cells is restricted to S- or G2 -phase of the cell cycle as it relies on the presence of a sister chromatid as a template for DNA repair, though the homologous chromosome can also be used as a template with a much lower frequency (Kadyk and Hartwell, 1992; Takata et al., 1998). HR in somatic cells is very well described (Li and Heyer, 2008; Wright et al., 2018; Scully

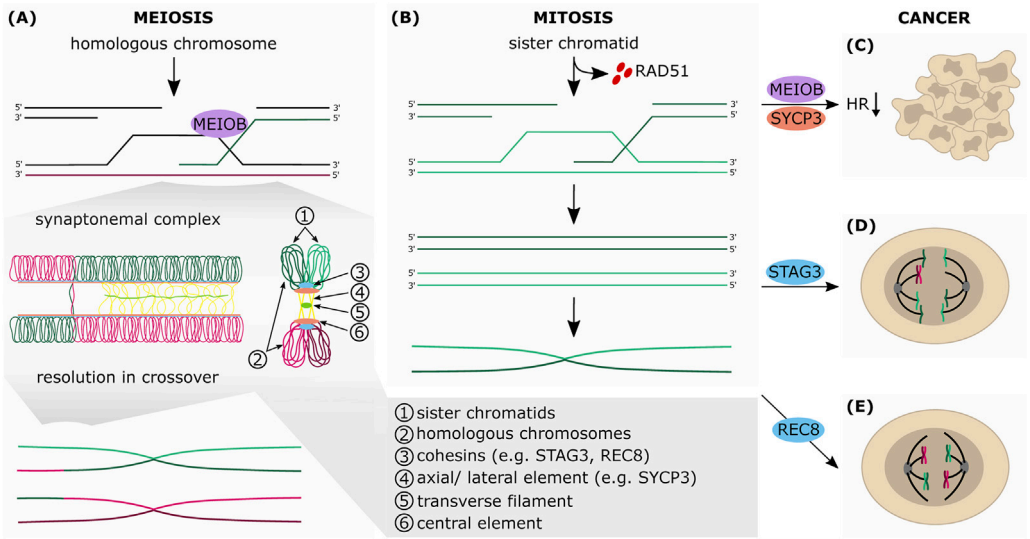

FIGURE 3 | The aberrant expression of meiotic genes in somatic cancers affects HR-dependent-DNA repair. Simplified schematic representation of the final steps of HR in meiosis **(A)** and mitosis **(B) (A)** During meiosis, the sister chromatids (1) are connected with each other by the cohesin complex (3). Cohesins are also essential for the formation of the synaptonemal complex (SC), which connects the homologous chromosomes (2). The SC consists of the axial elements (AEs) of the chromosomes (4), which are connected to each other by transverse filaments (5) and the central element (6). Upon formation of the SC, AEs are turned into lateral elements (LEs), which consist of two different SC proteins: SYCP2 and SYCP3. Between the AEs of the two homologous chromosomes SYCP1 builds parallel dimers that stabilize the positioning of the homologous chromosomes and favor crossovers. The exonucleolytic activity of MEIOB allows the formation of a double Holliday junction between two homologous chromosomes which can be resolved in both non-crossover and crossover formation **(B)** The RAD51-ssDNA filaments search for homologous DNA template by invading the sister chromatid, which leads to the formation of the Holliday junctions and finally to DNA synthesis of the missing sequence that was lost at the break point. After synthesis, the junctions are resolved by endonucleolytic cleavage, the invading strand is released and anneals to the other side of the break. The repair is completed by ligation of the gaps by a DNA ligase **(C)** Aberrant expression of MEIOB and SYCP3 in somatic cancers inhibits HR-mediated DNA repair **(D)** STAG3 altered expression mediates chromosomal mis-segregation and genome reduction of cancer cells **(E)** Augmented expression of REC8 in somatic cancer cells promotes meiosis-like reductional segregation of homologous chromosome, which provides a survival advantage following DNA damage-inducing treatment.

et al., 2019) and will therefore not be discussed in further details in this review. Overall, HR in mitosis and meiosis share many similarities, but they do involve different key players (**Figures 2**, **3**). In this review we will describe in more detail the process of HR in human meiotic cells.

HR IN MEIOTIC CELLS

Marking of Hotspot Sites and Introduction of DSBs

In contrast to mitosis, DSBs in meiotic cells are introduced in a programmed way and many factors involved in the repair are uniquely expressed in meiosis. The program is initiated at recombination hotspots, which are preferentially targeted for DSB formation. In mice and humans, PRDM9 is the main protein catalyzing the epigenetic marking and thus the initiation of the break-inducing process (Tock and Henderson, 2018). The PRDM9 zinc-finger domain is able to bind specific DNA sequences, bringing the PR/SET domain in position to allow trimethylation of histone H3 on lysine 4 (H3K4me3) and histone H3 on lysine 36 (H3K36me3) (Parvanov et al., 2010; Grey et al., 2011; Powers et al., 2016). Epigenetic modifications of H3K4 are not only promoted by PRDM9, they are also commonly induced at promoters or enhancers by other methyltransferases (Brick et al., 2012; Baudat et al., 2013; Tock and Henderson, 2018). Therefore, it is not surprising that upon loss of PRDM9 DSBs are still introduced at PRDM9-independent H3K4me3 sites, even though they result in inefficient repair and meiotic arrest (Berg et al., 2010; Brick et al., 2012). These findings show that trimethylation of H3K4 is not sufficient to induce a successful recombination, even though the exact mechanism remains elusive (Baudat et al., 2013). In a next step, a DSB machinery consisting of SPO11, IHO1, MEI4, MEI1 and REC114 needs to be activated. These members are evolutionarily conserved among eukaryotes (Kumar et al., 2010; Baudat et al., 2013). Besides SPO11, which is the catalytically active unit, IHO1, MEI4, MEI1 and REC114 are crucial for the introduction of DSBs and the preferential interaction with the homologous chromosome instead of the sister chromatid (Libby et al., 2003; Kumar et al., 2010; Stanzione et al., 2016; Kumar et al., 2018). SPO11 is highly conserved among eukaryotes, suggesting an important role of this protein in meiotic DSB repair. It is responsible for the introduction of the strand break at the marked hotspots by performing a topoisomerase-like reaction: its tyrosine residue attacks a phosphorous on the DNA, which then triggers the formation of a tyrosyl phosphodiester linked to DNA. This in turn disrupts the

double-helix and introduces a DNA break (Keeney et al., 1997).

DNA end resection and initiation of the synaptonemal complex

To allow further processing of the DNA break site, degradation of the 5′ end is required. End resection occurs by a two-step mechanism. In a first step, CtIP activates the Mre11-Rad50-Nbs1 (MRN) complex to endonucleolytically cleave the 5′-terminated DNA strands close to where SPO11 is bound. This in turn, releases SPO11 with short oligonucleotides from the DNA ends bound to it (Neale et al., 2005; Keeney, 2008; Garcia et al., 2011; Symington, 2016). In a second step, EXO1 and/or DNA2 nucleases extend the resected tracts to produce long 3′-ssDNA overhangs, which favors homology search (Symington, 2016). While in prokaryotes RecA is the only protein involved in homology search and strand invasion, in eukaryotes two of its homologs are involved: RAD51, which is also active in mitotic HR, and DMC1, which is exclusively expressed in meiotic cells (Bugreev et al., 2011). Similarly to the process in somatic cells, BRCA2 is required for proper loading of DMC1 and RAD51, since BRCA2-deficient spermatocytes can induce DSBs but fail in completing recombination (Sharan et al., 2004). Successful binding of DMC1 proteins onto ssDNA allows the formation of a nucleoprotein filament (Sehorn et al., 2004), which in turn promotes the interaction between homologous chromosomes. This process was shown to be stimulated by five Rad51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3), which prime the nucleoprotein filaments for strand exchange with the template duplex (Taylor et al., 2015). Moreover, the protein heterodimer HOP2-MND1 acts in concert during this process favoring homology search and therefore resolution of the DSB (Tsubouchi and Roeder, 2002; Chen et al., 2004). Besides recruiting MND1 to the break sites, HOP2 favors the interaction between homologous chromosomes over sister chromatids (Leu et al., 1998). There are three main modes of action of the HOP2-MND1 complex. It orchestrates the localization of DMC1 on the ssDNA and stabilizes the nucleoprotein complex (1) (Pezza et al., 2007). This allows DMC1 to induce the formation of a D-loop and the synaptonemal complex (SC) and together with HOP2-MND1, it brings homologs in close juxtaposition (2) (Chen et al., 2004; Pezza et al., 2007). Finally, HOP2-MND1 enhances the homology search by the condensation of the dsDNA around the filament (3) (Pezza et al., 2010).

Sister Chromatid Cohesion

The role of cohesins is crucial for the next steps of meiosis. Cohesion is not specific to meiosis but also occurs during mitosis and is essential for DNA replication, DNA repair, gene expression and development (Brooker and Berkowitz, 2014). During meiosis I, sister chromatids associate with each other via cohesins along the chromatid arms and at the centromere. The meiosis-specific members of this complex are SMC1β, REC8, RAD21L and STAG3, while SMC1α, RAD21 and STAG2 have been reported to be active in germ cells as well as in somatic cells (Brooker and Berkowitz, 2014). The chiasmata formed upon HR links the homologous chromosomes and allows their localization in the metaphase plate. This specific localization of the chromosomes triggers the attachment of the microtubules from the spindle machinery in a syntelic manner: the sister kinetochores of the maternal centromeres are attached to microtubules with opposite orientation of the paternal centromeres (Peters et al., 2008; Brooker and Berkowitz, 2014; Ishiguro, 2019). Segregation of the homologous chromosomes in meiosis I is triggered by the cleavage of the cohesins along the sister chromatid arms and the resolution of the chiasmata. A crucial component of the meiotic cohesin complex is REC8. The separase enzyme cleaves REC8 only from the sister chromatid arms, leaving the cohesins at the centromeres (Marston and Amon, 2004). REC8 knockout mice are sterile and show SC-like formation between sister chromatids instead of the homologous chromosomes (Xu et al., 2005). These data are further supported by the finding that cohesin at centromeres influences the orientation of the kinetochores (Ogushi et al., 2021). This suggests that functional REC8 is crucial for HR and proper chromosome segregation in meiotic cells. How exactly cohesion at centromeres differs from the arm-cohesion remains to be elucidated.

Synaptonemal Complex: Formation and Resolution

Cohesins are important for the formation of the SC as they initiate the recruitment of the complex members. The SC consist of a tripartite proteinaceous structure that is able to hold homologous chromosomes in close juxtaposition and allows formation of synapsis (**Figure 3A**). The SC includes three different parts: 1) the axial elements (AEs), that are assembled along the cohesin on the sister chromatid arms, and are connected to each other by transverse filaments (TFs) (2) and the central element (CE) (3) (Page and Hawley, 2004). Upon formation of the SC, AEs are turned into lateral elements (LEs). They consist of two different proteins SYCP2 and SYCP3 that form heterodimers (Yuan et al., 1998; Yang et al., 2006). Between the AEs of the two homologous chromosomes SYCP1 builds parallel dimers (TFs), which stabilize the positioning of the homologous chromosomes and favor crossovers (de Vries, 2005). The CE forms a network between SYCE1, SYCE2 and TEX12 that also interacts with the TF component SYCP1 (Yang and Wang, 2008). The SC controls in a feedback loop the generation of DSBs: the assembly of the central region triggers removal of HORMA-domain proteins which are essential for the recruitment of, for example, IHO1 and thus, hinders the assembly of the DSB machinery (Wojtasz et al., 2009; Hollingsworth, 2020; Mu et al., 2020). As mentioned earlier, after capturing the homologous chromosome that is close enough to the DSB site, the formation of the D-loop is triggered by DMC1. This structure can be either resolved as a non-crossover (NCO) or as a crossover (CO) after conversion into a double Holliday

junction (dHJ). Following the D-loop formation and invasion of the ssDNA, the homologous non-sister chromatid can be used as a template to repair the break site. This process is defined as single-end invasion (Hunter and Kleckner, 2001). At this stage, the reannealing of the repaired end to its parental strand results in a NCO event but in some cases the D-loop is further processed into a dHJ if the second end of the DSB site is captured by the same homologous non-sister chromatid (Hunter and Kleckner, 2001; Petronczki et al., 2003). This complex structure can be resolved either as NCO, or CO if the cleavage is induced asymmetrically between the homologous chromosomes, thus generating reciprocal exchanges (Petronczki et al., 2003; Heyer, 2004). Luo et al. suggest that the conversion of the D-loop to dHJ and thus potential CO is dependent on MEIOB, which works in a complex with SPATA22 and RPA (Luo et al., 2013). Resolution of the dHJ is thought to be mediated by the resolvases MUS81-EME1, SLX1-SLX4 and GEN1 (triggering COs) or BLM (NCO dissolution) (Wyatt and West, 2014). Moreover, the presence of functional CE seems to be essential for successfully CO events (Baudat et al., 2013).

