DOUBLE-EDGED SWORDS: GENETIC FACTORS THAT INFLUENCE THE PATHOGENESIS OF BOTH METABOLIC DISEASE AND CANCER

EDITED BY: Che-Pei Kung, Maureen E. Murphy and Hua Lu
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DOUBLE-EDGED SWORDS: GENETIC FACTORS THAT INFLUENCE THE PATHOGENESIS OF BOTH METABOLIC DISEASE AND CANCER

Topic Editors:
Che-Pei Kung, Washington University in St. Louis, United States
Maureen E. Murphy, The Wistar Institute, United States
Hua Lu, Tulane University, United States

Metabolic diseases and cancers account for half of all mortalities in the world, underscoring the significance of understanding the etiology of these diseases and developing effective therapies.

Genomic research in the 21st century has brought cancer and metabolic disease, two once seemingly parallel ailments, as close to each other as they've ever been. Many genetic factors have been found to display functions regulating both cancer and metabolic disease. In this research topic: "Double-edged Swords: Genetic Factors That Influence The Pathogenesis of Both MetabolicDisease and Cancer", you will be introduced to individual genes, as well as genetic pathways that play important roles in influencing the progression of both metabolic disease and cancer.

By no means covering an exhaustive list of genes qualified, this collection of articles rather serves as a precursor of what is yet to come in biomedical research. It paints the big picture of one of the major fields contributing to the future of "precision medicine".

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EDITORIAL
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Editorial: Double-Edged Swords: Genetic Factors That Influence the Pathogenesis of Both Metabolic Disease and Cancer

Che-Pei Kung[1,2]*, *Maureen E. Murphy*[3] and *Hua Lu*[4]

[1] Washington University School of Medicine, Saint Louis, MO, United States, [2] Division of Molecular Oncology, Department of Medicine, Siteman Cancer Center, Washington University School of Medicine, Saint Louis, MO, United States, [3] Program in Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA, United States, [4] Department of Biochemistry and Molecular Biology, Tulane Cancer Center, Tulane University School of Medicine, New Orleans, LA, United States

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Editorial on the Research Topic

Double-Edged Swords: Genetic Factors That Influence the Pathogenesis of Both Metabolic Disease and Cancer

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Edited and reviewed by:
Antonino Belfiore,
University of Catania, Italy

***Correspondence:**
Che-Pei Kung
patkung@wustl.edu

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Our understanding of cancer development has been marked by milestone discoveries in genetics, including the identification and cloning of oncogenes and tumor suppressor genes (1). Successful interventions of cancer, such as hormone therapies of breast cancers and vaccination of HPV, were aided in their development by our knowledge of these critical etiological factors underlying cancer. In contrast, metabolic disease was once thought to be a by-product of modern excessive lifestyles (2). Our increased ability to interrogate massive amounts of genetic information has strengthened the connection between genetics and metabolic disorders. As such, genetics-based therapy for metabolic disease has, at last, become more likely.

In this special research topic, we aim to highlight versatile genetic factors capable of regulating both cancer and metabolic disorders. By examining the existing literature, this collection of review articles provides both comprehensive overview and critical discussion about genetic factors/pathways that are involved in pathogenic mechanisms of multiple diseases, often through under-appreciated aspects of their functions.

The tumor suppressor TP53 is well-known for its role in maintaining genome stability and preventing cancer progression. Recent studies unveiled p53 functions in regulating metabolic homeostasis and diseases, such as diabetes (3). In Zwezdaryk et al. detail our current understanding of how p53 regulates functions of adipose tissues, often paradoxically, to influence the pathogenesis of obesity and cancer. In Gnanapradeepan et al. explain how p53-mediated regulation of gene expression contributes to a novel form of programmed cell death, ferroptosis (iron- and lipid-peroxide-mediated cell death), in the context of metabolic dysfunctions and cancer.

In the nucleus, the stability and transcriptional activity of p53 is promoted by binding to the regulatory region of another tumor suppressor gene, PTEN (4). In Chen et al. elegantly summarize data indicating that, in addition to collaborating with p53, PTEN also regulates tumor metabolism and insulin sensitivity through its phosphatase activity, suggesting PTEN as a potential target for treatment of both cancer and diabetes.

It is now appreciated that cancer cells often repurpose metabolic pathways shared by normal cells to facilitate pro-tumorigenic functions. Goetzman and Prochownik capture this essence with a comprehensive review. In their essay, they thoroughly describe how the

oncoprotein c-Myc regulates the metabolic balance in normal, cancerous, and metabolically-defective cell states.

In addition to oncogenes and tumor suppressors, non-coding RNAs also emerge as critical regulators of cellular homeostasis (5). Kong et al. introduce one of the long non-coding RNA (lncRNA), ANRIL. ANRIL resides in a genetic locus that is proximal or overlapping to several cancer-associated genes, including p16-INK4a, ARF (CDKN2a), and p15-INK4b. Potential connections between structural and functional characteristics, as well as unique SNPs (single nucleotide polymorphisms) of ANRIL and their impact on the development of cancer and metabolic diseases such as cardiovascular disease or diabetes are addressed.

Compared to the roles of cancer-associated genes in metabolic disorders, our understanding of how metabolic-disease genes regulate tumorigenesis is only now emerging. Deng et al. focus their mini-review, on one such gene, fat mass and obesity-associated protein (FTO), which acquired its name from epidemiological connections between its SNPs and obesity. Combining the new investigations linking FTO to tumorigenesis and its nature as an mRNA demethylase, development of FTO inhibitors is in full swing to treat both cancer and metabolic disease (6, 7).

Besides individual gene products, classes of genetic factors and pathways that regulate both tumorigenesis and metabolism are also discussed. In Nagarajan et al. summarize our current knowledge about the role of Heparan Sulfate Proteoglycans (HSPGs) in cancer development. Understanding the functions of these "protein-carbohydrate" conjugates, as well as factors regulating their metabolism, offers opportunities to develop treatments against cancers with deregulated HSPGs. Due to the diverse nature of HSPGs, it is not surprising that they are also involved in metabolic disorders like atherosclerosis and obesity (8).

Mechanisms to maintain the balance between biosynthesis and degradation of deoxyribonucleotide triphosphates (dNTPs), the building blocks of DNA, are keys to biological functions. Buj and Aird outline the intensively studied connections between dNTP metabolism and cancer development. They also discuss the recently-identified associations between dNTP homeostasis and metabolic diseases, and the potential novel therapeutic strategies targeting these interconnections for cancer and metabolic disorders.

As the organelle to produce ATP, the mitochondrion is a critical player in maintaining dNTP homeostasis. However, mitochondria also play multiple and important roles in human physiology through a variety of pathways, such as oxidative phosphorylation, production of reactive oxygen species (ROS), and inflammation (9). In Williams and Caino describe how the mitochondrial dynamics (shape and localization) in a cell contributes to cellular homeostasis, and how dysregulation of this network leads to cancer and type II diabetes.

Epigenetic alterations have been implicated in most human diseases, including cancer and metabolic disorders (10). In the review article, Kung et al. conduct a comprehensive review of the literature for the impact of RNA editing enzymes in the development of cancer and metabolic diseases. Importantly, in the era of genome editing, investigations of RNA-editing pathways could lead to promising therapeutic strategies.

To understand the complex nature of human diseases, a great window of opportunity is given by studying versatile players involved in multiple diseases. This collection of well-written reviews offers readers with an updated grand view on the double edged sword roles of genetic factors in connecting cancer with metabolic disorders. For their outstanding works, we sincerely thank all the authors as well as the reviewers/review editors for offering their time and effort for this project, and the editorial team at Frontiers, especially Dr. Emilie Schrepfer, for their helps and professionalism as demonstrated throughout this process.

AUTHOR CONTRIBUTIONS

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REVIEW
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The p53/Adipose-Tissue/Cancer Nexus

Kevin Zwezdaryk*[1]*, *Deborah Sullivan*[1]* and *Zubaida Saifudeen*[2]

[1] Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, LA, United States,
[2] Department of Pediatrics, Section of Nephrology, Tulane University School of Medicine, New Orleans, LA, United States

Obesity and the resultant metabolic complications have been associated with an increased risk of cancer. In addition to the systemic metabolic disturbances in obesity that are associated with cancer initiation and progression, the presence of adipose tissue in the tumor microenvironment (TME) contributes significantly to malignancy through direct cell-cell interaction or paracrine signaling. This chronic inflammatory state can be maintained by p53-associated mechanisms. Increased p53 levels that are observed in obesity exacerbate the release of inflammatory cytokines that fuel cancer initiation and progression. Dysregulated adipose tissue signaling from the TME can reprogram tumor cell metabolism. The links between p53, cellular metabolism and adipose tissue dysfunction and how they relate to cancer, will be presented in this review.

Keywords: p53, adipokines, obesity, cancer, white adipose tissue, metabolism

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Edited by:
Che-Pei Kung,
School of Medicine, Washington University in St. Louis, United States

Reviewed by:
Eva Surmacz,
Temple University, United States
Michaela Ruth Reagan,
Maine Medical Center Research Institute, United States

****Correspondence:***
Kevin Zwezdaryk
kzwezdar@tulane.edu
Deborah Sullivan
dsulliva@tulane.edu
Zubaida Saifudeen
zubisaif@tulane.edu

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INTRODUCTION

Cancers associated with obesity are estimated to account for up to 40% of all cancers diagnosed in the US (Centers for Disease Control and Prevention, CDC). Per CDC reports, the incidence of non-obesity related cancers showed a decline from 2005 to 2014, while the rates of obesity-related cancers increased (1). Causes of obesity and cancer are multifactorial with significant contribution from genetics and environmental factors. *TP53* (p53) is the most commonly mutated gene in cancer with nearly half of all human cancers showing protein loss or mutation (2). Of the cancers that do not have mutations in the p53 gene locus, the majority exhibit mutations or altered levels of negative regulators of p53 (3, 4). Classically, p53 is known as a tumor suppressor, but recent work highlights the diverse functions of p53, including p53's contribution to metabolic and adipose tissue regulation. As increasing evidence links obesity to the onset of cancer, in this review, we discuss the crosstalk between adipose tissue and metabolism in cancer and the central role of p53 therein.

P53 OVERVIEW

p53 is best known as a tumor suppressor that maintains genomic stability and inhibits cell proliferation pathways (5–11). Its significant role in tumor suppression is dependent on its activity as a transcription factor regulating expression of genes in cell cycle regulation, apoptosis, DNA repair, differentiation, and senescence pathways (**Figure 1**). Under conditions of mild stress, p53 initiates cell cycle arrest and DNA repair pathways. However, in response to catastrophic stress that inflicts irreparable damage, p53 triggers an apoptotic response designed to limit propagation of impaired cells.

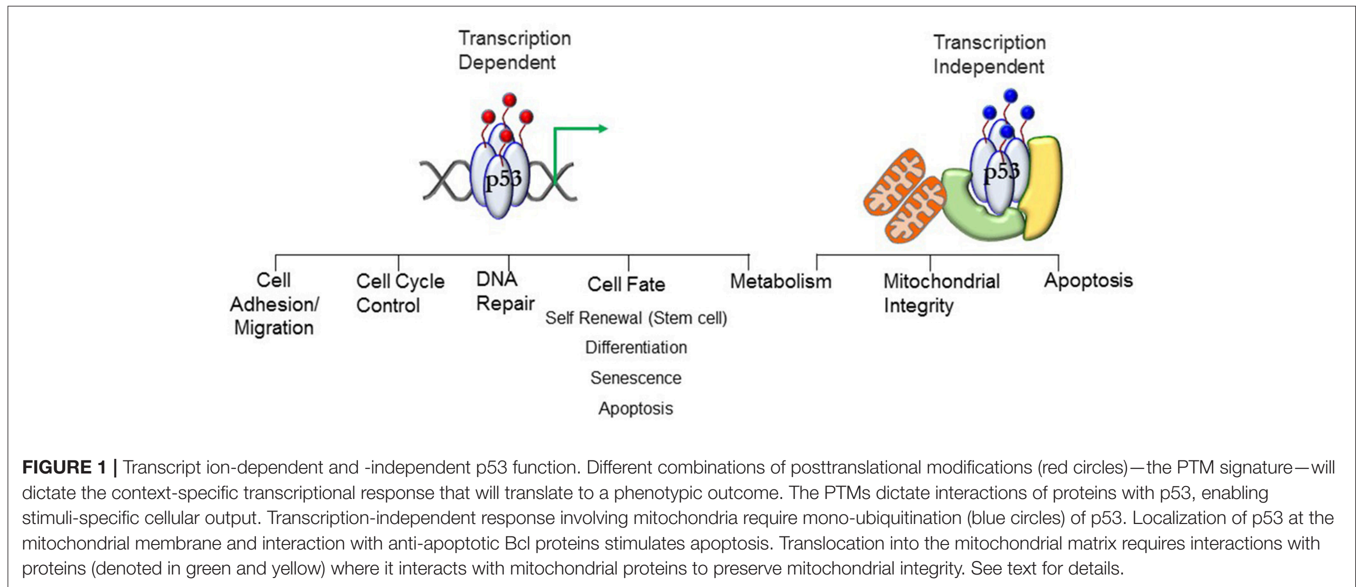

FIGURE 1 | Transcript ion-dependent and -independent p53 function. Different combinations of posttranslational modifications (red circles)—the PTM signature—will dictate the context-specific transcriptional response that will translate to a phenotypic outcome. The PTMs dictate interactions of proteins with p53, enabling stimuli-specific cellular output. Transcription-independent response involving mitochondria require mono-ubiquitination (blue circles) of p53. Localization of p53 at the mitochondrial membrane and interaction with anti-apoptotic Bcl proteins stimulates apoptosis. Translocation into the mitochondrial matrix requires interactions with proteins (denoted in green and yellow) where it interacts with mitochondrial proteins to preserve mitochondrial integrity. See text for details.

p53 protein levels are ubiquitously high in early embryogenesis in germ layer progenitors and embryonic stem cells until nearly mid-gestation (5, 12, 13), after which time expression is restricted to specific tissues during organogenesis as development progresses. Protein levels decrease postnatally to follow the recognized expression pattern of stabilization under cellular stress (5, 12, 14). Stimuli-induced post-translational modifications (PTM) stabilize the protein (15–21). In the absence of stress stimuli, negative regulation of p53 function is mediated by Mdm2 and Mdmx (22, 23). Different combinations of PTMs—the PTM signature—drive context-specific pathway activation. Protein stability and function are controlled by: (a) phosphorylation (b) acetylation (c) poly-ubiquitination (d) sumoylation (e) neddylation and (f) methylation (17, 21, 24–28). The *N*-terminus contains the transcription activation domain (TAD). In addition to stabilizing p53, the PTMs dictate the interactions of proteins with p53, enabling stimuli-specific cellular output. For example, p53 interacts with histone modifying enzymes and chromatin remodelers [e.g., HATs p300/CBP (29, 30), lysine-specific demethylase LSD1 (31)] which alter chromatin structure, along with interactions with proteins in the basal transcription machinery complex [TBP (32), and TBP-associated factors such as TFIIA and TAF1 (33, 34)] to regulate gene transcription (33, 35, 36). Transcription-dependent functions of p53 play a key role in cell-fate decisions by regulating expression of genes that control cell cycle arrest, DNA repair, apoptosis, senescence, and autophagy to limit the propagation of cells with damaged genomes (33–36).

Research in the last decade has revealed a critical role for p53 well beyond its role in tumor suppression. These roles include preserving stem cell health and differentiation in embryonic life, development of senescence and maintaining mitochondrial function in aging (5, 7, 37–41). Recent evidence strongly implicates p53 in the regulation of metabolism, linking p53 to metabolic abnormalities observed in aging, obesity, inflammation, and cancer (37, 42).

P53-MEDIATED REGULATION OF INTERMEDIARY METABOLISM

Choice of metabolic pathway usage is determined by the cell's energy, biomass and metabolite demands. Many cancer cells depend on glycolysis, even under aerobic conditions (Warburg Effect) (43, 44). The shift to aerobic glycolysis is an active reprogramming event that enables anabolic growth. Intermediates from the glycolytic pathway serve as precursors for biomass synthesis that are necessary for proliferation. Additionally, the pentose phosphate pathway (PPP) produces precursors for the synthesis of nucleotides that are essential for DNA replication. In contrast, differentiated cells preferentially utilize mitochondrial oxidative phosphorylation (OXPHOS) (45).

Consistent with its role as a tumor suppressor, p53 inhibits multiple steps of glycolysis and the PPP while promoting OXPHOS (46). Expression of glucose transporters Glut1 and Glut4 are downregulated by p53, resulting in the inhibition of glucose uptake. Induction of the phosphatase TP53-induced glycolysis and apoptosis regulator (TIGAR) decreases the production of fructose-2,6-bisphosphate (F2, 6BP) which allosterically activates phosphofructokinase 1 (PFK1) to increase glycolytic flux (9). By inhibiting expression of the negative regulator of the pyruvate dehydrogenase complex that is responsible for the transfer of cytosolic pyruvate to the mitochondria, p53 promotes OXPHOS by directing pyruvate to acetyl CoA rather than lactate (47). Increased lactate levels in the cell due to transcriptional repression of monocarboxylate transporter 1 (*mct1*) expression, a p53 target gene which

transports lactate out of the cell, also decreases glycolytic flux (48).

p53 is a critical regulator of mitochondrial morphology, mitochondrial genomic integrity, mitophagy, aerobic metabolism and cellular redox state (38, 41, 49). In contrast to inhibitory effects on anabolic glycolysis, p53 drives catabolic mitochondrial respiration via induction of key genes such as mitochondrial glutaminase (*Gls2),* Synthesis of cytochrome *c* oxidase 2 (*Sco2*) and Complex 1 proteins that are involved in fueling the tricarboxylic acid (TCA) cycle and driving electron transport (50, 51). p53 was demonstrated to adaptively regulate OXPHOS in Drosophila Myc+ cells and maintain their super-competitive status by enhancing the metabolic flux (52). In contrast to increased proliferation observed in cancer cells upon the loss of p53, the response of Drosophila Myc+ cells to p53 loss is impaired metabolism and reduced viability, suggesting a cell-context dependent regulation of cellular processes. By inducing expression of the mitochondria-eating protein (Mieap), p53 functions as a guardian of mitochondrial health, facilitating the removal of damaged mitochondria by mitophagy (53). Mitochondrial p53 physically interacts with TFAM, the factor that is responsible for mitochondrial DNA transcription, replication, and repair (11). Accordingly, decreased mitochondrial DNA content or mitochondrial DNA mutations are detected in fibroblasts from Li-Fraumeni patients (54).

p53 also plays a critical role in both normal and pathological lipid metabolism (55, 56). Generally, p53 is a negative regulator of lipid synthesis and activates fatty acid oxidation (FAO) via induction of expression of carnitine acetyltransferase genes (CPT1) that transport fatty acids to the mitochondria for oxidation. However, chronic p53 activation by nutrient stress (obesity) leads to hepatic steatosis, insulin resistance, and diabetes, pointing to the complexity of the homeostatic response (57–59). Dysregulated cell metabolism is an accepted hallmark of cancer and p53 can influence the function of many metabolic pathways (60). Obesity is also recognized as a state of dysregulated cell metabolism, and p53 is influential in adipose tissue differentiation, accumulation, and cytokine secretion.

ADIPOSE TISSUE

Adipose tissue is broadly subdivided into white and brown adipose tissue. The largest component of white adipose tissue is the large, spherical adipocyte with a unilocular lipid droplet occupying most of the cell volume. The primary role of white adipose tissue is to store energy in the form of triglycerides. When hormones signal the need for energy, fatty acids and glycerol are released through lipolysis. White adipose tissue is subdivided into unique depots highlighting the function and location of the adipose tissue. Visceral adipose tissue surrounds organs, subcutaneous adipose tissue forms a layer between the muscle and dermal fascia, and intramuscular adipose tissue protects tissue and supplies nourishment. Approximately 80% of human adipose tissue is deposited in subcutaneous depots. However, visceral adipose tissue is more metabolically active, and its accumulation is more prognostic of obesity-related mortality (61, 62). Both white adipose tissue depots store excess energy, but visceral fat also protects organs from physical trauma. White adipose tissue is capable of significant expansion that can lead to the accumulation of excess adipose tissue and thus increased propensity for obesity and related metabolic disorders (63).

In contrast to white adipose tissue, brown adipose tissue is specialized to burn sugars and lipids to generate heat and to help maintain body temperature through adaptive thermogenesis. Brown adipose tissue is abundant in neonates but undergoes rapid involution with age in humans. Consequently, adult human brown adipose tissue is relatively limited in mass and restricted to depots near the aorta and within the supraclavicular region of the neck (64). Brown adipose tissue is densely innervated by the sympathetic nervous system and is highly vascularized. Brown adipocytes contain multilocular lipid droplets and large numbers of mitochondria. The hallmark of brown adipose tissue function is the presence and activation of mitochondrial uncoupling protein 1 (UCP1) which uncouples OXPHOS from ATP synthesis in the inner mitochondrial membrane, thereby dissipating chemical energy as heat (65). A third adipose tissue type termed beige or "brown-in-white" (brite) adipose, has recently been characterized. Beige adipocytes can be induced by cold and a broad spectrum of pharmacological substances and, therefore, they are also known as "inducible brown adipocytes." These depots can be induced to appear morphologically similar to brown adipose tissue, but appear in classical white adipose tissue depots and are derived from a non-classical brown adipose tissue lineage (66, 67).

Recently, the bone marrow has been identified as a unique adipose depot. Although the bone marrow contains few adipocytes at birth, the number increases with age, and by adulthood, bone marrow adipose tissue constitutes over 10% of the total fat mass in lean, healthy humans. There are two types of bone marrow adipose tissue classified as "regulated" that may influence hematopoiesis and "constitutive" that is important during early vertebrate development (68). The ontogeny of bone marrow adipose tissue is not well defined. Bone marrow adipose tissue differs in diet response, phenotype, gene expression and physiological actions from other adipose depots [reviewed in (69)]. For example, during conditions of starvation bone marrow adipose tissue volume increases whereas white adipose tissue volume decreases.

It is now clear that all adipose tissue acts in an autocrine/paracrine and endocrine manner. Adipocytes secrete an array of signaling molecules such as leptin, adiponectin, plasminogen activator inhibitor (PAI-1), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-6, collectively referred to as adipokines, that communicate with other organs such as the brain, liver, muscle, the immune system, and adipose tissue itself. An example is metabolic symbiosis that occurs between tumor cells and adjacent adipose tissue during cancer progression. Adipokines and lipids are released from mature adipocytes and taken up by cancer cells. Paracrine factors from adipose tissue-derived stromal and immune cells that have infiltrated tumors, are secreted into the tumor microenvironment (70).