Checkpoint Surveillance

The completion of meiosis I requires the coordination of different events. First of all, one CO event per homologous chromosome pair is inevitable. Second, the CO frequency has to be regulated and COs need to be evenly spaced along the chromatids. Overall, the interaction with homologous chromatids should be favored over sister chromatids (Baudat et al., 2013). One group of proteins that mainly serves as checkpoint controls in meiosis I are the HORMA (Hop1, Rev7, Mad2)-domain proteins HORMAD1 and HORMAD2. Loading of HORMAD1 was found to be initiated by REC8 and RAD21L, two members of the cohesin complex. Both HORMAD proteins cluster along AEs until the assembly of the SC, where they are removed and regulate DSB induction (Wojtasz et al., 2009; Fujiwara et al., 2020; Mu et al., 2020). While HORMAD1 plays a role in homology search by increasing the number of ssDNA ends as well as in the synaptonemal complex formation, HORMAD2 is exclusively responsible as a checkpoint control element (Shin et al., 2010; Daniel et al., 2011; Wojtasz et al., 2012). Shin et al. observed that in the absence of HORMAD1, more inter-sister chromatid repair takes place, suggesting that HORMAD1 promotes the use of homologous DNA over sister DNA for repair of DSBs (Shin et al., 2013). Furthermore, HORMAD1 recruits IHO1 to unsynapsed regions which in turn triggers DSB formation by SPO11 and its auxiliary proteins (Stanzione et al., 2016). Another control checkpoint at this stage is the detection of unsynapsed chromosomes. HORMAD2 seems to play a key role in this process. It recruits ATR kinases along unsynapsed axes and induces phosphorylation of H2AX (Turner et al., 2005; Wojtasz et al., 2012). This leads to meiotic silencing of unsynapsed chromatin as protecting mechanism (Turner et al., 2005).

Hence, to ensure the programmed crossover of genetic information during Meiosis I, germ cells express a toolkit of specific genes involved in the induction of DSBs and their repair.

MEIOTIC DNA REPAIR GENES ABERRANTLY EXPRESSED IN CANCERS

Intriguingly, several of these genes have been found to be aberrantly expressed in mitotic cancer cells, and they are thought to contribute to driving genomic instability and carcinogenesis. Here we describe the main genes involved (**Figure 2** and **Figure 3**).

HORMADs

HORMAD1 is one of the most studied meiotic genes implicated in carcinogenesis and genomic instability. In physiological conditions, HORMAD1 expression is restricted to meiotic cells in testes and ovaries. However, many studies have shown that HORMAD1 is significantly upregulated in several cancers where it correlates with increased genomic instability and poor patient prognosis (Adelaide et al., 2007; Watkins et al., 2015; Chen et al., 2018; Gao et al., 2018; Nichols et al., 2018; Gantchev et al., 2020). Increased expression of HORMAD1 has been detected in patient samples isolated from breast cancer (including triple-negative breast cancer (TNBC) and basal-like breast cancer (BLBC)) (Adelaide et al., 2007; Yao et al., 2014; Chen et al., 2018), lung cancer (lung adenocarcinoma (Yao et al., 2014; Nichols et al., 2018), lung squamous cell carcinoma (Yao et al., 2014), small cell lung cancer, NSCLC (Chen et al., 2005)), esophageal, endometrial, bladder, colon (Chen et al., 2005), epithelial ovarian carcinoma (Shahzad et al., 2013), gastric cancer (Aung et al., 2006), head and neck squamous cell carcinoma, melanoma (Yao et al., 2014) and cutaneous T-cell lymphoma (CTCL) (Tsang et al., 2018).

Recent studies have demonstrated that the positive correlation between increased HORMAD1 expression and genomic instability in tumors is due to its ability in modulating DNA damage repair (Watkins et al., 2015; Gao et al., 2018; Nichols et al., 2018; Liu et al., 2020). These studies suggest distinct hypothesis on how HORMAD1 affects HR-mediated DNA repair. The group of Andrew N. J. Tutt was the first to demonstrate that the positive correlation between HORMAD1 expression and chromosomal instability observed in TNBC is the consequence of the disruption of HR-mediated repair (Watkins et al., 2015). Using a panel of TNBC cell lines, as well as non-transformed cells, the authors showed that overexpression of HORMAD1 suppresses RAD51-dependent HR. This drives the error-prone 53BP1-dependent non-homologous end joining (NHEJ) DNA repair pathway. In addition, HORMAD1 expression correlated with a better response to HR defect-targeting agents (such as poly ADP-ribose polymerase inhibitors PARPi or poly ADP-ribose polymerase (PARP) inhibitors olaparib and BMN673) in both TNBC cell lines and clinical trial data. With their data, the authors provided a possible mechanism for the increased levels of allelic-imbalanced copy-number aberrations (AiCNA) that are abundant in TNBC.

In contrast, two distinct studies demonstrated that HORMAD1 promotes HR in models of lung adenocarcinomas, providing a selective survival advantage for cancer cells (Gao et al., 2018; Nichols et al., 2018). HORMAD1 loss enhanced sensitivity to irradiation (IR), camptothecin and PARP inhibition, and significantly reduced tumor growth *in vivo*. Mechanistically, Gao and colleagues showed that HORMAD1 re-distributes to nuclear foci and co-localizes with the DSB marker yH2AX in response to IR and chemotherapeutic agents (Gao et al., 2018). Both studies demonstrated that HORMAD1 expression promotes DSB repair by HR, thus offering a mechanistic explanation for the reduced sensitivity to the PARP inhibitor Rucaparib in the work of Wang and colleagues (Wang et al., 2018). The conflicting data on the modulation of HR by HORMAD1 reported in these studies (Watkins et al., 2015; Gao et al., 2018; Nichols et al., 2018), could be explained by the different cellular models that have been used. HORMAD1 might have opposing effects on HR in different cancers due to tissue-specific expression of HR pathway regulators targeted by HORMAD1. This may explain why HORMAD1 inhibits HR in TNBC and stimulates HR in lung adenocarcinomas.

More recently, HORMAD1 was shown to modulate another DNA repair pathway besides HR. The group of Yidan Liu showed that aberrant expression of HORMAD1 compromises DNA mismatch repair in cancer cells (Liu et al., 2007). Mechanistically, HORMAD1 interacts with the MCM8-MCM9 complex and prevents its efficient nuclear localization. Consequently, HORMAD1-expressing cancer cells have reduced MLH1 chromatin binding and DNA mismatch repair defects. HORMAD1 expression is also associated with an increased mutation load and genomic instability in a human cancer samples cohort from the TCGA dataset (Liu et al., 2020).

Even though the homologous protein HORMAD2 was found to be aberrantly expressed in lung cancer tissues (Liu et al., 2012), its potential role in modulating DNA repair in cancer cells is less clear. In one study, aimed at investigating the impact of candidate genes on thyroid carcinoma (THCA), the authors found that HORMAD2 was significantly hypermethylated in THCA cells. Treatment with the DNA hypomethylating agent 5-Azacitidine, suppressed THCA cells' viability, motility and invasiveness (Lin et al., 2018). However, follow-up studies are needed to investigate a direct involvement of HORMAD2 in promoting cancer cell' growth.

HOP2-MND1

The group of Greenberg and colleagues discovered that the HOP2-MND1 heterodimer functions in cancer cells to promote an alternative lengthening of telomeres (ALT) mechanism in the absence of telomerase activity (Nam Woo Cho et al., 2014). Similar to meiotic recombination, this process involves the generation of DSBs to initiate the recombination between homologous DNA sequences on non-sister chromatids. Mechanistically, telomeres behave like a broken chromosome and serve as a substrate for DNA replication-dependent *de novo* telomere elongation, a process that is dependent on the ability of HOP2-MND1 to stimulate non-sister chromosome interactions (Nam Woo Cho et al., 2014). This discovery added a new class of factors to the mix of germline genes that become activated during oncogenesis. A role for HOP2 in tumors is also supported by several studies that have described *HOP2* germline mutations in familial breast and ovarian cancers (Peng et al., 2013a; Peng et al., 2013b; Yang et al., 2016). These mutations caused defective alternative splicing and truncated the open reading frame of the *HOP2* gene, generating an isoform that is expressed in the cytoplasm and it is often detected in tumor stromal cells. The splice variants act as dominant negatives to counteract wild type *HOP2* activity in transcription and to abolish Rad51 foci formation after IR-induced DNA damage. The constitutive expression of the HOP2 cytoplasmic isoform, but not the wild type, induced tumor growth in nude mice (Peng et al., 2013b). Another study from the same group found that mutant HOP2 protein production in the breast tumor microenvironment induced VEGF expression by enhancing VEGF promoter activity and potentially promote angiogenesis and adipogenesis (Yang et al., 2016). These results suggest that mutated HOP2 protein production in the tumor stroma may contribute to carcinogenesis and therefore could be used as a biomarker to define mutant reactive breast cancer stroma. *HOP2* mutations were also observed in cases of early onset familial breast and ovarian cancer and a *HOP2* mutation in the C-terminus (*HOP2 p.del201Glu*, is associated with XX ovarian dysgenesis (Zhao and Sung, 2015). Lastly, the group of I.V. Litvinov reported that the HOP2 protein is also ectopically expressed in cutaneous T-cell lymphomas (CTCL), suggesting that HOP2 expression is not unique to breast, ovarian and fallopian tube cancers (Tsang et al., 2018).

Although less is known about MND1 in carcinogenesis, its aberrant expression has been reported in ovarian cancers and lung adenocarcinoma (Yeganeh et al., 2017; Zhang et al., 2019; Wei et al., 2021; Zhang et al., 2021). By performing a differential mRNA expression analysis of normal versus malignant ovarian tumors, P.N. Yeganeh and colleagues identified *MND1* as one of the most significantly dysregulated genes in the malignant tissues (Yeganeh et al., 2017). In a recent study, genomic data from the GEO database that were further validated with clinicopathological data from the TCGA database revealed MND1 as a differentially expressed gene that significantly associated with overall survival of lung adenocarcinoma patients. The authors of the study therefore concluded that MND1 could be used as a prognostic biomarker and a molecular curative target for lung adenocarcinoma (Wei et al., 2021). However, in all these studies, the underlying molecular mechanism of how aberrant expression of MND1 contributes to carcinogenesis has not been reported. Using a genome-wide insertional mutagenesis screen in somatic cancer cells, we identified MND1 as a factor which increases cellular fitness following exposure to irradiation (IR) (Francica et al., 2020). Similarly, in somatic *Arabidopsis thaliana* cells, the homologue of MND1, AtMnd1, is induced by IR and its loss causes IR sensitivity, suggesting that AtMnd1 is required for DSB repair in somatic cells

(Domenichini et al., 2006). Hence, MND1 may be an interesting drug target to sensitize somatic cancers to DSB-inducing therapy.

SPO11

The human *SP O 11* gene is located in chromosome 20q13.2-13.3, a region that is amplified in multiple breast cancers and associated with genomic instability (Tanner et al., 1994; Courjal et al., 1996; Collins et al., 1998). However, there are limited studies to date that have investigated the potential role of SPO11 in carcinogenesis. The aberrant expression of *SP O 11* has been reported in patients samples of melanoma (Koslowski et al., 2002), colorectal cancer (Eldai et al., 2013), cervical cancer (Koslowski et al., 2002) as well as in Acute Myeloid Leukemia (AML) (Atanackovic et al., 2011), CTCL (Litvinov et al., 2014) and lung cancer (Koslowski et al., 2002) cell lines. High-resolution cytogenetic microarray data of 15 tumor-normal paired colorectal cancer samples revealed a gain in chromosome copy number of the *SP O 11* gene (Eldai et al., 2013). Increased *SP O 11* expression was also detected in patients with CTCL compared to expression in normal skin and benign inflammatory dermatoses (Litvinov et al., 2014). Based on the function of SPO11 in the induction of DSBs, it would be interesting to investigate whether its expression contributes to the genomic instability by promoting translocations, insertions and deletions.

PRDM9

PRDM9 is recurrently mutated in head and neck squamous cell carcinoma (Stransky et al., 2011), and an excess of rare *PRDM9* alleles has been reported in aneuploid and infant B-cell precursor acute lymphoblastic leukemia patients (Hussin et al., 2013). Based on its function, altered *PRDM9* expression could create vulnerable DNA regions that are favorable to the formation of chromosomic lesions. Indeed, new evidence has recently emerged to suggest a link between PRDM9-driven meiotic recombination hotspots and genomic instability (Houle et al., 2018; Kaiser and Semple, 2018). In a study where *PRDM9* expression was analyzed in 1879 cancer samples, *PRDM9* was unexpectedly found to be expressed in 20% of these tumors. Intriguingly, *PRDM9* expression correlated with areas of chromosomal instability and in samples with aberrant *PRDM9* expression, structural variant breakpoints frequently neighbor the DNA motif recognized by PRDM9 (Houle et al., 2018). This might suggest that PRDM9 generates chromatin regions that become more fragile and could favor genomic instability. All this evidence has raised the interest for targeting meiotic genes that are aberrantly expressed in somatic cancer cells. In a recent study, Allali-Hassani and colleagues reported the discovery of a potent and selective PRDM9 inhibitor (MRK-740) (Allali-Hassani et al., 2019). In HEK293T cells, MRK-740 specifically and directly inhibited PRDM9 catalytic activity on chromatin, reducing H3K4 methylation at intragenic and intergenic target sites. However, MRK-740 did not reveal any significant effect on proliferation of several cancer cell lines tested, indicating that at least for the cell lines tested their proliferation was not PRDM9-dependent (Allali-Hassani et al., 2019).