Differentiation of preadipocytes to mature adipocytes requires transcription regulators such as the peroxisome proliferator-activated receptor gamma (PPARγ) and members of the CCAAT/enhancer-binding protein family (C/EBPs) (71). p53 is a negative regulator of PPARγ expression, and concomitantly of white adipocyte differentiation both *in vivo* and *in vitro* (72). p53 inhibits an adipogenic program in 3T3-L1 preadipocytes and mouse embryonic fibroblasts (MEFs) (73, 74). Knockdown of p53 by specific shRNA enhances the adipogenic capacity in both mouse and human cell lines, indicated by increased levels of adipogenic markers such as PPARγ, AP2, and adiponectin even without hormonal induction (74). Moreover, differentiation of p53-null MEFs into adipocytes is more robust compared to wild-type cells in an adipogenic medium (73–75). Accordingly, transgenic mice overexpressing active p53 demonstrate decreased adipose tissue deposition and reduction in body mass (76). However, p53 is a positive regulator of brown adipocyte differentiation (75). Also, using a murine model of diet-induced obesity (DIO) weight gain was reduced in p53-null mice, and the mechanism was through an increase in UCP1 expression, both in brown and white adipose tissue (77).

ADIPOSE TISSUE DYSFUNCTION—PROMOTED BY P53?

As adipose tissue expands, adipogenesis is upregulated, mature adipocytes enlarge, and angiogenic processes promote neovascularization. In obese states, enlarged adipocytes experience hypoxic conditions due to larger distances from the vasculature (78), as cardiac output and total blood flow do not increase with increased obesity (79). In association with these changes, the adipose tissue starts to produce chemotactic factors, such as monocyte chemoattractant protein (MCP)-1, that attract monocytes/macrophages into adipose tissue (80). Murine studies have demonstrated that excess adiposity increases the proportion of proinflammatory M1 to anti-inflammatory M2 macrophages in white adipose tissue (81). As the adipose tissue becomes inflamed, production of inflammatory cytokines increases and production of adiponectin decreases, resulting in the inability to store surplus free fatty acids (FFAs) leading to further adipose tissue dysfunction (82). *In vitro* and *in vivo* studies by Shimizu et al. indicated that increased release of FFAs led to ROS-induced DNA damage and upregulation of p53 in adipose tissue (59) (**Figure 2**). Activation of p53 upregulated the expression of proinflammatory adipokines via the NF-κB signaling pathway, and promoted adipose tissue inflammation, insulin resistance, and diabetes, whereas inhibiting p53 activity attenuated the inflammation (59). These changes in p53 expression related to obesity have been observed in both murine models and obese human subjects (55, 58, 83–86). The chronic inflammation associated with dysfunctional adipose tissue is thought to contribute to a favorable microenvironment for tumor growth and progression (**Figures 2**, **3**).

In addition to data indicating p53 stimulation in dysfunctional adipose tissue exacerbates the pathology of adiposity, recent studies implicate p53 as a primary mediator of adiposity. As demonstrated by Kung et al. mice harboring the proline-to-arginine 72 (P72R) variant of p53 developed more severe obesity and glucose intolerance on a high-fat diet than mice with proline 72 variant (87). Further evidence supporting the adverse effect of high p53 activity in promoting obesity was demonstrated in mutant $MDM2^{C305F}$ mice that have impaired p53 regulation of lipid metabolism (88). The mutation disrupts ribosomal protein-MDM2 interaction that serves to sequester MDM2 and allow p53 activation. Also, pharmacological inhibition of p53 was demonstrated to prevent high-fat diet-induced weight gain observed in control mice (89). In summary, the data suggest that high p53 levels, whether induced in response to or as an inducer of adiposity, are likely counter-productive in maintaining adipose tissue homeostasis.

P53, ADIPOSE TISSUE, AND METABOLISM—AN UNEXPLORED LINK IN CANCER

Secretion of adipokines (leptin, adiponectin, endotrophin, etc.) and growth factors from AT promote tumor growth. There are more than 600 different adipokines currently identified and many cancers, such as breast cancer, have adipokine receptors present on the cancer cells (90, 91). Adipokine-linked cancer progression may occur through increased proliferation, migration, inflammation and anti-apoptotic mechanisms. Leptin secretion from adipose tissue near tumors is increased, but not in adipose depots that are distant from the tumors (92). Interestingly, leptin is a known regulator of p53 expression (93). Leptin binding to its receptor enhances the proliferation and growth of breast cancer cells through numerous signaling pathways including estrogen receptor, JAK/STAT3, and PI3K/Akt pathways (94–96) (**Figure 2**). Aberrant signaling through these pathways activates expression of genes that contribute to cancer cell survival, proliferation, and migration (97–99). Moreover, signaling pathway activation can reprogram cellular metabolism to support the specific metabolite demands of proliferating cells. Thus, ectopic activation of these pathways promotes tumor progression (**Figure 2**). Leptin was also shown to induce aromatase and this correlated positively with BMI, leading to increased risk for breast cancer (100) (**Table 1**). Given the participation of p53 in adipose tissue inflammation (as discussed above in section Adipose Tissue Dysfunction—Promoted by p53?) that promote proliferative pathways vs. the known involvement of p53 as a tumor suppressor restricting proliferation and cell growth, the role of p53 in adipose tissue-driven tumorigenesis remains to be elaborated.

Wild-type p53 in an inactivated or dysfunctional form accumulates in the cytoplasm whereas stable p53 binds to target genes in the nucleus. Expression of the p53 transcript, nuclear localization of the protein and phosphorylation at Ser15 was decreased in ASCs due to the effect of prostaglandins (PGE_2) (101). Wang et al. showed that the decrease in p53 protein expression and activity is through an inhibitory effect of PGE_2 on AMP-activated kinase (AMPK). AMPK can no

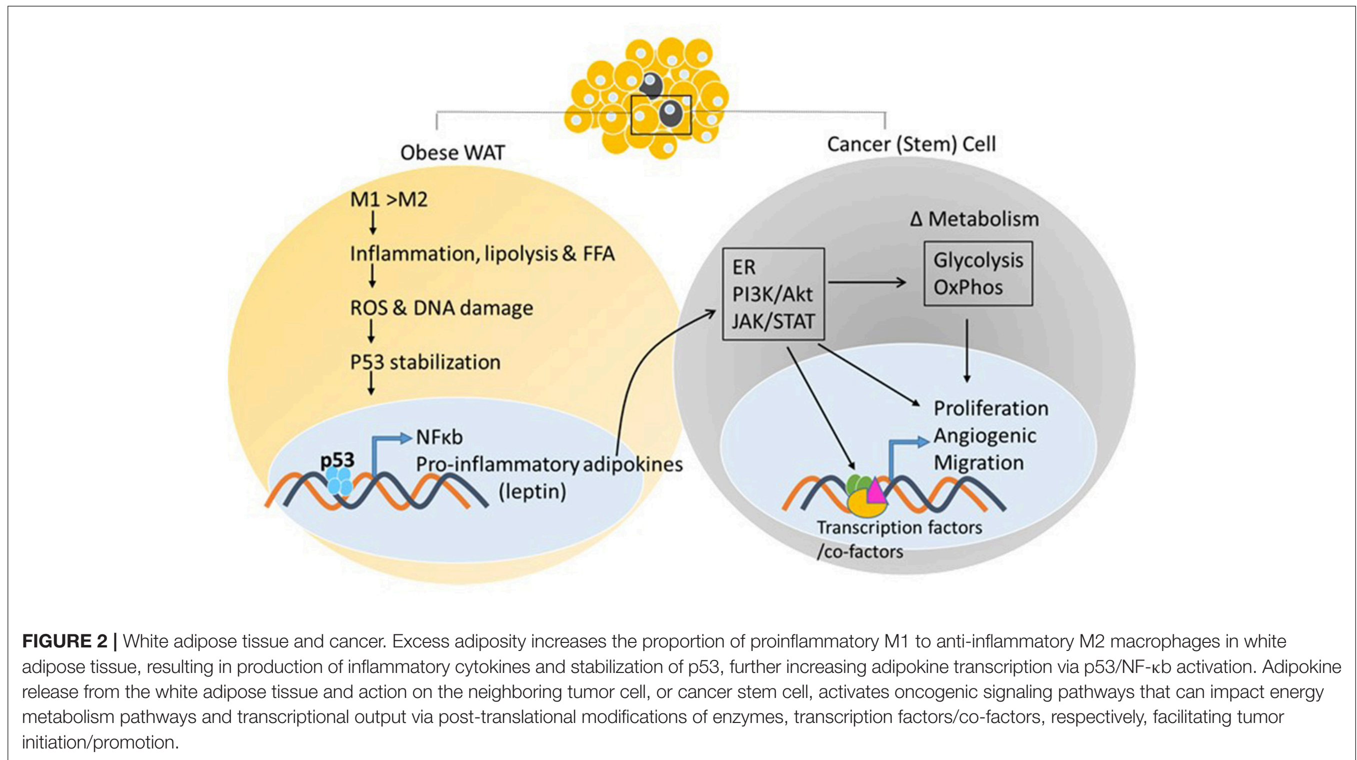

FIGURE 2 | White adipose tissue and cancer. Excess adiposity increases the proportion of proinflammatory M1 to anti-inflammatory M2 macrophages in white adipose tissue, resulting in production of inflammatory cytokines and stabilization of p53, further increasing adipokine transcription via p53/NF-κb activation. Adipokine release from the white adipose tissue and action on the neighboring tumor cell, or cancer stem cell, activates oncogenic signaling pathways that can impact energy metabolism pathways and transcriptional output via post-translational modifications of enzymes, transcription factors/co-factors, respectively, facilitating tumor initiation/promotion.

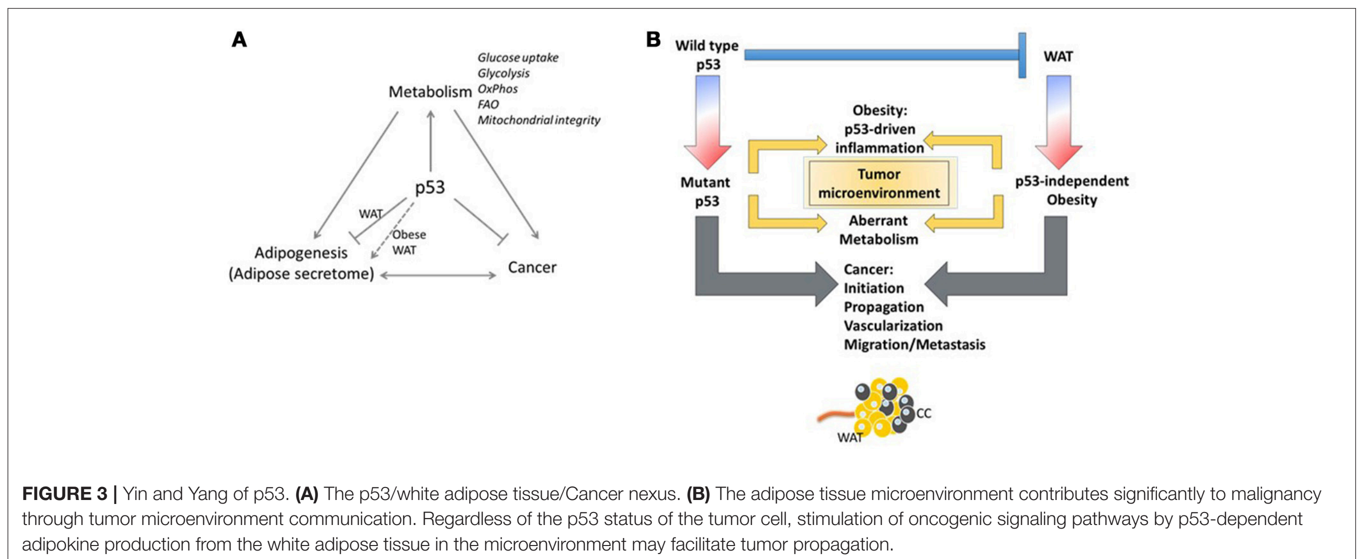

FIGURE 3 | Yin and Yang of p53. **(A)** The p53/white adipose tissue/Cancer nexus. **(B)** The adipose tissue microenvironment contributes significantly to malignancy through tumor microenvironment communication. Regardless of the p53 status of the tumor cell, stimulation of oncogenic signaling pathways by p53-dependent adipokine production from the white adipose tissue in the microenvironment may facilitate tumor propagation.

longer phosphorylate p53 at Ser15 (103, 104), resulting in decreased nuclear localization and transcriptional activity of p53. In clinical samples of breast cancer, tumor-associated ASCs had reduced nuclear p53 staining and increased perinuclear staining compared to normal ASCs (101). This is important as increased PGE_2 is linked with many cancers and PGE_2 associated inflammation is specifically associated with obesity and breast cancer (105). PGE_2 and TNFα may contribute to the Warburg effect due to stimulation of GLUT1 and GLUT3 in ASCs (106). Again, this mechanism is through adipose-derived inflammation altering the metabolic microenvironment resulting in reduced p53 nuclear localization. A mechanism to the observed obesity-associated increase in aromatase and its link to breast cancer has been suggested (100). Adipose or ASC leptin secretion resulted in activation of PKC/MAPK signaling pathways and inhibition of p53. Furthermore, HIF1α and PKM2 were stabilized, resulting in increased expression of aromatase, and an increased risk of estrogen-dependent breast cancer. Conversely, p53 related mechanisms have been shown to promote hepatocellular carcinoma cell apoptosis. Omentin-1,

TABLE 1 | p53 and adipose tissue metabolism.