DMC1

Similarly to other genes involved in meiotic recombination, DMC1 was found to be ectopically expressed in various cancer cell lines including cervical (Erenpreisa et al., 2009), colon (Ianzini et al., 2009), breast (Salmina et al., 2019), glioblastoma (Rivera et al., 2015) and lymphoma cancer cell lines (Kalejs et al., 2006) as well as in CTCL biopsy samples (Gantchev et al., 2020). Interestingly, the upregulation of DMC1 was reported in a number of studies to drive the resistance of cancer cells to various cytotoxic and genotoxic agents (Kalejs et al., 2006; Erenpreisa et al., 2009; Ianzini et al., 2009; Rivera et al., 2015; Salmina et al., 2019). When challenged with high doses of ionizing radiation, tumor cells can escape cell death by transient endopolyploidisation (Illidge, 2000). While most of these polyploid cells will undergo cell death following aberrant mitosis (mitotic catastrophe), some will undergo genome reduction giving rise to viable tumor cells with reduced ploidy that can resume the mitotic cell cycle and are resistant to the treatment (Illidge, 2000). Experiments conducted with the large-scale digital cell analysis system, show that meiosis-specific genes such as DMC1, are expressed in the polyploid cells during depolyploidization allowing them to escape radiation-induced cell death (Ianzini et al., 2009). The study suggests that tumor cells might take advantage of the temporary change from a pro-mitotic to a pro-meiotic division regimen to facilitate depolyploidization and restore the proliferative state of the tumor cell population (Ianzini et al., 2009). A few years later, another study investigated the aberrant activity of DMC1 in glioma and showed that loss of DMC1 inhibited the activation of the DNA damage response and increased radiosensitivity. Furthermore, loss of DMC1 reduced tumor growth and prolonged survival *in vivo* (Rivera et al., 2015). These data suggest that the activation of meiotic repair genes in neoplastic cells selectively provides tumor cells with a repair mechanism to evade cell death caused by DNA damage, while at the same timeincreasing genetic diversity to drive clonal evolution (Rivera et al., 2015).

MEIOB

Analysis of multiple independent transcriptome databases containing both normal and tumor samples, identified the aberrant activation of MEIOB in lung adenocarcinomas (Wang et al., 2016). In the same study its meiotic partner, *SPATA22,* was also found to be aberrantly activated and co-expressed with *MEIOB*. Expression of MEIOB was also greatly enhanced in several lung cancer cell lines after treatment with the DNA methylation inhibitor 5-Aza-2′-deoxycytidine, known to induce the expression of certain meiotic genes by the demethylation of promoter CpG islands (De Smet et al., 1999). More recently, MEIOB aberrant expression was reported *in vitro* and *in vivo* models for TNBCs as well as in patients, where it correlated with poor survival (Gu et al., 2021). The authors of the study showed that MEIOB significantly promoted the proliferation of TNBC cells as well as DSBs repair. However, in contrast to its function in meiosis, MEIOB expression mediated homologous recombination deficiency (HRD) through the activation of polyADP-ribose polymerase (PARP). Furthermore, MEIOB was shown to confer sensitivity to PARP

inhibitors *in vitro*, as well as in a PDX model of TNBC (Gu et al., 2021). Together this suggests that MEIOB expression could be useful as a predictive biomarker of PARP inhibitor response in TNBC.

Genes of the Cohesin Complex

Consistent with roles in chromosome segregation and regulation of gene expression, aberrant expression and malfunctioning of cohesins is expected to be associated with cancer development (Losada, 2014). Indeed, several studies reported that meiosis-specific cohesins are aberrantly expressed in different types of somatic cancers.

STAG3

As most meiosis-specific genes, STAG3 is silenced in somatic cells by methylation of histone H3 on lysines 9 and 27 (Storre et al., 2005). However, reactivation of the cancer testis antigen STAG3 has been reported in cancers. For instance, mutations on the *STAG3* gene in cases of colorectal cancers have been identified (Barber et al., 2008). While it has still to be clarified whether the aneuploidy and tumorigenesis observed in these cancers are due to altered gene expression or due to chromosome mis-segregation (or both), the authors suggest that these mutations may lead to chromosome instability. Aberrant expression of STAG3 was also reported in patient-derived lymphocytes isolated from a CTCL patient as well as in skin biopsy samples from Sézary Syndrome patient (Tsang et al., 2018; Gantchev et al., 2020). Microarray analysis associated *STAG3* gene expression with tumorigenicity in ovarian cancer cell lines (Notaridou et al., 2011) while another study reported that multiple meiotic genes, including *STAG3*, are aberrantly activated during mitotic catastrophe in lymphoma cells after irradiation and may mediate chromosomal mis-segregation and genome reduction (Kalejs et al., 2006).

REC8

One of the first indications of a role for REC8 in cancer progression comes from a study that revealed REC8 upregulation in *Tp53*-mutated lymphoma cells after irradiation. REC8-augmented expression induced mitotic catastrophe and the generation of endopolyploid tumor cells (Kalejs et al., 2006). Similar findings were reported in additional endopolyploid p53-deficient tumor cells, where REC8 upregulation upon irradiation induced pseudomeiotic chromosome segregation events that enabled them to survive genotoxic treatment (Erenpreisa et al., 2009). A few years later, the work of Grewal et al. in fission yeast significantly contributed to the understanding of the mechanistic role of REC8 in cancer progression (Folco et al., 2017). The authors found that upregulation of REC8 expression was caused by the dysregulation of the Mmi1 pathway, which plays a crucial role in suppressing meiotic genes during mitotic proliferation (Harigaya et al., 2006). This causes high levels of chromosome mis-segregation events in mitotically dividing diploid cells, including high levels of uniparent disomy (UPD), a phenomenon that is linked to congenital disorders (Mobasheri et al., 2007)and various cancers (Tuna et al., 2009; Andersen and Petes, 2012), where it can drive loss of heterozygosity. Strikingly, REC8 overexpression in mitotically dividing diploid cells was sufficient to induce UPD, suggesting that the expression of a single meiotic cohesin gene is enough to promote meiosis-like reductional segregation of homologues in mitotic cells. In contrast to other meiotic genes, reactivation of REC8 in mitotic cells was also shown to play a tumor suppressor role in certain cancer cell lines, such us gastric cancer cells where induced overexpression of REC8 inhibited cell proliferation, invasion and migration (Yu et al., 2017; Zhao et al., 2018). However, the role of REC8 as a tumor suppressor remains elusive and further studies are needed to decipher how reactivation of a cohesin protein could protect cells from cancer progression.

Genes of the Synaptonemal Complex

The formation of the SC is mediated by proteinaceous axial structures, which include the central SYCP1 and the two lateral SYCP2 and SYCP3 components. Remarkably, re-expression of synaptonemal complex genes has been implicated in cancer to modulate the level of genome integrity (Gantchev et al., 2020; Hosoya and Miyagawa, 2021).

SYCP1

Aberrant expression of SYCP1 was first reported in melanoma, breast cancer, glioma, stomach cancer, NSCLC and renal carcinoma (Tureci et al., 1998). Subsequently, elevated SYPC1 expression was also reported in other types of tumors and cancer cell lines including gastric (Mashino et al., 2001), hepatocellular (Chen et al., 2001), pancreatic adenocarcinomas (Kubuschok et al., 2004), head and neck squamous cell carcinoma (Atanackovic et al., 2006), meningiomas, astrocytomas and oligodendrogliomas (Sahin et al., 2000), medulloblastomas (Oba-Shinjo et al., 2008) and testicular germ cell tumors (Zhang et al., 2005). SYCP1 expression was also detected in various hematological malignancies such as myelomas, acute lymphatic leukemia (AML), chronic myeloid leukemias (Lim et al., 1999), acute lymphocytic leukemias (Niemeyer et al., 2003), chronic lymphocytic leukemia, B-Cell lymphomas, Burkitt's lymphomas, lymphoblastic lymphomas (Xie et al., 2003) and non-hodgkin's lymphomas (Huang et al., 2002). Despite the expression of SYCP1 in a vast variety of tumors, there is currently no solid evidence describing the consequence of ectopic expression in somatic cancer cells or the underlying mechanism of action.

SYCP3

SYPC3 expression has also been documented in various cancers, including NSCLC (Kitano et al., 2017), acute lymphoblastic leukemia (Niemeyer et al., 2003), breast cancers, brain, gastrointestinal, skin tumors (Mobasheri et al., 2007) and cervical cancers (Hanbyoul Cho et al., 2014). It was reported that SYCP3 expression can be induced in the colorectal carcinoma cell line DLD1 after treatment with the demethylating agent 5-azacytidine, indicating that SYCP3 expression in mitotic cells is regulated by a demethylation-dependent process, similarly to other meiotic genes (Hosoya et al., 2012). The clinical relevance of SYCP3 expression was described in cervical cancer and NSCLC. Cho et al., examined SYCP3 expression in tumor specimens from 181 cervical cancer and 400 cervical intraepithelial neoplasia (CIN) patients by immunohistochemistry and analyzed the correlation between SYCP3 expression and clinicopathologic factors or survival. High expression of SYCP3 was significantly associated with late stage and high grade. At a molecular level, SYCP3 expression positively correlated with pAKT protein levels, suggesting that SYCP3 role in carcinogenesis may be mediated by an

activated AKT signaling (Hanbyoul Cho et al., 2014). In NSCLC, there are two studies describing the clinical relevance of SYCP3 expression. Immunohistochemical and tissue microarray analysis of NSCLC patient samples revealed high cytoplasmic SYCP3 expression, which correlates with early stage NSCLC, lymph node metastasis, pleural invasion and poor survival (Chung et al., 2013). Consistent with these data, increased SYCP3 expression was also detected in another immunohistochemical analysis in NSCLC cases with lymph node metastasis (Kitano et al., 2017). In this study, SYPC3 expression positively correlated with VEGF-C and VEGF-D expression, which are both involved in NSCLC lymphangiogenesis and metastatic spread to lymph nodes (Kitano et al., 2017). Mechanistically, SYCP3 expression outside the meiotic context has been shown to disrupt the activity of the tumor-suppressing recombination regulator BRCA2 (Hosoya et al., 2012). In SYCP3-expressing somatic cells, the BRCA2-mediated recruitment of RAD51 to the break site is in fact inhibited, resulting in defective sister-chromatid recombination. The authors of the study further show that expression of SYCP3 inhibits homologous recombination, inducing hypersensitivity to DNA-damaging agents such as PARP inhibitors and chromosomal instability. These findings highlight a new mechanism for genomic instability and extend the range of PARP-inhibitor sensitive tumors to those expressing SYCP3 (Hosoya et al., 2012).

SUMMARY

From these studies, it emerges that the ectopic activation of meiotic genes is detected in a wide variety of cancers, where it drives genomic instability and cancer progression. Even if cancer cells are not dependent on these genes for normal growth, they may become essential in tumors (but not in healthy tissues) to tackle endogenous DNA damage or DNA lesions induced by anticancer therapies. Indeed, most of the meiotic genes that are aberrantly expressed in cancer cells have a direct or indirect effect on pathways that are responsible for the repair of the DSBs induced by anticancer therapies. Examples include the HORMAD1/2, MND1, MEIOB and SYCP3 genes, which directly influence the HR activity of cancer cells. Their loss may induce sensitivity to agents that put more pressure on a functional HR pathway, such as PARP inhibitors. Other genes, including DMC1, STAG3 and REC8, allow somatic cancer cells to escape radiation-induced cell death without directly affecting the intracellular DNA repair pathways. Instead, they appear to promote meiosis-like reductional segregation of homologues in polyploid cells and thereby restore the proliferative state of the tumor cell population. For SPO11 and PRDM9, which induce DNA strand breaks and create crossover events in cancer cells, one can speculate that their activation in cancer cells drives genomic instability and might therefore increase the sensitivity of these cells to DNA-damaging agents.

The expression of meiotic genes in somatic cells appears to provide an evolutionary advantage for cancer initiation and progression. Such re-expression occurs via different mechanisms, including gain in copy number, increased expression following a genotoxic stress, and most frequently, via demethylation of meiotic gene promoters. In addition to promoting genomic instability, the activation of germ cell genes in mitotic cells influences how cells handle genomic instability.