Type of cancer	Model	Mechanism	Cancer-related outcome	References
Breast cancer	Primary breast adipose stromal cells	Prostaglandin E2 (PGE2) decreases p53 expression and increases aromatase levels.	Increased aromatase is associated with increased estrogen production	(101)
Breast cancer	Primary preadipocytes	Leptin-mediated induction of aromatase was dependent on PKC/MAPK signaling and inhibition of p53	Increased aromatase is associated with increased estrogen production	(100)
Hepatocellular cancer	HepG2 and HuH-7 cell line	Omentin-1 upregulated p53 through sirtuin1-dependent deacetylation of p53	Apoptosis	(102)

an adipokine, was added to hepatocellular carcinoma cells and resulted in an inhibition of proliferation and an induction of apoptosis (102). It was shown that omentin-1 upregulated p53 through sirtuin1-dependent deacetylation of p53. This is in contrast to the actions of most reports on adipokines and cancer, which show promotion of metastatic potential and cancer cell survival.

Obesity has long been linked to increased local inflammation. As discussed above, obesity also reprograms metabolism systemically and can lead to increased levels of glucose and dyslipidemia in the blood (107). Although these examples are associated with obesity, the distribution of adipose tissue results in proximate or direct contact of tumors with adipose tissue, both in obese and non-obese conditions. This growing field of study suggests that the adipose tissue microenvironment contributes significantly to malignancy through tumor-microenvironment communication (**Figure 3**). In an invasive ductal carcinoma breast cancer model, increased lymph node metastasis was reportedly linked to adipose tissue invasion at the tumor margin (108). Tumor cells have been reported to induce delipidation of adipocytes and promote lipolysis in the tumor microenvironment (109). Regardless of the p53 status of the tumor cell, stimulation of oncogenic signaling pathways by p53-dependent adipokine production from the white adipose tissue in the microenvironment may facilitate tumor propagation (**Figure 3**).

Finally, bone marrow adipose tissue (BMAT) has recently been shown to affect metastatic progression and drug resistance in prostate and breast cancer. The mechanisms involved in this new adipose depot are currently being resolved. Fairfield et al. used a 3D culture model of BMAT to show that BMAT adipocytes, when co-cultured with tumor cells, undergo delipidation (110). This supports the model of exogenous lipid dependency by tumor cells for metabolic flexibility within the metastatic niche. Lipids from adipocytes in the tumor microenvironment could potentially regulate metabolic and signaling pathways in cancer cells, providing them with a survival advantage. A role for p53 in bone marrow adipose tissue has not yet been investigated.

CONCLUSIONS AND FUTURE DIRECTIONS

An increased risk of cancer development and a poorer cancer prognosis is associated with increased obesity (107, 111–114). Cancer survivors with a higher body mass index are more likely to experience a cancer recurrence (115). The mechanisms linking increased adiposity to malignancy are not entirely understood. Altered interactions between adipose tissue and systemic or neighboring tissue, changing endocrine hormone and adipokine secretion that would facilitate tumor invasion and metastasis are hypothesized to drive metabolic reprogramming in tumor cells and provide metabolites and lipids required for tumor progression and growth. Although brown adipose tissue is metabolically more active than white adipose tissue, the link between chronic metabolic diseases and brown adipose tissue is unknown. Given the differential regulation by p53 of white vs. brown adipose tissue, it will be interesting to compare the influence of these different adipose depots for their potential to contribute to cancer. A thorough understanding of the crosstalk between cancer cells and the adipose microenvironment may well reveal novel therapeutic targets for cancer treatment.

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

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REVIEW
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The p53 Tumor Suppressor in the Control of Metabolism and Ferroptosis

*Keerthana Gnanapradeepan[1,2], Subhasree Basu[1], Thibaut Barnoud[1], Anna Budina-Kolomets[1], Che-Pei Kung[3] and Maureen E. Murphy[1]**

[1] Program in Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA, United States, [2] Graduate Group in Biochemistry and Molecular Biophysics, The Perelman School of Medicine, The University of Pennsylvania, Philadelphia, PA, United States, [3] Department of Internal Medicine, School of Medicine, Washington University in St. Louis, St Louis, MO, United States

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Haim Werner,
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****Correspondence:***
Maureen E. Murphy
mmurphy@wistar.org

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The p53 tumor suppressor continues to be distinguished as the most frequently mutated gene in human cancer. It is widely believed that the ability of p53 to induce senescence and programmed cell death underlies the tumor suppressor functions of p53. However, p53 has a number of other functions that recent data strongly implicate in tumor suppression, particularly with regard to the control of metabolism and ferroptosis (iron- and lipid-peroxide-mediated cell death) by p53. As reviewed here, the roles of p53 in the control of metabolism and ferroptosis are complex. Wild-type (WT) p53 negatively regulates lipid synthesis and glycolysis in normal and tumor cells, and positively regulates oxidative phosphorylation and lipid catabolism. Mutant p53 in tumor cells does the converse, positively regulating lipid synthesis and glycolysis. The role of p53 in ferroptosis is even more complex: in normal tissues, WT p53 appears to positively regulate ferroptosis, and this pathway appears to play a role in the ability of basal, unstressed p53 to suppress tumor initiation and development. In tumors, other regulators of ferroptosis supersede p53's role, and WT p53 appears to play a limited role; instead, mutant p53 sensitizes tumor cells to ferroptosis. By clearly elucidating the roles of WT and mutant p53 in metabolism and ferroptosis, and establishing these roles in tumor suppression, emerging research promises to yield new therapeutic avenues for cancer and metabolic diseases.

Keywords: p53, metabolism, ferroptosis, apoptosis, tumor suppressor

INTRODUCTION

The tumor suppressor gene *TP53* has been the most heavily studied human gene since its discovery nearly 40 years ago (1). The main reason behind this status is the critical role p53 plays in preventing cancer development, and it is widely regarded as the "guardian of the genome." For some time it has been generally believed that p53's role in tumor suppression is by virtue of its ability to induce the apoptosis, cell cycle arrest, and senescence of pre-cancerous cells (2). However, it is now increasingly clear that p53 regulates many other pathways in the cell and that these other pathways also play roles in p53's ability to function as a tumor suppressor (3). In particular, p53's role in the regulation of genes involved in metabolism and ferroptosis has been implicated in its ability to suppress tumor development. Ferroptosis is a novel cell death pathway first characterized in 2012 and can be best described as an iron-dependent, caspase-independent form of cell death driven by the formation of

lipid peroxidation (4). Specifically, two mouse models containing engineered mutations in p53 that eliminate the ability of p53 to induce apoptosis and senescence both retain the ability to suppress spontaneous tumor development; both of these mutants retain the ability to transactivate genes in metabolism and ferroptosis (5, 6). A summary of the data implicating p53 in the regulation of metabolism and ferroptosis is detailed below.

WILD-TYPE (WT) p53 POSITIVELY REGULATES OXIDATIVE PHOSPHORYLATION AND SUPPRESSES GLUCOSE METABOLISM

Wild-type p53 regulates the metabolic versatility of cells by favoring mitochondrial respiration over glycolysis, in part *via* the transactivation of *SCO2* (cytochrome *c* oxidase assembly), which plays a direct role in oxidative phosphorylation (7). p53 also directly regulates the transactivation of *GLS2* (Glutaminase 2); this enzyme allows glutamine usage as an energy source for the mitochondria (8). In addition, WT p53 negatively regulates glycolysis by transcriptionally repressing the glucose transporters *GLUT1* and *GLUT4*, and by transactivating *RRAD* and *TIGAR*; both are inhibitors of glycolysis (9–11). Finally, p53 also directly binds and inhibits the enzyme glucose-6-phosphate dehydrogenase, thus suppressing glucose metabolism (12). It is clear from these and other studies that in normal, unstressed organisms, p53 directly regulates the metabolic state in a cell (**Figure 1**). Not surprisingly, this gene and many of its regulators are implicated in metabolic diseases, including obesity and diabetes (13).

MUTANT p53 POSITIVELY REGULATES WARBURG METABOLISM (AEROBIC GLYCOLYSIS)

In contrast to the function of WT p53, mutant p53 in tumor cells favors aerobic glycolysis, in part by enhancing the trafficking of the glucose transporter GLUT1 to the plasma membrane, hence increasing glucose import (14, 15). Following the mutation of p53, the reduced levels of *SCO2* and *GLS2* and the increased levels of *GLUT1* and *GLUT4* favor aerobic glycolysis over oxidative phosphorylation. In this manner, mutant p53 is believed to contribute to the propensity of tumor cells to utilize aerobic glycolysis in favor of oxidative phosphorylation, or so-called Warburg metabolism (15). One of the hallmarks of cancer is deregulated metabolism, generally demonstrated by this switch from aerobic glycolysis to oxidative phosphorylation. Though this results in a lower and less efficient ATP yield, it is believed that cancer cells benefit by diverting glycolytic intermediates to biosynthetic pathways necessary for rapid cell division (16). This metabolic switch also leads to decreased mitochondria-mediated apoptosis and more efficient signaling through available metabolites in cancer cells (17).