While the re-expression of meiosis-specific genes promotes cancer progression, it may provide a new vulnerability that can be exploited therapeutically. As ectopic expression of meiotic genes has been shown to affect the response of tumor cells to anticancer therapies, it might be used as a predictive biomarker of therapy response and thus guide treatments' decision in the clinic. Further, meiotic genes represent promising candidate targets for cancer immunotherapy with little risk of side effects, due to high tumor specificity and immunogenicity. Since germ cells in adults lack HLA-class I molecules and cannot present the antigens to T cells, meiotic genes expressed in cancer cells have the capacity to promote immune responses that are strictly cancer specific. There are currently two immunotherapy strategies that are being tested in clinical settings, which exploit meiotic genes as cancer antigens: adoptive transfer, where recombinant T-cell receptors specific for cancer antigen epitopes are inserted into patient T cells and transferred back to patients, and vaccination, which stimulates the patient's intrinsic immune response to cancer antigens thanks to the use of immunogenic peptides (Gjerstorff et al., 2015). The therapeutic function of these two approaches is currently being tested in a variety of clinical settings and recent clinical trials have provided encouraging results (Gjerstorff et al., 2015).

We therefore think that studying the role of meiotic genes in somatic cancers is an interesting area to further explore, particularly in the context of DSB repair. We may also find out that several of the genes that we link to meiosis-specific exchange of genetic information actually have an additional and thus far unknown role in homology-directed DNA repair in somatic cells, even in non-transformed ones. It may not be remnants of embryonic tissue, but rather remnant DSB repair pathways that are reactivated to promote cancer growth.

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The Pathogenic R3052W BRCA2 Variant Disrupts Homology-Directed Repair by Failing to Localize to the Nucleus

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The BRCA2 germline missense variant, R3052W, resides in the DNA binding domain and has been previously classified as a pathogenic allele. In this study, we sought to determine how R3052W alters the cellular functions of BRCA2 in the DNA damage response. The BRCA2 R3052W mutated protein exacerbates genome instability, is unable to rescue homology-directed repair, and fails to complement cell survival following exposure to PARP inhibitors and crosslinking drugs. Surprisingly, despite anticipated defects in DNA binding or RAD51-mediated DNA strand exchange, the BRCA2 R3052W protein mislocalizes to the cytoplasm precluding its ability to perform any DNA repair functions. Rather than acting as a simple loss-of-function mutation, R3052W behaves as a dominant negative allele, likely by sequestering RAD51 in the cytoplasm.

Keywords: BRCA2, R3052W, homology-directed repair, nuclear localization, DNA repair, DSS1, RAD51

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INTRODUCTION

BReast CAncer Susceptibility Gene 2 (BRCA2), identified in the early 1990s, is a hereditary breast and ovarian cancer gene which codes for a 3,418 amino acid protein with several identifiable functional and structural domains and numerous interacting partners (Wooster et al., 1994; Wooster et al., 1995; Easton et al., 1997) (**Figure 1A** and reviewed in the work of Jimenez-Sainz and Jensen, 2021). The BRCA2 DNA binding domain (DBD) was crystallized in complex with DSS1 (PDB ID: 1IYJ, 736 amino acids) illuminating an alpha-helical domain, tower domain, and three tandem oligonucleotide/oligosaccharide-binding folds (OB-folds 1–3) (Yang et al., 2002). DSS1 is a small acidic protein (70 amino acids) proposed to drive nuclear localization of BRCA2 and promote BRCA2 protein stability (Gudmundsdottir et al., 2004; Kojic et al., 2005; Li et al., 2006). BRCA2, together with DSS1, facilitates the exchange of replication protein A (RPA) for RAD51 on resected single-stranded DNA (ssDNA) (Yang et al., 2002; Zhao et al., 2015). The crystal structure also revealed that BRCA2 can bind an ssDNA oligonucleotide making several contacts with the OB2 and OB3 folds located in the DBD (Yang et al., 2002). Our previous study demonstrated that a BRCA2 protein fragment encompassing the DBD and C-terminal domain (CTD) can localize to the nucleus, bind 3′tail DNA, and is capable of minimally stimulating RAD51-mediated DNA strand exchange (Chatterjee et al., 2016).

BRCA2 possesses several putative nuclear localization (NLS) and nuclear export (NES) sequences (Spain et al., 1999; Yano et al., 2000; Han et al., 2008; Jeyasekharan et al., 2013) distributed throughout the domains of the protein. Three of the NLS (NLS1, NLS2, and NLS3) located at the C-terminus of BRCA2 have previously been proposed as the primary NLSs for the nuclear

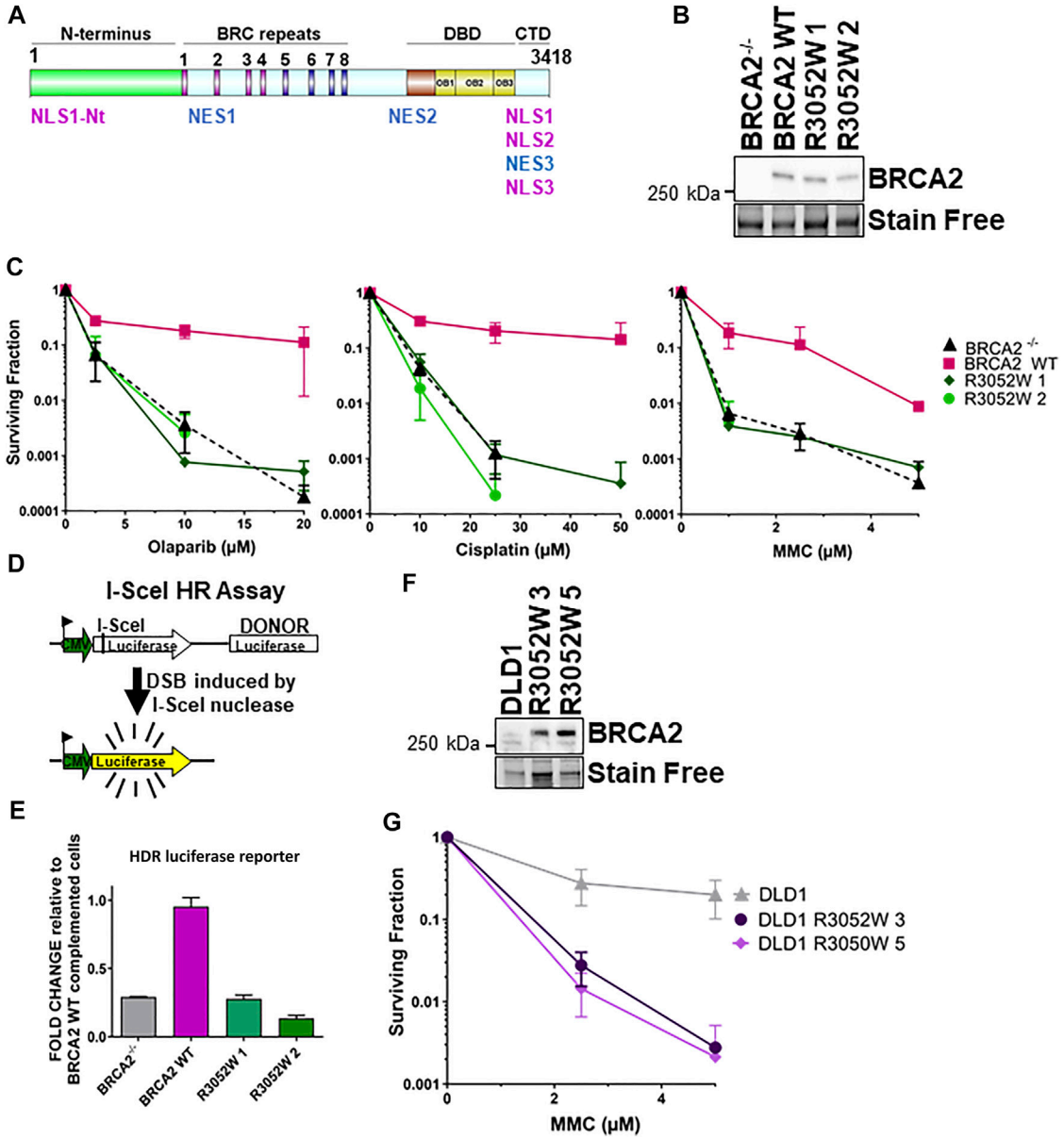

FIGURE 1 | The BRCA2 R3052W mutation fails to complement chemotherapeutic sensitivity and homology-directed repair functions in BRCA2 knockout cells. Overexpression of R3052W in DLD1 parental BRCA2 wild-type cells confers sensitivity to MMC DNA damage. **(A)** BRCA2 protein schematic depicting domain organization: N-terminus, BRC repeats DNA binding domain (DBD), and C-terminal domain (CTD). BRCA2 nuclear localization and export sequences are listed. NLS1-Nt (433–436 aa), NLS1 (3,265–3,270 aa) NLS2 (3,311–3,317 aa), NLS3 (3,381–3,385 aa), NES1 (1,383–1,392 aa), NES2 (2,682–2,698 aa) and NES3 (3,270–3,280 aa). **(B)** Western blot of total cellular lysates from DLD-1 BRCA2$^{-/-}$ cells stably transfected with full-length BRCA2 cDNA constructs: BRCA2 Wild Type (WT) and BRCA2 R3052W (1 and 2 correspond to two independent clones). BRCA2 cDNAs contain a 2XMBP tag on the N-terminus and were detected with an MBP antibody. 2XMBP-BRCA2 (470 kDa). Stain Free is a protein-loading control. **(C)** Clonogenic survival analyses of BRCA2 WT versus the two independent R3052W clones after treatment with Olaparib, cisplatin, and mitomycin C. **(D)** Schematic of I-SceI nuclease-induced DSB HDR luciferase assay. **(E)** Quantification of luciferase activity (normalized to BRCA2 WT as 1). Error bars are SD (n = 5). **(F)** Western blot of total cellular lysates from DLD-1 parental cells (these cells express a wild-type allele of BRCA2) stably transfected with R3052W (3 and 5 correspond to two independent clones) full-length 2XMBP-BRCA2 cDNA constructs. BRCA2 was detected with an MBP antibody. **(G)** Clonogenic survival analyses of DLD parental clones upon treatment with mitomycin C.

localization of BRCA2 (Spain et al., 1999) (**see Figure 1A**). BRCA2 nuclear localization is essential for the protein to carry out homology-directed repair (HDR), replication fork protection, and cell cycle checkpoint functions (Scully and Livingston, 2000; Venkitaraman, 2002; Schlacher et al., 2011; Mijic et al., 2017; Taglialatela et al., 2017; Eckelmann et al., 2020; Rickman et al., 2020). Importantly, truncating mutations upstream of NLS1, 2, and 3 are predicted to lead to loss of nuclear localization and, thus, are associated with HDR dysfunction and cancer predisposition (Spain et al., 1999; Sakai et al., 2009).

The R3052 residue of BRCA2 is located in exon 23 situated between OB folds 2 and 3 in the DBD. The R3052W mutation (c.9154 C > T) co-segregates with BRCA2-related disease (breast, ovarian, and prostate cancer) in several families (Farrugia et al., 2008; Gomez Garcia et al., 2009) and has been classified as pathogenic according to the ClinVar and Breast Cancer Information Core (BIC) databases (Variation ID: 52763, rs45580035, ExAC 0.006%).

Prior functional studies implicated R3052W as pathogenic due to HDR impairment and the inability to rescue cell viability in a mouse embryonic stem cell model (Farrugia et al., 2008; Kuznetsov et al., 2008; Guidugli et al., 2013; Cunningham et al., 2014; Hendriks et al., 2014; Shimelis et al., 2017; Guidugli et al., 2018; Hart et al., 2019). In this study, we analyzed the functional effects of the R3052W missense mutation incorporated into our full-length BRCA2 construct and expressed the mutant protein in a viable BRCA2 deficient human cell model. The full-length BRCA2 R3052W protein appears stable as expression levels are similar to the WT protein. We find that R3052W is unable to rescue the sensitivity of BRCA2 deficient cells to crosslinking agents and PARP inhibitors, is defective in executing HDR, and these defects arise due to the mislocalization of the protein in the cytosol. To our surprise, expression of the R3052W mutant in a BRCA2 WT background exacerbates genomic instability and sensitivity to DNA damage suggesting a dominant negative effect rather than a simple loss-of-function mutation. We postulate that cytosolic localization of R3052W could be due to protein aggregation or nuclear import defects, but not to the loss of DSS1 binding leading to active nuclear export, as we confirm that DSS1 binding remains intact in the R3052W mutant.

MATERIALS AND METHODS

Constructs

Point mutation R3052W was cloned into the phCMV1 2XMBP-BRCA2 and phCMV1 2XMBP-BRCA2 DBD + CTD only sequences, *via* site-directed mutagenesis (cloning strategy and primers available upon request). We verified the putative recombinant clones through restriction digestion and sequencing analysis. The previously described 2XMBP tag (Jensen et al., 2010) was placed in-frame at the N-terminus of all proteins separated by an Asparagine linker and the PreScission Protease cleavage sequence. To clone N-terminal fusions of GFP and mCherry to BRCA2 WT and R3052W, 2XMBP tag was removed from the constructs and PCR products of GFP and mCherry were digested with KpnI/NotI and inserted in phCMV1 BRCA2 and R3052W constructs. All the constructs were verified by sequencing analysis.