A COMMON GENETIC VARIANT IN TP53 INFLUENCES ITS FUNCTION IN METABOLISM

There is a common coding region polymorphism of p53 at codon 72, encoding for either proline (P72) or arginine (R72). This amino acid variation can impact p53 function with regard to cell fate after stress. In response to DNA damage, the P72

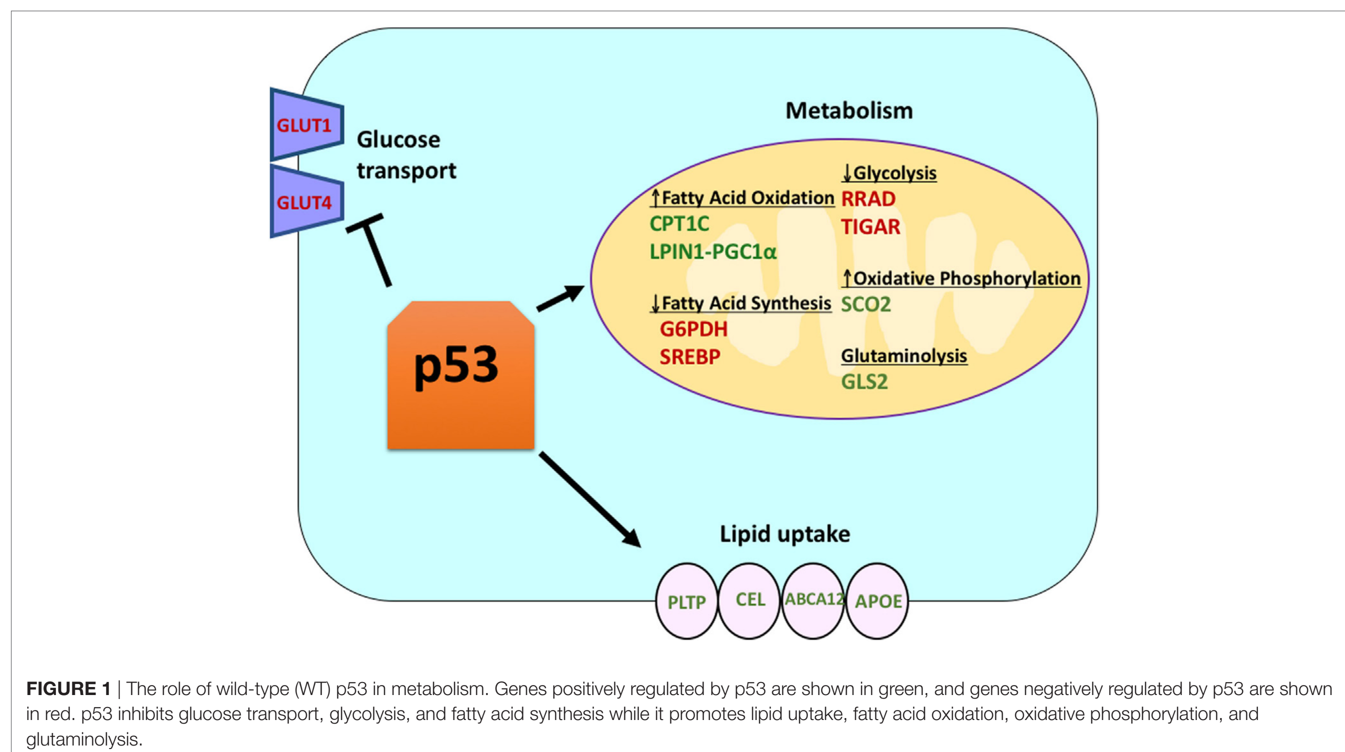

FIGURE 1 | The role of wild-type (WT) p53 in metabolism. Genes positively regulated by p53 are shown in green, and genes negatively regulated by p53 are shown in red. p53 inhibits glucose transport, glycolysis, and fatty acid synthesis while it promotes lipid uptake, fatty acid oxidation, oxidative phosphorylation, and glutaminolysis.

variant of p53 predominantly triggers cell cycle arrest, while the R72 variant predominantly induces cell death, or apoptosis (18, 19). Despite these differences in function, the codon 72 variation has not been consistently associated with cancer susceptibility (20). By contrast, in human studies this polymorphism is significantly associated with increased body mass index and risk for diabetes (21, 22). This premise is supported by studies in mice, where a mouse model for these codon 72 variants shows increased high-fat diet-induced diabetes in mice with the R72 variant, compared to P72. In these studies, the p53 target genes *TNF*α and *NPC1L1* were identified as critical regulators in the increase in diet-induced obesity in R72 mice (23). Interestingly, the R72 variant has also been shown to confer increased survival of cells in response to nutrient deprivation (24). These findings have led to the hypothesis that the R72 variant of p53 arose and was selected for as populations migrated north, where cold weather would require increased fat accumulation, but where survival in response to nutrient deprivation would also be under selection (24).

p53 REGULATES LIPID METABOLISM

Though p53 is well known for regulating glycolysis and the citric acid cycle, p53 also has been shown to play a role in regulating lipid metabolism (25). It is believed that WT p53 enhances fatty acid oxidation while inhibiting fatty acid synthesis, thus acting as a negative regulator of lipid synthesis (25). There are several p53 target genes with roles in lipid metabolism. Sanchez-Macedo and colleagues demonstrated that carnitine palmitoyltransferase 1C (CPT1C) is transcriptionally regulated by p53; this enzyme aids in the transport of activated fatty acids to the mitochondria. In support of a role for this p53-regulated gene in cancer, this group showed that *Cpt1c*-deficient mice display delayed tumor development and higher survival rates (26). Lipin 1 (*LPIN1*) is another p53 target gene; LPIN1 is necessary for proper adipocyte development and is induced under low nutrient conditions (27). Finck and colleagues showed that LPIN1 interacts with PGC-1α, another known p53 target gene with a role in metabolism, and that this interaction activates the expression of genes involved in promoting fatty acid oxidation (28).

In addition to directly regulating the transcription of genes involved in lipid metabolism, p53 can also regulate lipid metabolism in a manner involving direct protein–protein interaction. For example, glucose-6-phosphate dehydrogenase, which is the rate-limiting enzyme in the pentose phosphate pathway, binds to and is directly inhibited by p53, resulting in decreased NADPH production and consequently decreased fatty acid synthesis (12). The sterol regulatory element-binding proteins (SREBP) family of transcription factors modulate the expression of genes involved in cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis (29–31). WT p53 represses SREBP function (32), while mutant forms of p53 bind directly to SREBP and enhance their transcriptional function, leading to increased SREBP activity in human tumors (33, 34). Consequently, mutant p53 is correlated with higher expression of sterol biosynthesis genes in human breast tumors (34, 35). Finally, AMP-activated protein kinase (AMPK) is an enzyme that is activated under low nutrient levels or energy stress and is known to inhibit fatty acid synthesis by interacting with acetyl-CoA-carboxylase and SREBP-1 (36, 37). Zhou and colleagues demonstrated that mutant p53 preferentially binds to and inhibits AMPK, leading to increased fatty acid synthesis. As a result, mutant p53 proteins lead to increased AMPK signaling, contributing to invasive cell growth of tumor cells (33). A lesser explored area is the role of p53 in lipid transport. It has been shown that p53 transcriptionally regulates apolipoprotein B (apoB) and apoB editing enzyme complex 1, indicating the role of p53 in regulating atherogenic lipoproteins (38). Microarray analysis of human liver-derived cells identified phospholipid transfer protein, ATP binding cassette A12, and carboxyl ester lipase as three p53 target genes that all play a role in lipid transport (39, 40). Overall, though it is clear that p53 plays a key role in mediating lipid synthesis and metabolism, the contribution of this pathway, and these p53 target genes, to tumor suppression by p53 remains to be determined (**Figure 1**).

FERROPTOSIS IS A NOVEL CELL DEATH PATHWAY DRIVEN BY LIPID PEROXIDATION

In 2012, Dixon and colleagues discovered a novel form of regulated cell death called ferroptosis. Ferroptosis is an iron-dependent, caspase-independent form of cell death resulting from the accumulation of oxidized lipids (4, 41). This process is driven by the inactivation of glutathione peroxidase 4 (GPX4), an enzyme that is responsible for converting lethal lipid hydroperoxides to non-toxic lipid alcohols, which requires glutathione in order to function (41). It is believed that peroxidation of polyunsaturated fatty acids (PUFAs) is the driving impetus for cell death by ferroptosis. PUFAs contain bis-allylic protons that can easily be abstracted and produce radicals that will react with oxygen, creating more radicals and resulting in a chain reaction of lipid reactive oxygen species (42). The exact mechanism of cell death by ferroptosis remains unknown, but one hypothesis is that the lipid damage leads to the destruction of the plasma membrane (43). It has been speculated that ferroptosis could be a mechanism of tumor suppression that works by eliminating cells that are nutrient deprived or have been exposed to an environmental stress or infection.

PHARMACOLOGIC REGULATION OF FERROPTOSIS

Ferroptosis can be induced using inhibitors of system x_c^- such as erastin, or analogs such as glutamate and sorafenib, which inhibit the import of cystine, resulting in depleted glutathione and subsequent inactivation of GPX4. Alternatively, ferroptosis can be induced by (1S,3R)-RSL3 (hereafter referred to as RSL3), which directly binds to and inhibits GPX4 (4, 5, 42). Buthione sulfoximine, FIN56, FINO2, CCl_4, and cisplatin are other agents that have been demonstrated to induce ferroptosis in cells. Death by ferroptosis can be prevented by suppressing lipid peroxidation, which can be accomplished by using lipophilic antioxidants, such as ferrostatin-1, liproxstatin-1, or vitamin E. Iron chelators such

as deferoxamine or cicloprox are another tool used to suppress ferroptosis by reducing the levels of iron. Depleting PUFAs or adding monounsaturated fatty acids to cell culture media can also rescue cells from ferroptosis (42, 44).

FERROPTOSIS IS IMPLICATED IN p53-MEDIATED TUMOR SUPPRESSION

In 2012, Gu and colleagues developed a mouse model in which three normally acetylated lysine residues in the DNA-binding domain of p53 were mutated to arginine, and therefore could not be acetylated; this mouse is referred to as the 3KR mouse. Notably, cells from the 3KR mouse are unable to undergo p53-dependent apoptosis, cell cycle arrest, or senescence, and indeed the 3KR mutant of p53 fails to transactivate the majority of p53 target genes. Interestingly, this mouse model does not spontaneously develop cancer, implying that p53 could suppress tumor development independent of senescence or apoptosis (45). This group found that the mutant 3KR protein retains the ability to undergo ferroptosis and regulate cystine metabolism by regulating the expression of the cystine importer *SLC7A11*; this suggested that ferroptosis might be one pathway that underlies p53-mediated tumor suppression. When wild type and 3KR MEFs were treated with the ferroptosis inducer Erastin, almost 50% cell death was observed whereas p53 null MEFs exhibited 20% cell death; this indicates that p53 sensitizes cells to ferroptosis, and also that other key regulators also play a role in ferroptosis (5). Subsequently, Gu and colleagues identified an additional acetylation site at lysine 98 of p53, and they generated a mouse model in which all four acetylation sites were mutated to arginine (4KR). Interestingly, the 4KR mutant was unable to regulate genes involved in ferroptosis like *SLC7A11*, and unlike the 3KR mutant was unable to suppress tumor development (46). Though at present correlative, these data implicate the role of p53 in ferroptosis in its ability to suppress tumor development.

IN NON-TRANSFORMED CELLS, p53 POSITIVELY REGULATES FERROPTOSIS

In addition to *SLC7A11*, several other direct p53 target genes have been discovered to play a role in ferroptosis. These include *GLS2*, *PTGS2*, and *SAT1*. Studies from two separate groups support the role of *GLS2* in ferroptosis, which is known to decrease glutathione and increase cellular ROS levels. Jiang and colleagues used ferroptosis inhibitors combined with glutaminolysis inhibitors to inhibit Erastin-induced ferroptosis, thereby demonstrating that ferroptosis requires glutaminolysis and *GLS2* (47). Murphy and colleagues showed that a polymorphic variant of p53 was able to induce growth arrest and senescence in both human and murine cells but failed to repress *SLC7A11* or transactivate *GLS2*. This variant was markedly impaired at inducing ferroptosis and suppressing tumor development, thus again implicating the role of p53 in ferroptosis-mediated tumor suppression (48). Another p53 target gene with a role in ferroptosis is *PTGS2*, a gene encoding the enzyme cyclooxygenase-2. Stockwell and colleagues first showed that the induction of ferroptosis using Erastin and RSL3 led to the upregulation of *PTGS2* (41). Notably, *PTGS2* was not upregulated by ferroptosis inducers in p53-null cells, suggesting that this regulation is p53 dependent (5). Presently, the upregulation of *PTGS2* is widely used as a ferroptosis marker (5, 41).