Cell Culture Transient Transfections

All culture media were supplemented with 10% fetal bovine serum (FBS). HEK293T cells were cultured in DMEM (source Jensen et al., 2010); DLD1 cells were cultured in RPMI. Transient transfections were carried out with Turbofect (Thermo Scientific) (2 μg of DNA, 6-well plates) in HEK293T cells and with JetOptimus (Polyplus Transfection) in DLD1 cells following the manufacturer's protocol. DLD1 BRCA2$^{-/-}$ cells 50–60% confluent in 24-well plates (15000 cells per well) were transiently transfected with 0.5 μg of 2XMBP DBD + CTD BRCA2 WT or 2XMBP DBD + CTD R3052W construct with jetOPTIMUS reagent (Polyplus). Then, 48 h post-transfection cells were processed for immunofluorescence. Calcium Phosphate (25 μg of DNA, 15 cm^2 plate, see BRCA2 purification section) was used on large scale in HEK293T cells (Jensen et al., 2010). All cell lines were tested regularly for mycoplasma (Mycoalert, Lonza) and confirmed through STR profiling.

Generation of Stable Cell Lines

Human colorectal adenocarcinoma DLD-1 BRCA2$^{-/-}$ and DLD1 parental cells [Horizon Discovery, originally generated by (Hucl et al., 2008)] were stably transfected with 2 μg of DNA using Lipofectamine 3000 (Invitrogen). After 48 h, the cells were trypsinized and diluted 1:2, 1:4, and 1:8 into 100 mm plates containing 1 mg/ml G418. Single-cell colonies were picked into 96-well plates and subsequently cultured into 24-well plates, 12-well plates, and 6-well plates. Positive clones were isolated, and protein expression was detected by western blot and immunofluorescence analyses.

Western Blots and Amylose Pulldowns

Human embryonic kidney HEK293T cells 70% confluent in 6-well plates were transiently transfected with 2 μg of the phCMV1 mammalian expression vector containing a 2XMBP fusion to the full-length of BRCA2 using TurboFect reagent (Thermo Scientific) (Jensen et al., 2010). 1ug of HA-DSS1 was transfected into the cells as described before (Zhao et al., 2015). 0.5 μg of 2XMBP empty construct was transfected into these cells and an untransfected well was also seeded as a negative control. The cells were lysed 48 h after transfection in 200 μL of lysis buffer: 50 mm HEPES (pH 7.5), 250 mm NaCl, 5 mm EDTA, 1% Igepal CA-630, 3 mm $MgCl_2$, 10 mm DTT and protease inhibitor cocktail (Roche). Cell extracts were batch bound to amylose resin (NEB) for 2 h to capture the 2XMBP tagged BRCA2 proteins. Total cellular lysate aliquots were taken before batch binding for control analysis. Total cellular lysates and amylose pulldown samples were run on a 4–15% gradient SDS-PAGE TGX stain-free gel (Bio-Rad 456-8086), which was subsequently transferred to an Immobilon-P membrane (Merck Millipore IPVH00010) in 1X Tris/glycine buffer (diluted from 10X Tris/glycine buffer, Bio-Rad 161-0771). The membrane was blocked in 5% milk in 1X TBS-T (diluted from 10X TBS-T: 0.1 M Tris base, 1.5 M NaCl, 0.5% Tween-20). Washes and antibody incubations were done with 1X TBS-T. Primary mouse antibodies against MBP (NEB E8032L, 1:5,000), RAD51 (Novus Biologicals 14b4, 1:1,000), and primary rabbit antibody against BRCA2 (Abcam, ab27976), GAPDH (Sta Cruz Biotechnology 0411m #sc-47724, 1:1,000) and HA (Cell Signaling C2974 mAB#3724, 1:1,000) were used for western blotting. Membranes were then incubated with secondary mouse and rabbit antibodies (HRP-conjugated, Santa Cruz Biotechnology sc-516102, and sc-2004, respectively). Protein expression was visualized by incubating with Clarity Western ECL substrate (Bio-Rad 170-5061) for

3 min and scanning with a ChemiDoc MP imaging system (Bio-Rad).

Clonogenic Survival Assay

Stable cell clones generated from DLD-1 BRCA2$^{-/-}$ cells were serially diluted and seeded into 6-well plates at concentrations of 100 and 500 cells per well in triplicate for plating efficiency. Simultaneously, cells were seeded for treatment in 6-well plates at 1,000 and 10,000 cells per well in triplicate per treatment dosage. 24 h after seeding, cells were treated with indicated doses of mitomycin C (MMC, 1.5 mm stock in water) cisplatin (100 mm stock in DMSO) for 1 h in serum-free media, and Olaparib (50 mm stock in DMSO) for 24 h. Following treatment, media was aspirated, and cells were washed with 1X PBS and re-fed with fresh media containing FBS. Cells were cultured for 14 days to allow colony formation, after which they were stained with crystal violet staining solution (0.25% crystal violet, 3.5% formaldehyde, 72% methanol). Colonies containing 50 or more cells were scored and surviving fractions were determined.

Homology-Directed Repair Luciferase Assay

The HDR luciferase reporter gene (gWiz.Lux-5′-3′Luc) was constructed from the parental vector gWiz Luciferase (Genlantis) as previously described (Chatterjee et al., 2016). An I-SceI site was created in the luciferase ORF by ligating the following annealed oligonucleotides: 5′SCEILUC (5′-CGC TAG GGA TAA CAG GGT AAT-3′) and 3′SCEILUC (5′-CGA TTA CCC TGT TAT CCC TAG-3′) into a BstBI site. The 7 amino acid insertion at amino acid 56 of the luciferase ORF ablates luciferase activity. A second luciferase ORF was ligated into the XmnI site 700 bp downstream from the first luciferase ORF (see **Figure 1D**). The second luciferase ORF lacks a promoter but can be utilized as a donor in homology-directed repair of the first luciferase ORF upon generation of a double-strand break by expression of the I-SceI nuclease. The pSce-MJ mammalian I-SceI expression vector was a kind gift from Dr. Fen Xia.

To perform the assay, cells were seeded into 6-well plates at 2.5 × 10^5 cells/well. 24 h later, cells were transfected with 200 ng of gWiz.Lux-5′-3′Luc vector and 1 μg of the I-SceI expression vector using Lipofectamine 3,000. gWiz.Lux-5′-3′Luc or gWiz.Lux vectors were transfected alone and used as negative and positive controls, respectively. Cells were harvested 48 h post-transfection in 200 μL of lysis buffer described above prior to luminometer analysis. Luminescence was measured using an integration time of 5 s with 40 μL of the lysate plus 100 μL of luciferin substrate (One-Glo luciferase assay, Promega). Luciferase values were measured as independent duplicates in each experiment. The raw data were normalized to protein levels and the values plotted were calculated by setting the full-length BRCA2 mean value to 100%. The data presented the average of three independent experiments.

Laser Microirradiation

Sterile gridded glass coverslips (#1.5H D263 Schott glass cat. #10816; ibidi inc.) were coated with collagen (cat. #125-50, Millipore-Sigma) by incubation at 37°C for 1 h. Subsequently, exponentially growing DLD1$^{-/-}$, WT BRCA2, and R3052W stable cells were plated into coverslips and incubated overnight. After overnight incubation cells were treated with 10 μM BrdU (19–160; EMD Millipore) and incubated for additional 48 h prior to irradiation. Shortly before the irradiation, coverslips with cells were washed with PBS and placed in a 50 mm glass bottom (#1.5 glass) dish (P50G-1.5-30-F, Mattek Corp.) in a low-absorption medium (RPMI; 31053; Thermo Fisher Scientific). A dish with cells was mounted on a Leica TCS SP8 X microscope system (Leica Microsystems) inside an incubator chamber at 37°C with 5% CO2 supplementation. For laser microirradiation, a Leica HC PL APO 40 ×/1.30 Oil CS2 objective with a zoom factor of 0.75 was used to establish the expected field of irradiation, which encompassed up to 200 cells in a single frame. 40 horizontal stripe masks (5 px wide) were placed in a 512 px × 512 px view field. The cells were irradiated 55 times with a 405 nm diode laser at 95% with FRAP booster with a pixel dwell time of 3.75 μs. One full frame irradiation lasted 1.985 s, with a total irradiation time of 109.175 s for 55 iterations (130 Hz bidirectional, frame rate 0.503/sec). A laser power exiting the objective was equal to 1.14 J/sec. Consequently, each pixel of the irradiation masks was exposed to 4.275 μJ per iteration resulting in a total energy of 235.125 μJ. Our experimental conditions induce ssDNA and dsDNA breaks. The cells were not synchronized so only 20% of the cellular population showed RAD51 recruitment to the micro-irradiated area. After irradiation, cells were fixed and permeabilized with 3% formaldehyde, 0.2% Triton X-100 and 8% sucrose for 15 min at RT and were subject to immunofluorescent staining protocol.

Immunofluorescence Staining, Imaging, and Quantification

Stable cell clones generated from DLD-1 BRCA2$^{-/-}$ cells were grown on coverslips at 10^5 cells/well in a 24-well plate for 24 h. Cells were washed twice with 1X PBS, fixed in 1% paraformaldehyde-2% sucrose in 1X PBS for 15 min at room temperature, washed twice with 1X PBS, permeabilized with methanol for 30 min at −20°C, then washed two more times with 1X PBS, and finally incubated with 0.5% triton in PBS for 10 min. Samples were then blocked with 5% BSA in 1X PBS for 30 min at room temperature followed by subsequent incubation with primary antibodies against MBP (NEB E8032L, 1:200), gammaH2AX (Millipore, Ser 139, clone JBW301, 05-636, 1:100) and RAD51 (Proteintech 14961-1-AP, 1:100 or Abcam ab63801) in 5% BSA-0.05% TritonX-100 at 4°C overnight. The next day, cells were washed three times with 1X PBS and incubated with goat anti-rabbit and anti-mouse secondary antibodies conjugated to the fluorophores Alexa-488 and Alexa-546 (Thermo Fisher Scientific A11034 and A11003, respectively; 1:1,000). Coverslips were washed three times with 1X PBS, incubated with 30 nm DAPI for 5 min, and mounted on slides with FluorSave reagent (Calbiochem 345789). Immunofluorescence images were taken using a Keyence BZ-X800E All-in-One Fluorescent Microscope with a 40x or 60x objective lens. Cells were either untreated as control or irradiated

at 12 Gy using an X-Rad 320 Biological Irradiator and cells were collected at 6- and 24-hours post-irradiation for immunofluorescence protocol. For subcellular localization analysis of BRCA2 (red), RAD51 (green in Figures 3A, 4A and red in **Figure 5**) and DSS1 proteins, cells with nuclei (N), nuclei/cytosol (N/C) and cytosol (C) distribution of the proteins were scored and divided by the total number of cells (DAPI, blue) to obtain a ratio between 0 (no signal) to 1 (positive signal) and the ratios were represented. For micronuclei formation analysis all the micronuclei, as well as the total number of cells, were counted with DAPI staining and the percentage of cells with micronuclei was represented. At least 300 cells (3-5 microscope images in three independent experiments) were counted. For gammaH2AX and RAD51 foci quantification at least 300 cells (3-5 microscope images in three independent experiments) were counted. In the case of RAD51 foci analysis, cells with more than 5 foci within the nuclei were considered positive and in the case of gammaH2AX, cells with more than 10 foci within the nuclei were considered positive. Graph Pad PRISM version 9.3.1 was used to generate all graphs.

For microirradiation immunofluorescence staining cells were incubated for 2 h at RT in a blocking buffer solution containing: 5% normal goat serum (10000C; Invitrogen), 8% Sucrose, and 0.2% Triton X-100 in PBS. Subsequently, cells were incubated overnight at 4°C with primary antibodies: gammaH2AX (Millipore, Ser 139, clone JBW301, 05-636, 1:100) and RAD51 (Abcam ab63801). Following three washing steps with PBS +0.5% Triton X-100, cells were stained with secondary goat antibodies anti-rabbit Alexa-488 and anti-mouse Alexa-546 as indicated above. Next, cell nuclei were stained with DAPI 2.5 μg/ml; 1816957; Thermo Scientific) for 15 min at RT. Samples were washed three times with PBS +0.5% Triton X-100 for 5 min and then rinsed one time with PBS before mounting with DAKO Fluorescence Mounting Medium (S3023; Dako—Agilent Technologies).

Subsequently, images were acquired with a Nikon Eclipse Ti fluorescence microscope with a CFI Plan Apochromat Lambda 60x/1.4 Oil, WD 0.13 mm objective (Nikon Corporation), a CSU-W1 confocal spinning disk unit (50 μM disk pattern, Yokogawa), an iXon Ultra 888 EMCCD camera (Andor Technology), MLC 400B laser unit (Agilent Technologies) and NIS Elements 4.30 software (Nikon Corporation). Images were taken with three-quarters of the maximum intensity without overexposure. The pictures were saved as 1024 pixels × 1024 pixels, 16-bit multi-channel. nd2 files with no further editing. For stripes data quantification. nd2 files were additionally exported to 16-bit OME-TIFF format. For quantification of fluorescent intensities within damaged areas and nuclei overall, the ImageJ-based tool Stripenator was used (Oeck et al., 2019). For RAD51 protein fluorescent intensities, damaged area/background intensity ratios were calculated for each cell to normalize the damage intensity values to their own background. Consequently, if the damaged and background area have similar intensities because of no change in protein localization to the damaged site, a value of 1 would be obtained versus higher values if the damage was more intensely stained than the background and values lower than 1 if damage area was less intensely stained than the background. For the automatic quantification of RAD51 staining in the stripes, all cells in the same field were included so the difference in the average of RAD51 recruitment is smaller than expected due to the mixture of ssDNA and dsDNA breaks and the lack of cell cycle synchronization. Additionally, mean fluorescent intensities (MFIs) of RAD51 inside and outside of the cell nuclei were calculated using the ImageJ-based tool Focinator (Oeck et al., 2017). These ratios were normalized and are presented with respect to the area of field of view (220.16 microns × 220.16 microns) of each evaluated picture since on each analyzed picture was the different number of cells.

Nuclear Export Analysis

For nuclear export inhibitor experiments, stable cell clones generated from DLD-1 BRCA2$^{-/-}$ cells were grown on coverslips at 1 × 10^5 cells/well in a 24-well plate for 24 h. Then, cells were treated with 50 ug/ml of leptomycin B for different time points (0, 2, 4, 6 h) and processed as in the immunofluorescence imaging section. YFP-c-abl (gift from Dr. Anthony Koleske) and Rev 1.4 MP2K2 GFP (gift from Dr. Ane Olazabal) constructs were included as positive controls of nuclear retention upon Leptomycin B treatment (Henderson and Eleftheriou, 2000). In CRM1 silencing experiments, stable cell clones generated from DLD-1 BRCA2$^{-/-}$ cells were grown at 5 × 10^5 cells/well in a 6-well plate for 24 h. Then, 25 nM siRNA for CRM1 (ON-TARGETplus siRNA Dharmacon Set of four: 09, 10, 11, 12) were introduced into the cells with Dharmafect. After 24 h, cells were reseeded on coverslips 1 × 10^5 cells/well in a 24-well plate and kept for 24 h. The next day, cells were processed as in the immunofluorescence imaging section above. Non-targeting control was used as a negative control. siRNA for GAPDH was used as a positive control.

RESULTS

The BRCA2 R3052W Variant Does Not Rescue Chemotherapeutic Sensitivity or Homology-Directed Repair Deficiency

Several families in the BIC database with evidence of genetic linkage indicate that the BRCA2 R3052W variant is a pathogenic mutation with a high probability of future cancer risk (Farrugia et al., 2008;Gomez Garcia et al., 2009;Mohammadi et al., 2009; Capanu et al., 2011;Cunningham et al., 2014). The crystal structure of the carboxy terminus of BRCA2 (Yang et al., 2002) places the R3052 residue at a potentially critical interface between OB folds 2 and 3 (Kuznetsov et al., 2008) in the DBD. The R3052 residue (see **Supplementary Figure S1** for sequence alignment) is conserved amongst several different species suggesting that deviations from this amino acid are not tolerated throughout evolution. To gain further insight into the mechanistic nature of this variant, we expressed an N-terminal 2XMBP tagged full-length BRCA2 construct incorporating the R3052W mutation in a human DLD1 BRCA2 knockout cell line to directly compare against our previously generated wild-type (WT) complemented cell line (Chatterjee et al., 2016). Single-cell-

derived stable R3052W clones had comparable BRCA2 protein expression levels to the WT BRCA2 complemented cell line (**Figure 1B**). BRCA2 deficient cells are sensitive to crosslinking agents and PARPi (Davies et al., 2001;Bryant et al., 2005; Chatterjee et al., 2016). To determine if the R3052W mutation could rescue BRCA2 deficient cells similar to the WT protein, survival assays were performed (**Figure 1C**). Examination of two independently derived stable clones expressing the BRCA2 R3052W protein resulted in the same level of surviving cellular fraction as the BRCA2 knockout cells (empty vector) suggesting a complete loss-of-function in response to PARPi and crosslinking agents (**Figure 1C**). Similarly, the R3052W expressing cells were unable to repair a split luciferase reporter construct designed to measure HDR repair of a single I-Sce-induced DNA DSB (Scheme **Figure 1D**) (**Figure 1E**). Together, these results suggest that the R3052W mutation is incapable of providing the canonical HDR cellular functions of BRCA2. Studies in other DNA repair proteins (e.g., ATM) postulated that missense variants could exert dominant negative effects, however, it was unknown if BRCA2 missense variants previously described could elicit such an effect and increase cancer risk in the heterozygous state or in the ectopic expression state (Tavtigian et al., 2009). To test whether the R3052W mutation could impact functions of the WT BRCA2 protein, we expressed R3052W (**Figure 1F**) in DLD1 parental cells (containing one endogenous BRCA2 WT allele) and interrogated the cellular sensitivity to mitomycin C. As shown in **Figure 1G**, R3052W ectopic expression decreased the survival of DLD1 parental cells suggesting a dominant negative effect. Our results point toward a pathogenic component of the R3052W mutation that somehow disrupts the normal cellular functions of WT BRCA2, rather than a simple loss-of-function allele.

R3052W Increases Genomic Instability and Decreases RAD51 Foci Formation Upon Irradiation

Previous studies in a conditional BRCA2 knockout mouse embryonic stem cell model and VC8 BRCA2 mutant hamster cells demonstrated that R3052W can not rescue cell viability or HDR activity (Farrugia et al., 2008; Kuznetsov et al., 2008). However, information regarding the role of R3052W in maintaining genomic integrity and HDR activity in human cells has been lacking. To address this gap, we irradiated human cells stably expressing either the WT or R3052W protein with 12 Gy to track micronuclei formation. Micronuclei contain chromosomes, or damaged chromosome fragments, not incorporated into the nucleus during cell division and have been previously used as a readout of genomic instability (Luzhna et al., 2013). Our results demonstrate that expression of R3052W increases the percentage of cells with micronuclei in both a BRCA2 deficient ($BRCA2^{-/-}$) and a BRCA2 proficient background (DLD1) (**Figures 2A,B**). Next, we visualized RAD51 and gammaH2AX (γH2AX) foci formation as surrogates of HDR activation and DNA damage response respectively (**Figure 2C**) (Heddle et al., 1983; Ban et al., 2001; Haaf et al., 1995; Rothkamm et al., 2015; Chatterjee et al., 2016). RAD51 and γH2AX foci peaked at 6 h and were mostly resolved by 24 h following 12 Gy of ionizing radiation in WT complemented cells and the DLD1 parental cells (**Figures 2C–E**). However, R3052W expression in BRCA2 knockout cells displayed no visible nuclear RAD51 foci following ionizing radiation damage while γH2AX foci persisted at the 24 h timepoint. Interestingly, stable R3052W expression in DLD1 parental cells (with endogenous BRCA2) displayed a significantly lower percentage of cells with RAD51 foci at the 6 h time point and a higher proportion of cells with γH2AX foci at the 24 h timepoint unresolved, suggesting an HDR defect leading to a sustained DNA damage response **(Figures 2C–E).**

R3052W Mislocalizes to the Cytosol

In order to execute genomic integrity functions in both HDR of DNA DSBs and replication fork protection, it is essential that BRCA2 localizes to the nucleus (Spain et al., 1999;Jeyasekharan et al., 2013). To date, studies with BRCA2 fragments by the Venkitaraman group have shown that missense mutations in the DBD, including the R3052W mutation, are mislocalized to the cytosol due to impairment of DSS1 binding and/or active nuclear export (Jeyasekharan et al., 2013; Lee et al., 2021). However, these studies did not fully explore the cellular localization of all variants tested using the full-length BRCA2 protein nor did they address any changes in localization following DNA damage. We directly examined the localization of stably expressed WT BRCA2 and the R3052W mutant in our human BRCA2 knockout cell model by immunofluorescence under basal conditions (no exogenous DNA damage) (**Figure 3A**, quantification **Supplementary Figure S2A,B)**. To our surprise, and in agreement with studies using a BRCA2 fragment (Lee et al., 2021), the full-length R3052W protein localized to the cytosol **(Figure 3A**, upper right panel) whereas WT BRCA2 localized to the nucleus as expected **(Figure 3A** upper center panel) in most cells. Furthermore, transient transfection of WT BRCA2 and R3052W constructs fused at the N-terminus with GFP or mCherry in human 293T cells verified nuclear localization of the WT protein and cytoplasmic localization of R3052W utilizing live cell imaging (**Figure 3B**, **Supplementary Figure S2C**). As previously described by Lee et al., we confirmed that BRCA2 DBD + CTD localizes to the nucleus whereas R3052W DBD + CTD localizes to the cytosol (**Supplementary Figure S2D**). Finally, we found that the cellular localization of RAD51 correlated with the cellular compartment occupied by BRCA2 as most RAD51 signal was nuclear in cells expressing WT BRCA2 whereas the majority of RAD51 signal was present in the cytoplasm in R3052W cells (**Figure 3A**, lower panels, and quantification in **Supplementary Figure S2B**). By immunofluorescence, RAD51 appears as a diffuse signal in BRCA2 knockout cells (**Figure 3A**, lower left panel and quantification in **Supplementary Figure S2B**) likely due to lower expression levels (Magwood et al., 2013; Chatterjee et al., 2016) and loss of compartmentalization regulated by BRCA2. These results suggest that a large cellular pool of RAD51 is complexed with BRCA2 as proposed previously (Reuter et al., 2014).

We utilized laser microirradiation (LMI) to determine whether RAD51 could still be recruited to DNA damage and

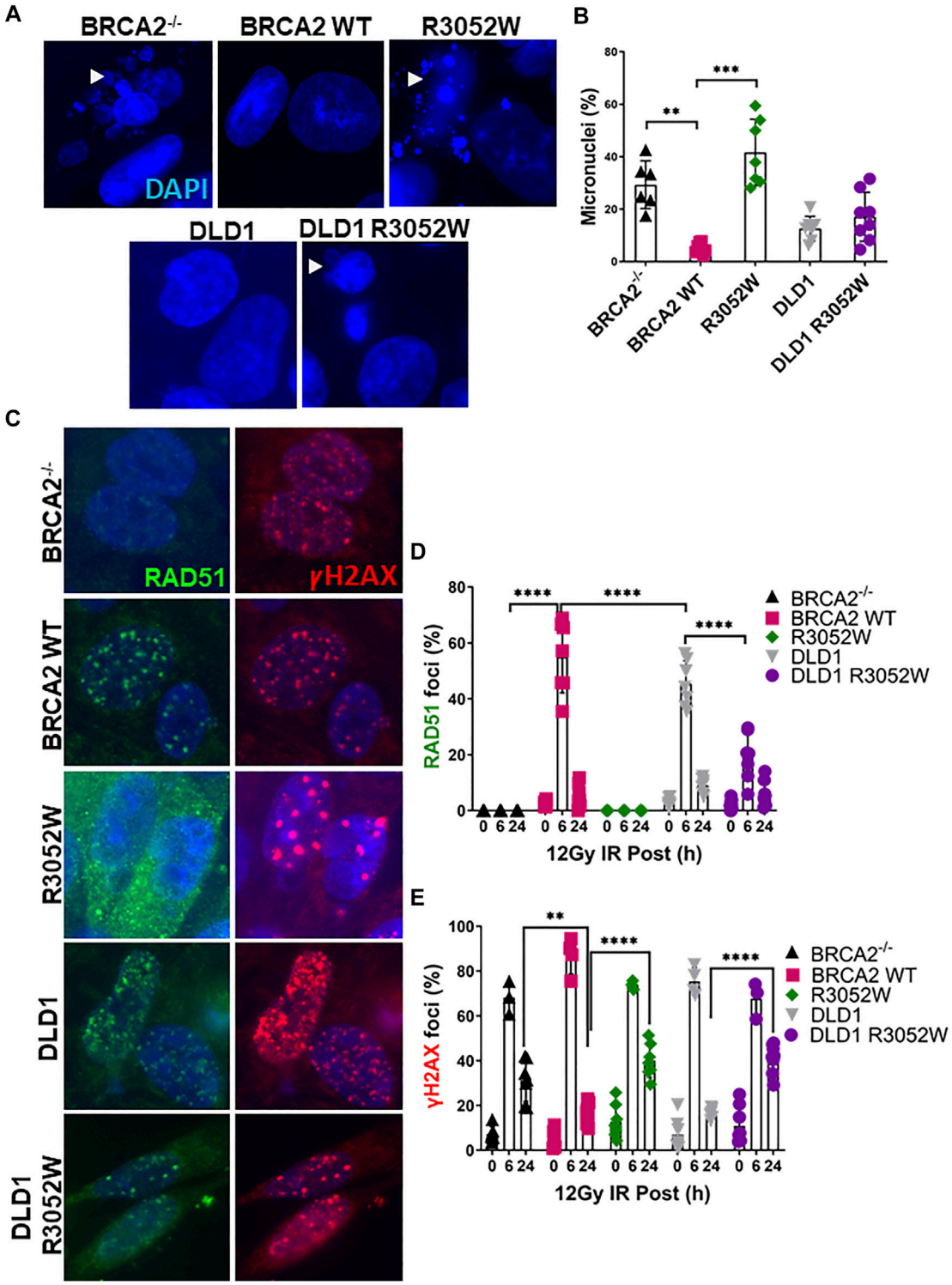

FIGURE 2 | The BRCA2 R3052W mutation exacerbates micronuclei formation and interferes with RAD51 foci formation in DLD1 parental and BRCA2 wild-type cells. **(A)** Representative immunofluorescent images of micronuclei (blue) in a DLD1 BRCA2 knockout cell line (top) expressing WT or the R3052W mutant and parental DLD1 cells (bottom) compared to a stable cell line expressing the R3052W protein. **(B)** Quantification of the percentage of cells with micronuclei. **(C)** Representative immunofluorescent images of RAD51 (green) and gammaH2AX (red) foci, and DAPI staining to visualize nuclei (blue). Cells were fixed and imaged 6 h post-IR (12 Gy). **(D)** Quantification of the percentage of cells with RAD51 foci. **(E)** Quantification of the percentage of cells with gammaH2AX. (Quantification represents 3 independent experiments and statistical analysis t-test and one-way ANOVA. ***p*-value < 0.01, ****p* value < 0.001, *****p* value < 0.0001).