A recent study by the Gu group showed that the p53 target gene *SAT1* regulates ferroptosis (49). The authors identified *SAT1* as a direct target of p53 and showed that silencing of *SAT1* reduced cell death induced by reactive oxygen species in cells with WT p53, but had no effect in p53-null cells. Mechanistically, this group showed that *SAT1* increases the level and activity of arachidonate 15-lipoxygenase, an iron-binding enzyme that oxidizes PUFAs and increases lipid peroxidation. Notably, this study showed that neither p53 nor SAT1 alone appear to be sufficient to induce ferroptosis. Instead, the combined data are more consistent with the premise that p53, by virtue of regulating genes that contribute to ferroptosis, regulates the sensitivity of cells to this pathway, rather than directly induces ferroptosis. Whether p53 regulates other genes involved in ferroptosis remains to be determined (**Figure 2**).

IN SOME CELLS, p53 NEGATIVELY REGULATES FERROPTOSIS

A study recently published by Tarangelo and colleagues shows that p53 negatively regulates ferroptosis in cancer cells (50). This group found that pre-treating cells with Nutlin-3, a compound that stabilizes p53 delays the onset of ferroptosis in several cell types. The delayed onset of ferroptosis was found to depend on *CDKN1A* (encoding p21), a critical p53 transcriptional target. The mechanism through which p21 delays ferroptosis has yet to be elucidated, but it is believed that the conservation of intracellular glutathione may be a contributing factor for reduced ferroptosis sensitivity. The authors conclude that the p53–p21 axis enables cancer cells to survive under conditions of metabolic stress, such as cystine deprivation, by suppressing the onset of ferroptosis (50). A recent study showed that p53 inhibits ferroptosis in colorectal cancer cells by binding to the enzyme dipeptidyl-peptidase-4 (DPP4), which is a modulator of ferroptosis and lipid metabolism. Mechanistically, this study showed that p53 antagonizes ferroptosis by sequestering DPP4 in a nuclear enzymatic inactive pool. In the absence of p53, DPP4 is free to interact with and form a complex with NOX1; this leads to increased lipid peroxidation and ferroptosis. Inhibition of DPP4 suppresses ferroptosis significantly, whereas overexpression of DPP4 triggers Erastin sensitivity, particularly in p53-depleted cells (51). The bidirectional control of ferroptosis by p53 through transcription-dependent and transcription-independent mechanisms may be context or cell-type dependent (**Figure 2**).

THE P47S POLYMORPHISM OF TP53 AFFECTS FERROPTOSIS AND TUMOR SUPPRESSION

In addition to missense mutations, there are several functionally significant single-nucleotide polymorphisms (SNPs) in the TP53 gene and other proteins known to regulate this pathway (such as

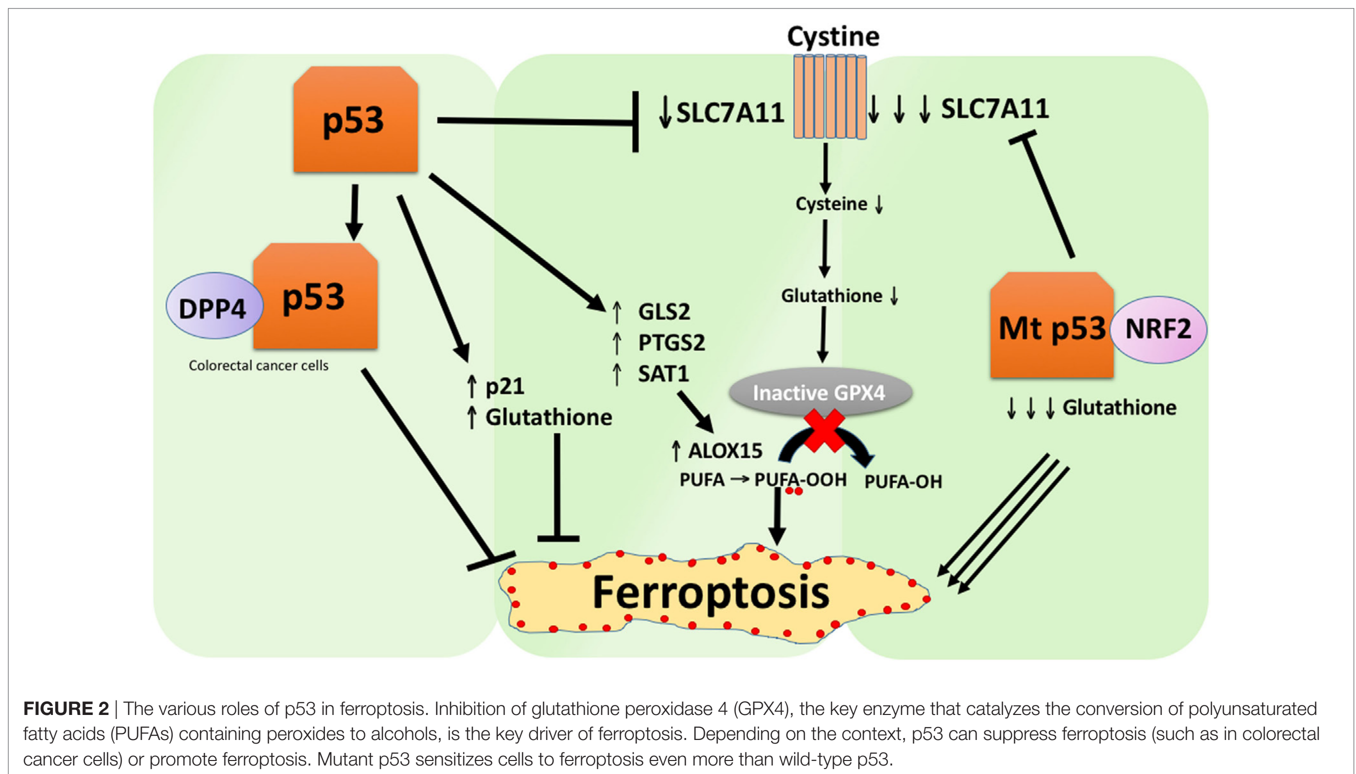

FIGURE 2 | The various roles of p53 in ferroptosis. Inhibition of glutathione peroxidase 4 (GPX4), the key enzyme that catalyzes the conversion of polyunsaturated fatty acids (PUFAs) containing peroxides to alcohols, is the key driver of ferroptosis. Depending on the context, p53 can suppress ferroptosis (such as in colorectal cancer cells) or promote ferroptosis. Mutant p53 sensitizes cells to ferroptosis even more than wild-type p53.

MDM2 and *MDM4*). The Pro47Ser variant (hereafter S47) is the second most common SNP found in the p53 coding region (after Pro72Arg) that alters the amino acid sequence of the protein. To better elucidate the impact of this variant on p53 function and cancer risk, the Murphy group generated a humanized p53 knock-in mouse model, in which exons 4–9 of murine p53 were replaced by human p53 exons containing either the wild type or the S47 variant (52–55). The majority of S47 mice spontaneously developed tumors of various histologic types, particularly liver cancer, between 12 and 18 months of age, unlike WT p53 mice (48). In mouse embryonic fibroblasts and human lymphoblastoid cell lines, the S47 variant showed impaired programmed cell death in response to cisplatin and other genotoxic stresses. Mechanistically, the S47 variant is defective for transactivation of genes involved in metabolism, such as *Gls2* (glutaminase 2) and *Sco2* (48). Consistent with the role of *Gls2* in ferroptosis, this group found that S47 cells were markedly resistant to the ferroptosis-inducing agents Erastin and RSL3 (47, 48). This defect may contribute to the tumor-prone phenotype observed in S47 mice.

MUTANT p53 SENSITIZES TUMOR CELLS TO FERROPTOSIS

Wild-type p53 negatively regulates the expression of the cystine importer *SLC7A11*, which inhibits sensitivity to ferroptosis (5). Although this regulation occurs in normal cells, in tumor cells, other mediators of *SLC7A11* appear to predominate in the regulation of this gene. For example, the master antioxidant transcription factor NRF2 can also regulate the expression of *SLC7A11* at the transcriptional level, and NRF2 has been implicated as a key player in protecting cancer cells against ferroptosis. For example, inhibition of NRF2 in hepatocellular cancer cells increases the anti-cancer activity of Erastin and Sorafenib *in vivo* (56). Mutant forms of p53 can inhibit NRF2 function by direct interaction, and one group found that tumors with mutant p53 contain very low levels of *SLC7A11*, and thus show increased sensitivity to ferroptosis. Notably, overexpression of *SLC7A11* in mutant p53 models led to drug resistance, suggesting that levels of *SLC7A11* expression must be considered when targeting mutant p53 driven cancers with ferroptosis-inducing compounds (57). In support of this premise, recent work in colorectal (CRC) cancer, where mutation or deletion of p53 is a frequent event, showed that human CRC cell lines harboring mutant p53 were far more sensitive to Erastin-mediated cell death when compared to CRC cells with WT p53. To validate these findings, they showed that knock in of a p53 hotspot mutation in both HCT116 and SW48 cells restored sensitivity to Erastin (51). These data highlight a novel mechanism by which cancers driven by mutant p53 can be exploited using targeted therapy.

CONCLUSION

The role of p53 in metabolism is quite clear and possibly even intuitively obvious: WT p53 limits glucose metabolism and lipid synthesis, while mutant p53 appears to do the opposite. The contribution of its metabolic role to tumor suppression by p53, and to the ability of mutant p53 to drive tumor progression, remains to be unequivocally proven. The role of p53 in the regulation of ferroptosis, and the contribution of this function, to tumor

suppression is even less clear. While compelling data from mouse models supports the premise that p53 regulates the sensitivity of cells to ferroptosis, this may be restricted to the ability of basal p53 to suppress spontaneous tumor development, and in oncogene-stressed mouse models, it is clear that senescence and apoptosis play the predominant role. Similarly, p53 may regulate ferroptosis sensitivity in a cell type-specific manner. More studies in animal models, with attention to ferroptosis in different tissues, need to be done to more fully understand the role of p53 in ferroptosis and ferroptosis in tumor suppression. Additionally, a clearer idea of what p53-target genes play a role in sensitivity to ferroptosis needs to be attained. Resolution of these questions should provide for much needed novel avenues to combat tumors with mutant p53.

AUTHOR CONTRIBUTIONS

KG, SB, TB, AB-K, C-PK, and MM each wrote one to two paragraphs of this article. KG and SB did the figure. KG and MM outlined the chapter.

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REVIEW
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PTEN: Tumor Suppressor and Metabolic Regulator

Chien-Yu Chen[1†], *Jingyu Chen*[1†], *Lina He*[1] and *Bangyan L. Stiles*[1,2*]

[1] Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA, United States, [2] Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States

Phosphatase and Tensin Homolog deleted on Chromosome 10 (PTEN) is a dual phosphatase with both protein and lipid phosphatase activities. PTEN was first discovered as a tumor suppressor with growth and survival regulatory functions. In recent years, the function of PTEN as a metabolic regulator has attracted significant attention. As the lipid phosphatase that dephosphorylates phosphatidylinositol-3, 4, 5-phosphate (PIP_3), PTEN reduces the level of $PIP_{3,}$ a critical 2nd messenger mediating the signal of not only growth factors but also insulin. In this review, we introduced the discovery of PTEN, the PTEN-regulated canonical and nuclear signals, and PTEN regulation. We then focused on the role of PTEN and PTEN-regulated signals in metabolic regulation. This included the role of PTEN in glycolysis, gluconeogenesis, glycogen synthesis, lipid metabolism as well as mitochondrial metabolism. We also included how PTEN and PTEN regulated metabolic functions may act paradoxically toward insulin sensitivity and tumor metabolism and growth. Further understanding of how PTEN regulates metabolism and how such regulations lead to different biological outcomes is necessary for interventions targeting at the PTEN-regulated signals in either cancer or diabetes treatment.