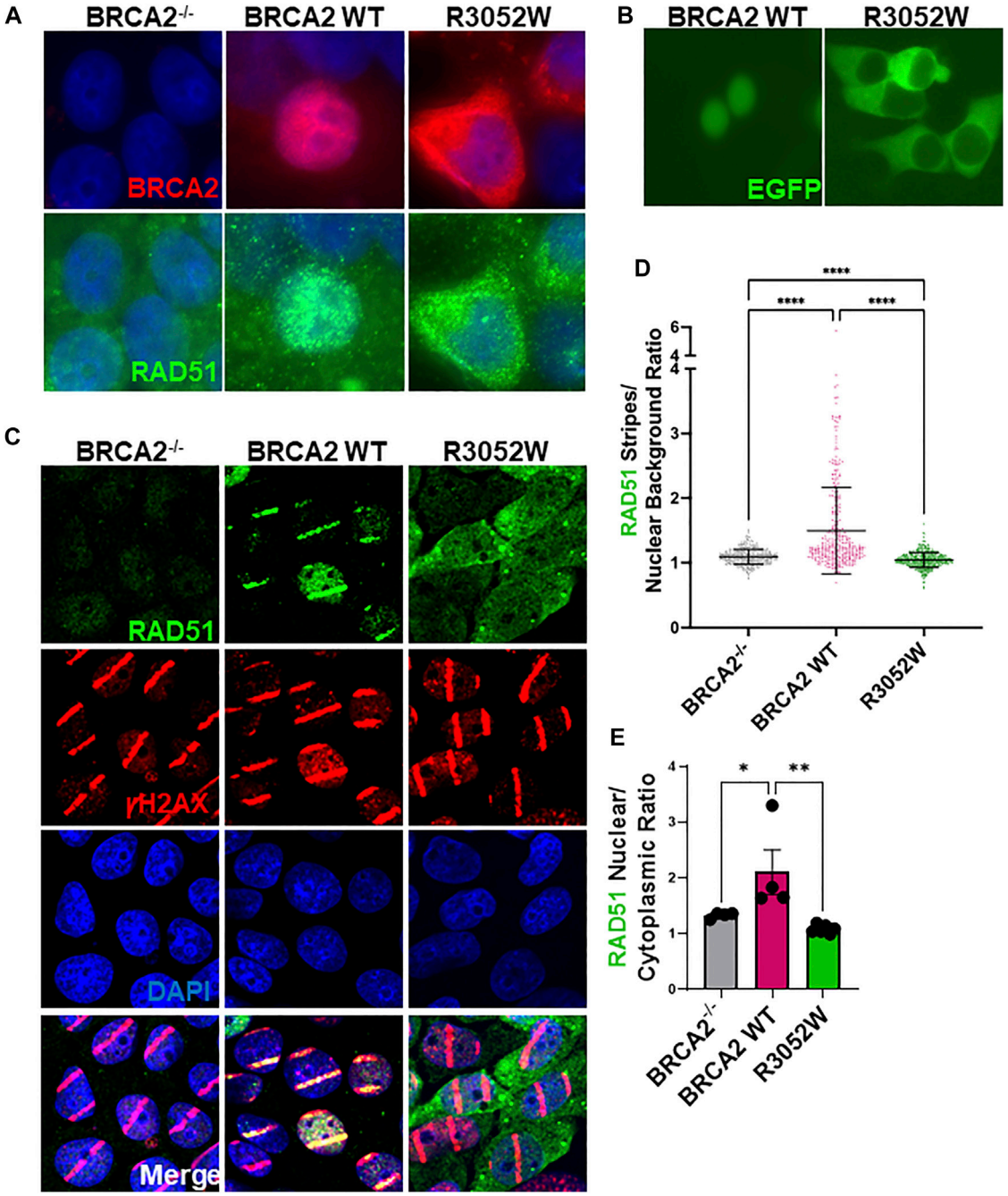

FIGURE 3 | The R3052W protein localizes to the cytosol. **(A)** Immunofluorescent localization of BRCA2 in untreated BRCA2 knockout cells (BRCA2$^{-/-}$), and stable cell lines expressing either BRCA2 WT or R3052W. Representative images of 2XMBP-BRCA2 (red, anti-MBP), RAD51 (green), and nuclei (blue). **(B)** Live images of BRCA2 knockout cells expressing either BRCA2 WT or R3052W fused to GFP at the N-terminus. **(C)** Representative immunofluorescence images of laser micro-irradiation experiments in BRCA2 knockout cells stably expressing BRCA2 WT or R3052W. DNA damage (stripes) are depicted in red (gammaH2AX), green (RAD51), and nuclei in blue (DAPI). **(D)** Quantification of RAD51 fluorescence intensity in damage areas (stripes) over the background in non-irradiated areas of respective laser micro-irradiated nuclei. Each data point represents a single analyzed nucleus, while the solid line is a mean value ± SD (Kruskal-Wallis test with Dunn's multiple comparison post hoc test; **** p value < 0.0001). **(E)** Quantification of RAD51 intensity in the nuclear and cytoplasmic compartments. Each data point represents a single analyzed area (220.16 microns × 220.16 microns), while bars represent mean ± SD (one-way ANOVA with Holm-Šídák's multiple comparisons post hoc test, * p-value<0.05; ** p-value<0.01).

directed to a discrete nuclear location inside cells expressing the R3052W mutant. LMI allows for the creation of designated DNA damage sites within cell nuclei, often referred to as a "stripe", and subsequent analysis of protein accumulation or modification. BRCA2$^{-/-}$ cells stably expressing WT BRCA2 or R3052W were pre-treated with BrdU for 48 h and subsequently micro-irradiated with a 405 nm laser. Several localized DNA damage areas (stripes) were obtained as monitored by γH2AX Ser 139 phosphorylation (**Figure 3C**). At 30 min -post-micro-irradiation, prominent recruitment of RAD51 to damaged sites was observed in WT BRCA2 cells, whereas cells lacking BRCA2 displayed no RAD51 signal despite equal amounts of DNA damage as visualized by γH2AX stripe intensity (**Figure 3C**). Intriguingly, in BRCA2$^{-/-}$ cells expressing R3052W, we observed diffuse nuclear/cytoplasmic staining of RAD51, similar to results under basal conditions (no exogenous DNA damage), with no discernible RAD51 signal localized to the γH2AX stripes (compare **Figures 3A,C**, RAD51 panels and quantification in **Supplementary Figure S2B**). We quantified intensities of protein recruitment to the laser-induced stripes using an ImageJ-based high-throughput tool in a minimum of 300 nuclei (Oeck et al., 2019). We normalized the fluorescence intensity of RAD51 protein staining present in each damaged area (stripe) to the overall background fluorescence in undamaged areas of the nucleus. Notably, the RAD51 stripe intensity was significantly higher in the WT BRCA2 cells than in BRCA2$^{-/-}$ or R3052W expressing cells (**Figure 3D**). Additionally, we measured the overall mean fluorescence intensities of the RAD51 protein signal in nuclear and cytoplasmic compartments upon laser microirradiation. Strikingly, the ratio of nuclear to cytoplasmic RAD51 protein fluorescent intensity was significantly higher for WT BRCA2 cells than for BRCA2$^{-/-}$ or R3052W expressing cells (**Figure 3E**). Overall, the results indicate that the R3052W mutation prevents significant accumulation of RAD51 in the nucleus and further impairs RAD51 recruitment to sites of DNA damage.

R3052W Binds DSS1 and Remains Cytoplasmic Despite Nuclear Export Inhibition

A previous report interrogating the mislocalization of another BRCA2 missense mutation, D2723H, demonstrated that loss of DSS1 binding led to the exposure of a masked nuclear export signal sequence resulting in nuclear export to the cytoplasm (Jeyasekharan et al., 2013). The prior study utilized a fragment of BRCA2 (amino acids 2,432-3,418) containing the D2723H mutation, nonetheless, we confirmed that the full-length BRCA2 D2723H protein does indeed mislocalize to the cytoplasm (**Figure 4A**) and does not bind DSS1 (**Figure 4B**). However, in contrast to the prior study, we found that the full-length BRCA2 D2723H protein remained cytoplasmic despite CRM1 (exportin 1) depletion using RNA interference or by treatment with leptomycin B or Selinexor, two nuclear export inhibitors

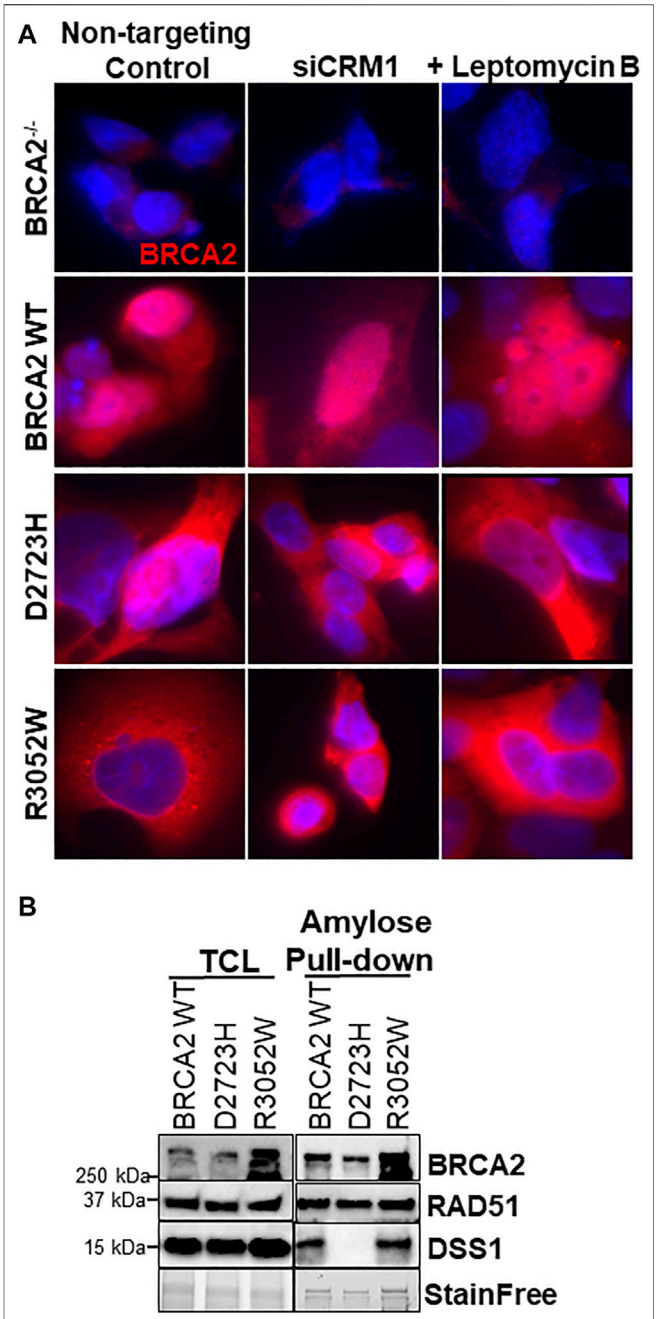

FIGURE 4 | R3052W cytoplasmic localization is not altered by CRM1 depletion or leptomycin treatment and retains binding to DSS1. **(A)** Immunofluorescent localization of BRCA2 in untreated stable cell lines expressing WT, D2723H, or R3052W BRCA2 proteins upon RNA interference-mediated depletion of CRM1 or treatment with the nuclear export inhibitor leptomycin B. Representative images of BRCA2 (red, MBP antibody) and DAPI staining to visualize nuclei (blue). **(B)** Western blots of total cellular lysates (TCL) and amylose pulldowns from HEK 293T cells transiently transfected with 2XMBP-BRCA2 WT, D2723H, or R3052W co-transfected with HA-DSS1. Anti-MBP antibody was used for BRCA2 detection, Anti-RAD51 antibody was used for endogenous RAD51 detection and Anti-HA antibody was used for DSS1 detection.

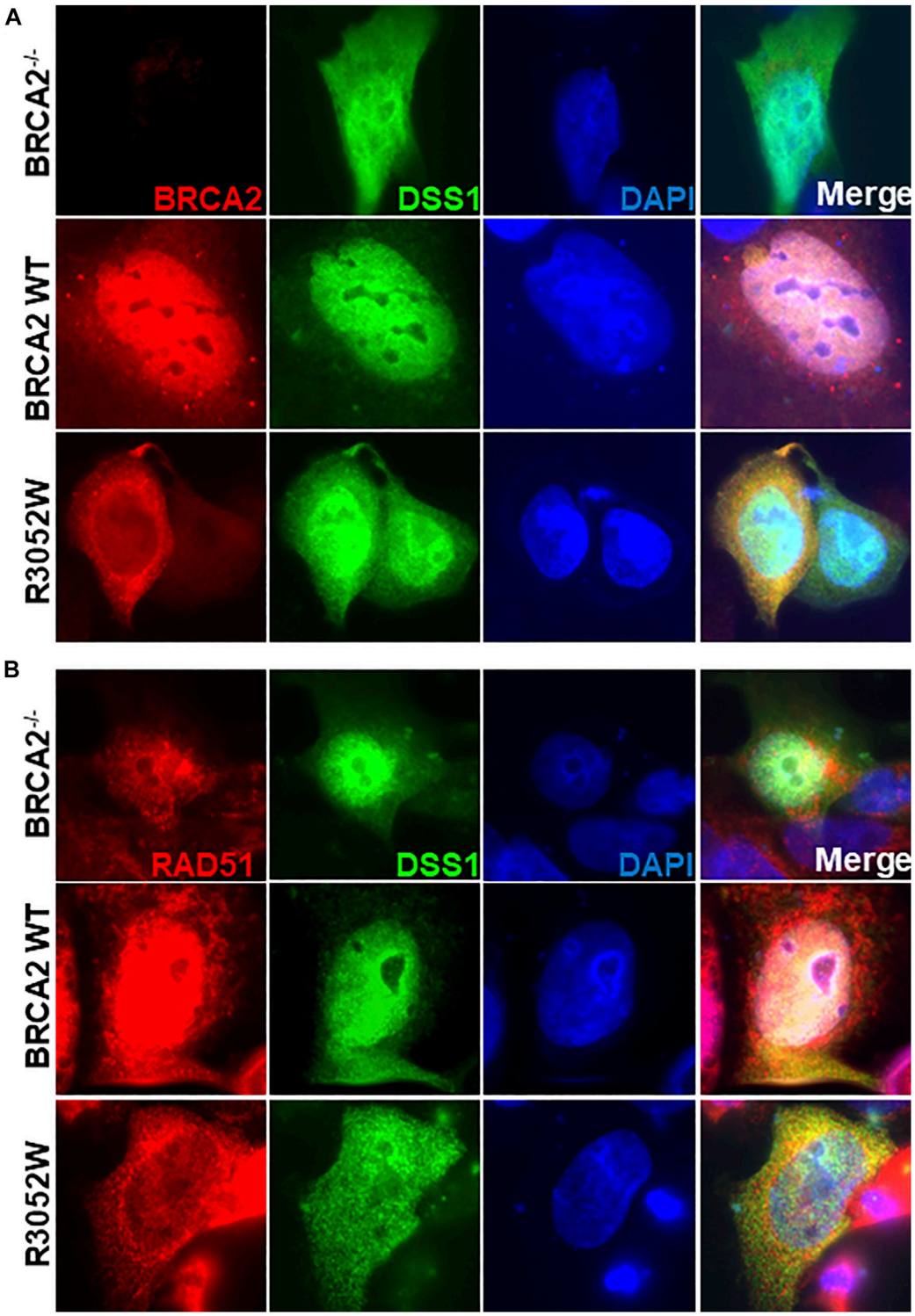

FIGURE 5 | Ectopic expression of DSS1 does not alter BRCA2 WT, R3052W or RAD51 cellular localization. **(A)** Immunofluorescent localization of BRCA2 and DSS1 in untreated BRCA2 knockout cells stably expressing WT BRCA2 or the R3052W mutant concurrent with transient expression of HA-DSS1. Representative images of BRCA2 (red, MBP antibody), DSS1 (green, HA antibody) and DAPI staining to visualize nuclei (blue). **(B)** Immunofluorescent localization of RAD51 and DSS1 in untreated BRCA2 knockout cells stably expressing WT BRCA2 or the R3052W mutant concurrent with transient expression of HA-DSS1. Representative images of RAD51 (red, RAD51 antibody), DSS1 (green, HA antibody), and DAPI staining to visualize nuclei (blue).

(**Figure 4A**, quantification in **Supplementary Figures S2E, S3**). To confirm the activity of leptomycin B in preventing nuclear export, we visualized nuclear retention of c-abl and Rev1.4 MP2K2 as positive controls (**Supplementary Figures S3A,B**). Likewise, the R3052W protein remained localized in the cytoplasm following the same treatments (**Figure 4A**, lower panel, and **Supplementary Figures S3C,D**). However, unlike D2723H, the R3052W protein retained binding to DSS1 (**Figure 4B**). We further over-expressed recombinant HA-DSS1 to determine if the R3052W protein could be re-directed to the nucleus, however, we failed to observe any significant movement (**Figure 5A** and quantification in **Supplementary Figure S4**). Interestingly, the cellular distribution of exogenously expressed HA-DSS1 appeared both nuclear and cytosolic (green panels in **Figures 5A,B**) in BRCA2$^{-/-}$ cells (and in R3052W expressing cells) whereas a much higher nuclear intensity was observed in cells expressing nuclear WT BRCA2. Thus, WT BRCA2, but not R3052W, localizes with DSS1 in the nucleus. Moreover, RAD51 cellular localization correlated with BRCA2 independently of DSS1 (**Figure 5B**). In summary, the results confirm that the BRCA2 R3052W mutant protein is mislocalized to the cytoplasm, but unlike the D2723H variant, our results point towards a different mechanism than the loss of DSS1 binding leading to nuclear export. Further investigation will be required to reveal the mechanism underlying the cytoplasmic mislocalization of R3052W.

DISCUSSION

Our results confirm and extend previous reports demonstrating that arginine to tryptophan substitution at the highly conserved 3,052 residues, located in the DNA binding domain of BRCA2, alters critical HDR functions (Kuznetsov et al., 2008; Guidugli et al., 2013; Hart et al., 2019; Ikegami et al., 2020). We conclude that R3052W is a pathogenic mutation unable to perform HDR or rescue sensitivity to DNA damaging agents. Moreover, our findings of increased genomic instability (micronuclei formation) and sensitivity to MMC upon ectopic expression of R3052W in a background of wild-type endogenous BRCA2 suggest R3052W is a dominant negative allele. Dominant negative effects have been attributed to other missense variants in DNA repair proteins such as p53 and BRCA1 (Willis et al., 2004; Vaclova et al., 2016), re-affirming that loss-of-heterozygosity leading to loss-of-function is not the only path to increased cancer risk. Further analysis of the R3052W allele in a heterozygous state by knock-in at the endogenous locus should aid clarification of the dominant negative impact, however, gene targeting of endogenous BRCA2 is currently an extremely difficult technical challenge. Notably, the BRCA2 R3052W mutant protein maintains all RAD51 binding sites intact. R3052W mislocalization to the cytoplasm likely explains the dominant negative effect as the mutant protein could antagonize the wild-type nuclear BRCA2 by sequestering a portion of the cellular pool of RAD51 resulting in sub-optimal RAD51 recruitment to DSBs.

Despite the sequence identification and previous characterization of several potential NLS sites in BRCA2, it remains unclear if one or multiple NLS are necessary and sufficient for nuclear localization (Spain et al., 1999; Yano et al., 2000; Han et al., 2008; Jeyasekharan et al., 2013). BRCA2 interactions with PALB2 acting collaboratively to deliver RAD51 to sites of DNA damage sites may indirectly play a role in the nuclear retention of the BRCA2 protein (Xia et al., 2006). Previous studies suggested that mutations in (or near) the binding pocket of DSS1 could unmask a nuclear export sequence misdirecting BRCA2 to the cytosol through active nuclear transport (Jeyasekharan et al., 2013). During the course of our studies, Lee et al. observed that the R3052W mutation, in the context of a fragment comprising the BRCA2 DBD + CTD domain, was indeed localized to the cytosol (Lee et al., 2021). Our findings utilizing the full-length BRCA2 R3052W protein corroborate this result, however, discrepancies have arisen regarding the underlying molecular mechanism. Our results suggest R3052W mislocalization is independent of active nuclear export or DSS1 expression/binding. We directly demonstrate that full-length BRCA2 R3052W protein binds DSS1 and silencing of exportin 1 (CRM1) or inhibition of nuclear export by Leptomycin B or Selinexor treatments do not change the cytoplasmic location of mutant BRCA2 (**Figure 4** and **Supplementary Figure S3**). Furthermore, ectopic expression of DSS1 does not alter the cellular location of the wild-type or mutant BRCA2 proteins but WT BRCA2 localizes with DSS1 in the nucleus (**Figure 5**).

We postulate that R3052W, and other pathogenic mutations in the BRCA2 DBD domain, are cytosolic due to either aggregation properties driven by protein misfolding or a breakdown in the recognition or regulation of the nuclear import machinery required to transport BRCA2 into the nucleus. Our analyses are ongoing and will hopefully shed light on the nature of the altered molecular mechanism. Immunohistochemical analyses capable of differentiating nuclear from cytoplasmic BRCA2 protein may present diagnostic opportunities to pathologically classify BRCA2 status in patient tumors.

As germline and tumor sequencing endeavors become incorporated into clinical cancer care, findings of missense variants, such as BRCA2 R3052W, with potentially uncertain functional consequences will be encountered more frequently (Thompson et al., 2001; Karchin et al., 2008; Guidugli et al., 2014). However, BRCA2 variants are present amongst healthy individuals and only a subset are causative of hereditary breast and ovarian cancer. The lack of classification standards and the rare incidence of individual variants can complicate the evaluation and diagnosis of patients. We advocate for the incorporation of functional assays into clinical practice to facilitate the correct classification of those BRCA2 missense variants where genetic linkage data and other traditional variant risk analyses are lacking. The ability to differentiate pathogenic from benign variants will enhance precision medicine efforts to stratify patients for increased cancer surveillance or targeted therapies such as PARP inhibitors.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JJ-S has made a substantial contribution to the concept and design of the article. JJ-S has acquired, analyzed, and interpreted the data of the article. AK has assisted with the LMI experiments, quantification, and interpretation of the data. JG has created the R3052W model in the PDB. JJ-S has written the manuscript with editorial support from RJ. All the authors have discussed the results, contributed to the final manuscript, revised the article critically for important intellectual content, and approved the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.884210/full#supplementary-material

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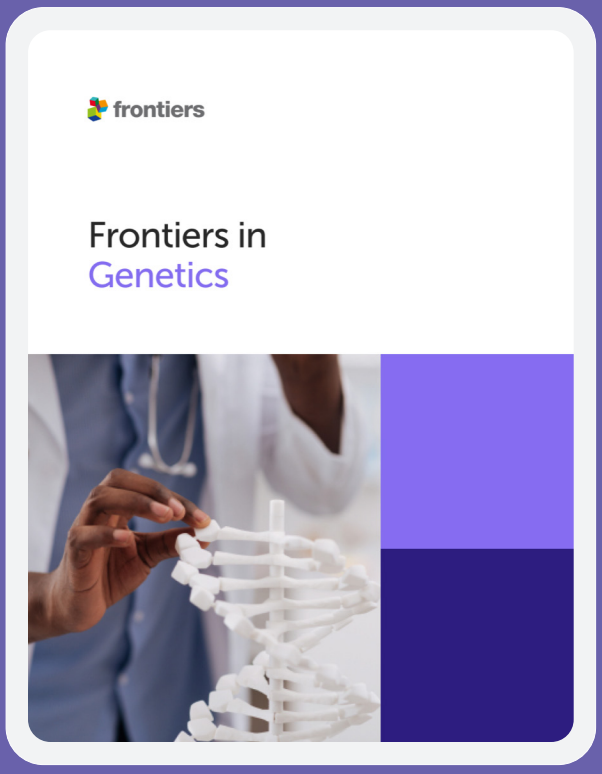

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