Keywords: PTEN, PI3K, AKT, cancer, metabolism

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Che-Pei Kung,
School of Medicine, Washington University in St. Louis, United States

Reviewed by:
Roger Moorehead,
University of Guelph, Canada
Jean-Yves Scoazec,
Institut Gustave Roussy, France

Correspondence:
Bangyan L. Stiles
bstiles@usc.edu

†These authors have contributed equally to the work.

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KEY CONCEPTS

- Phosphatase and Tensin Homolog deleted on Chromosome 10 (PTEN) is a dual phosphatase with both protein and lipid phosphatase activities.
- PTEN reduces the level of PI-3, 4, 5-$P_{3,}$ a critical 2nd messenger mediating the signal of not only growth factors but also that of insulin.
- In addition to the canonical PI3K/AKT signaling, PTEN also functions in the nucleus.
- PTEN regulates signals in metabolic regulation, includes the role of PTEN in glycolysis, gluconeogenesis, glycogen synthesis, lipid metabolism as well as mitochondrial metabolism.
- PTEN and PTEN regulated metabolic functions act paradoxically toward insulin sensitivity and tumor metabolism and growth.

INTRODUCTION

PTEN (phosphatase and tensin homolog deleted on chromosome 10) (also named MMAC1/TEP1) was discovered in 1997 independently by three laboratories as a tumor suppressor of which the expression is often lost in tumors (1–3). Later studies established that PTEN is a negative regulator of a major cell growth and survival signaling pathway, namely the phosphatidylinositol-3-kinase

(PI3K)/AKT signaling pathway (4, 5). It is now well established that PTEN plays a role in growth and survival. Studies in recent years also established a role of PTEN in metabolic regulation (6, 7). In this review, we summarized the roles of PTEN as both a tumor suppressor and a metabolic regulator and reviewed the biological functions of PTEN and its downstream target proteins. We also summarized the regulations of PTEN transcriptionally, post-transcriptionally and through regulation of its subcellular localization.

PTEN FUNCTION AND ITS REGULATION

PTEN: A Dual Phosphatase

PTEN is encoded on chromosome 10q23, a region where loss of heterozygosity frequently occurs in various types of cancer (8). The protein encoded by *PTEN* contains 403-amino acid where the amino-terminal region shares sequence homology with the actin filament capping protein TENSIN and the putative tyrosine-protein phosphatase AUXILIN (1, 6). Crystal structure of PTEN revealed a C2 domain that contains the affinity for phospho-lipids on membrane and a phosphatase domain that contains the CX5R signature motif for phosphatases (9) (**Figure 1**). *In vitro*, PTEN is capable of dephosphorylating phospho-peptides as well as phospho-lipids. Thus, PTEN is a dual lipid and protein phosphatase. The biological effects of PTEN, however are dominated by its ability to dephosphorylate the lipid substrate phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) whereas protein substrates for PTEN are still being discovered (5, 7, 10). The lipid phosphatase motif of PTEN dephosphorylates PIP_3 at the 3′ position and converts it back into PIP_2 (5), leading to reduced PIP_3 production and signals that depends on PIP_3 (11) (**Figure 2**). PI3K functions to catalyze the reaction from PIP_2 to PIP_3. It achieves this task by phosphorylating the hydroxyl group of the 3rd position on the inositol ring of Phosphatidylinositols (12). The enzymatic function of PTEN thus acts as a negative regulatory signal for the PI3K mitogenic signaling pathway (**Figure 2**).

While the lipid substrate is well characterized to be PIP_3, the identity of the protein substrates for PTEN *in vivo* has been illusive (13). However, *in vitro* study has revealed that PTEN is able to regulate cell migration by dephosphorylating itself, providing insights for investigating potential protein substrates for PTEN (14).

Regulation of PTEN

Localization of PTEN

Several non-canonical nuclear localization domains have been found on PTEN (15). A cytoplasmic localization signal has been identified for the N-terminus of PTEN that spans the residue from 19 to 25 (16). Mutations in these residues leads to increased nuclear localization of PTEN with unknown mechanisms. Studies suggest that ubiquitination controls the shuttling of PTEN between cytosol and nucleus (17–19). Monoubiquitination of lysine 289 (K289) is necessary for PTEN to move into the nucleus. Mutation of this site, K289E, is found in familial Cowden's syndrome that carries multiple mutations of the *PTEN* gene (17, 20, 21). A second ubiquitination site K13 and several other sites may also facilitate the nuclear transportation of PTEN (**Figure 1**). In addition, the localization of PTEN is regulated by $Ca2^{+}$-mediated interactions with the major vault protein (MVP) (22, 23). In the nucleus, PTEN is more stable and still capable of inhibiting AKT and inducing cell death. Structural analysis also reviewed that PTEN harbors a PDZ domain and two PEST sequences in the C-terminal region (24). The PDZ domain is thought to regulate PTEN's subcellular localization whereas the two PEST sequences regulate its protein stability (25) (**Figure 1**).

Interestingly, while monoubiquitination leads to its nuclear shuttling, poly-ubiquitination of PTEN leads to its degradation. Though disagreement exists, an E3 ubiquitin ligase for PTEN has been reported (18, 21). NEDD4-1 was reported to add ubiquitin to both K13 and K289 of PTEN molecule, leading to both mono- and poly-ubiquitination of PTEN. However, others suggest that NEDD4 is dispensable for the regulation of PTEN (21).

Transcriptional Regulation of PTEN

PTEN is also regulated on the transcriptional and post-transcriptional levels. Several transcriptional factors have been reported to control the transcription of PTEN, including the tumor suppressor p53, the early growth response protein 1 (EGR-1), a metabolic regulatory gene peroxisome proliferation-activator receptor γ (PPARγ) [for detail, see (6)] and active transcription factor 2 (ATF2) (26). PTEN is also transcriptionally repressed by SNAIL and SLUG (27). These two zinc finger-like transcriptional factors compete with p53 for PTEN promoter binding. In addition, the nuclear factor kappa B (NFkB), the AP-1 transcription factor subunit c-Jun and the Notch signaling coregulatory CBF-1 (C-promoter binding factor-1) also bind to the *PTEN* promoter to regulate its transcription (28, 29).

More recently, regulation of PTEN by RNA-RNA interaction is reported that include microRNAs and long noncoding RNAs. Several miRNA including miR-205, miR-122, miR-21, etc. were identified to bind to the 3′untranslated region of PTEN mRNA. Elevated levels of many miRNAs are correlated with a concomitant reduction of PTEN mRNA (30–33). The long noncoding RNA that is encoded by the PTEN pseudogene transcript PTENP1 shares sequence identity with PTEN mRNA (34, 35). This transcript binds to miRNAs that target PTEN, leading to stabilization of PTEN mRNA. The antisense transcript of this pseudogene binds to the promoter of PTEN and negatively regulates the transcription of PTEN (34, 36).

Post-translational Regulation of PTEN

Post-translationally, PTEN is modified by acetylation, oxidation and phosphorylation in addition to the ubiquitination discussed above [for detail, see (37)] (**Figure 1**). Phosphorylation of PTEN occurs on several clustered residues in the C-terminal domain of PTEN (38, 39). Several enzymes are responsible for these phosphorylations including casein kinase 2 (CK2), GSK3β, RhoA kinase, and P110δ subunits of PI3K (38–43). Phosphorylation of PTEN generally leads to the stabilization of the molecule but may reduce its activity (38, 39, 44). Recently, it was shown that ataxia-telangiectasia-mutated kinase (ATM) phosphorylates SUMOylated PTEN in response to γ-irradiation which leads to its nuclear exclusion (45). SUMOylation of PTEN typically

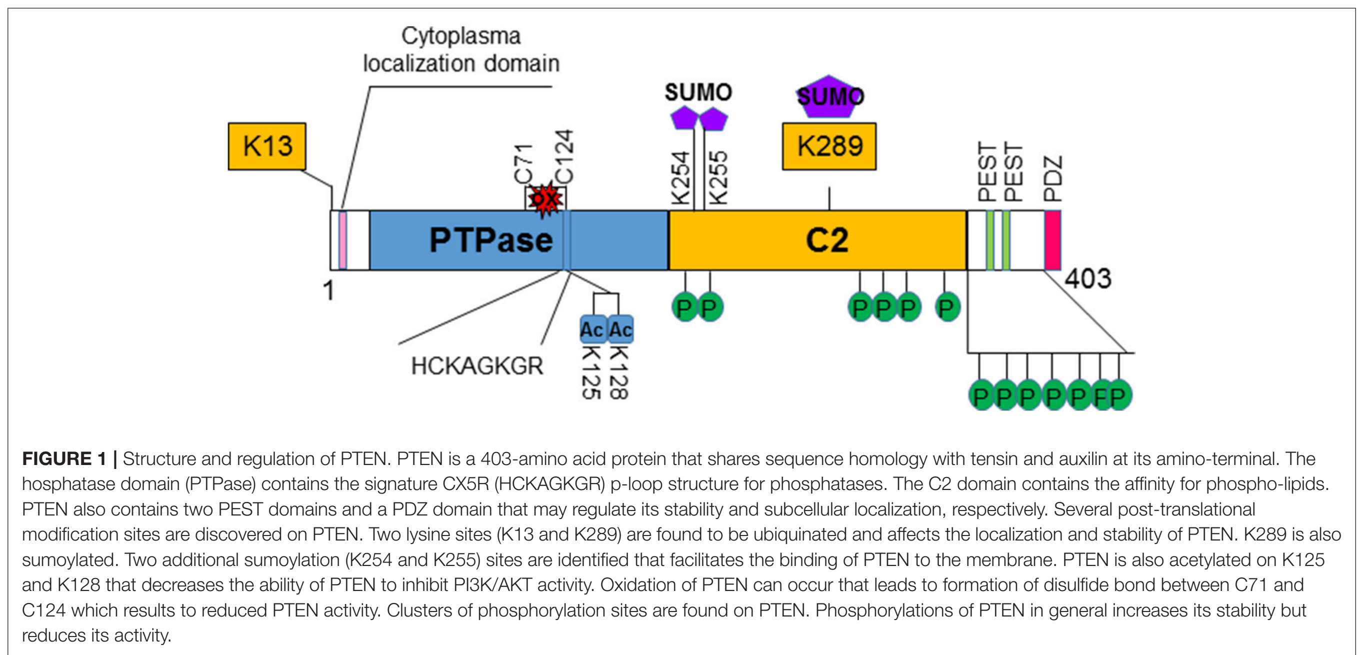

FIGURE 1 | Structure and regulation of PTEN. PTEN is a 403-amino acid protein that shares sequence homology with tensin and auxilin at its amino-terminal. The hosphatase domain (PTPase) contains the signature CX5R (HCKAGKGR) p-loop structure for phosphatases. The C2 domain contains the affinity for phospho-lipids. PTEN also contains two PEST domains and a PDZ domain that may regulate its stability and subcellular localization, respectively. Several post-translational modification sites are discovered on PTEN. Two lysine sites (K13 and K289) are found to be ubiquinated and affects the localization and stability of PTEN. K289 is also sumoylated. Two additional sumoylation (K254 and K255) sites are identified that facilitates the binding of PTEN to the membrane. PTEN is also acetylated on K125 and K128 that decreases the ability of PTEN to inhibit PI3K/AKT activity. Oxidation of PTEN can occur that leads to formation of disulfide bond between C71 and C124 which results to reduced PTEN activity. Clusters of phosphorylation sites are found on PTEN. Phosphorylations of PTEN in general increases its stability but reduces its activity.

occurs on K254 and K255 (46) which facilitates its binding to the membrane whereas modification on K289 is involved in its nuclear shuttling due to competition with ubiquitination modification. A couple of lysine residues at the catalytic domain of PTEN, lysine 125 and 128 are acetylated by PCAF (47). These acetylations lead to the diminished ability of PTEN to inhibit downstream events. PTEN is also regulated by the redox status of the cells. Two cysteine residues (124 and 71) form a disulfide bond in response to H_2O_2 treatment that leads to reduced activity of PTEN (48). Cys 124 is one of the "hot spots" that are often found mutated in human cancers.

PTEN AS A TUMOR SUPPRESSOR

Canonical Signaling Pathways Regulated by PTEN

Accumulation of PIP_3 serves as a major signal for growth factor stimulation. PIP_3 binds to the pleckstrin homology (PH) domain of downstream proteins (e.g., AKT) and provides a lipid moiety and recruits these proteins to the plasma membrane (49). Binding of PIP_3 to the PH domain also changes the confirmation of these proteins so they can later be activated by phosphorylation. By reducing the intracellular levels of PIP_3, PTEN inhibits the activation of downstream proteins of the PI3K pathway, including the serine/threonine kinase AKT and the protein kinase C (PKC).

A well-known downstream effector protein of the PTEN signal is AKT (50), which plays a critical role in regulating a number of cellular activities including cell growth, survival, cell migration and differentiation, cell and organ size control, metabolism, et al. [for detailed review, see (51)]. AKT, also known as Protein Kinase B (PKB) is a serine/threonine kinase. Following PI3K activation, accumulation of PIP_3 allows recruitment of AKT to the plasma membrane via direct interaction with its PH domain (11). This binding of AKT to PIP_3 not only allows AKT to be translocated to the membrane but also exposes sites on AKT where it can be further modified. It has been shown that AKT is phosphorylated by another PH domain-containing kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) at Thr308 (13, 52). This phosphorylation on Thr308 is important for initial activation of AKT whereas phosphorylation of Ser473 by mTORC2 is required for maximal AKT activation (53) (**Figure 2**).

Activated AKT phosphorylates a plethora of downstream targets including the regulations of kinases such as glycogen synthase kinases (GSK3α and β) (54), IκB kinases (IKKα and IKKβ) (55), apoptotic factors such as BAD (56), MDM2, a ubiquitin ligase for p53 (57), GTPases like Rac and Rho (58), cell cycle inhibitors p21 and p27 (59), and transcription factors such as forkhead transcription family (FoxO) members (**Figure 2**) (60–62). Phosphorylation by AKT regulates the functions of these molecules that are important for multiple cellular processes. For instance, phosphorylation of the pro-apoptotic factors BAD, caspases 3 and 9 by AKT renders them inactive and thus promotes cell survival (56, 63, 64). Phosphorylation of p21 on T145 and p27 on T157 leads to their nuclear exclusion and the inability of these cell cycle inhibitors to inhibit cell proliferation (65, 66). Likewise, AKT also directly phosphorylates MDM2 and MDMX (67, 68). The phosphorylated MDM2 and MDMX bind to 14-3-3 proteins, leading to stabilization of the MDM2-MDMX complexes which mediates the degradation of p53 to keep the level of p53 low in the cells.

AKT also phosphorylates forkhead transcriptional factors and induces their binding to 14-3-3 proteins (63). This process blocks their translocation to the nucleus. Several members of the forkhead transcriptional factor family are targets of AKT, including FOXO1 and FOXO3. The binding elements for these

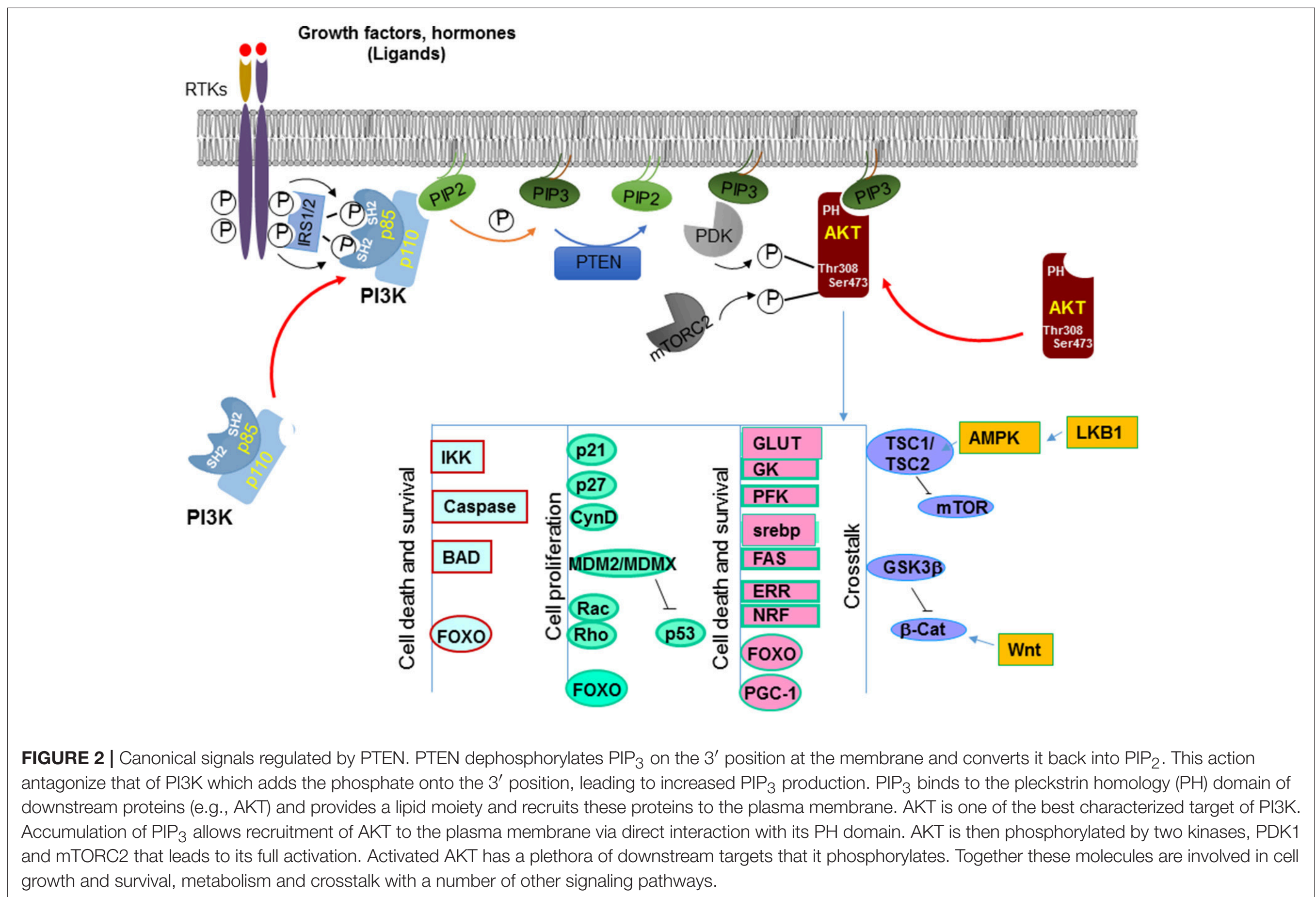

FIGURE 2 | Canonical signals regulated by PTEN. PTEN dephosphorylates PIP_3 on the 3′ position at the membrane and converts it back into PIP_2. This action antagonize that of PI3K which adds the phosphate onto the 3′ position, leading to increased PIP_3 production. PIP_3 binds to the pleckstrin homology (PH) domain of downstream proteins (e.g., AKT) and provides a lipid moiety and recruits these proteins to the plasma membrane. AKT is one of the best characterized target of PI3K. Accumulation of PIP_3 allows recruitment of AKT to the plasma membrane via direct interaction with its PH domain. AKT is then phosphorylated by two kinases, PDK1 and mTORC2 that leads to its full activation. Activated AKT has a plethora of downstream targets that it phosphorylates. Together these molecules are involved in cell growth and survival, metabolism and crosstalk with a number of other signaling pathways.

forkhead transcriptional factors are widely spread on promoter regions of genes that regulate cell proliferation, survival and metabolic changes (69). For example, FOXO3a binds to the promoters of Bim and PUMA and can initiate apoptosis cascades by inducing the transcription of these death genes (70, 71). FOXO1 transcriptionally activates p21 and p27 and inhibits cell proliferation through these actions (72, 73). Furthermore, these forkhead transcriptional factors are also responsible for many metabolic effects induced by insulin signaling through the PI3K/AKT signaling pathway (74). Additional evidence suggests that the forkhead transcriptional factors may play a key role in the feedback regulation of the Insulin/PI3K/AKT signaling pathway (75).

Two substrates of AKT, GSK3β and tuberous sclerosis complex TSC1/2, play important roles in mediating cross talks between PI3K/AKT signaling pathway and other signaling pathways (**Figure 2**). GSK3β is phosphorylated by AKT on Serine 21/9 which inhibits its activity (76). GSK3β is an important regulator in Wnt signaling. It phosphorylates β-catenin, resulting in its ubiquitin-mediated degradation. The crosstalk between PTEN and Wnt signaling may underlie some of the effects of PTEN on the regulation of stem cell maintenance (77–79) and G_0-G_1 cell cycle regulation (80–84). Another substrate of AKT is TSC1/2. TSC1/2 plays a key role in incorporating metabolism and cell size control, together with cell growth and proliferation regulation (85). The heterodimer of TSC1 and TSC2 is essential for suppressing the function of mTOR (mammalian target of rapamycin). TSC2 activity is inhibited when phosphorylated by AKT (86). Therefore, by acting on TSC2, AKT induces the activity of mTOR and the downstream events of mTOR activation that include metabolic changes, protein translation as well as cell proliferation. This regulation of mTOR by AKT-TSC-mediated signal allows the crosstalk of PTEN with another tumor suppressor LKB1 (87).

Nuclear PTEN

In earlier studies, PTEN was reported to be a protein that is exclusively localized in the cytoplasm. However, it is clear now that PTEN can be both cytoplasmic and nuclear (88–90). In more differentiated and resting cells, PTEN is often found in the nucleus even though it was originally identified to be a cytosolic protein (using primarily tumor cells) (90). Nuclear PTEN also plays other roles in addition to its lipid phosphatase activity and the nuclear function of PTEN is important for the ability of PTEN to inhibit tumor development (**Figure 3**). Nuclear PTEN is reported to play important roles in chromosome stability, DNA repair and cell cycle regulation. In the nucleus, PTEN promotes the stability and transcriptional activity of the

tumor suppressor p53 by directly associating with p53 (91–93). Forced expression of PTEN in the nucleus led to MAP kinase-dependent inhibition of cyclin D1 expression (15, 22). Nuclear expression of PTEN results in the dephosphorylation of MAP kinase. Whether this is a direct effect of the protein phosphatase activity of PTEN is not clear. In addition, PTEN is found to be associated with the centromere in the nucleus by direct binding to the centromere specific binding protein C (CENP-C) (94). Disruption of this binding leads to premature centromere separation. In addition, PTEN is also found to collaborate with E2F to induce the expression of Rad51 and thus enhance DNA repair (94). This relationship between PTEN and Rad51 may explain the observation that double-stranded DNA breakage rate is found to be increased when nuclear PTEN function is interrupted. PTEN also interacts with the anaphase-promoting complex (APC) to promote its association with its binding partner which together results in proteolysis of mitotic cyclins (95).

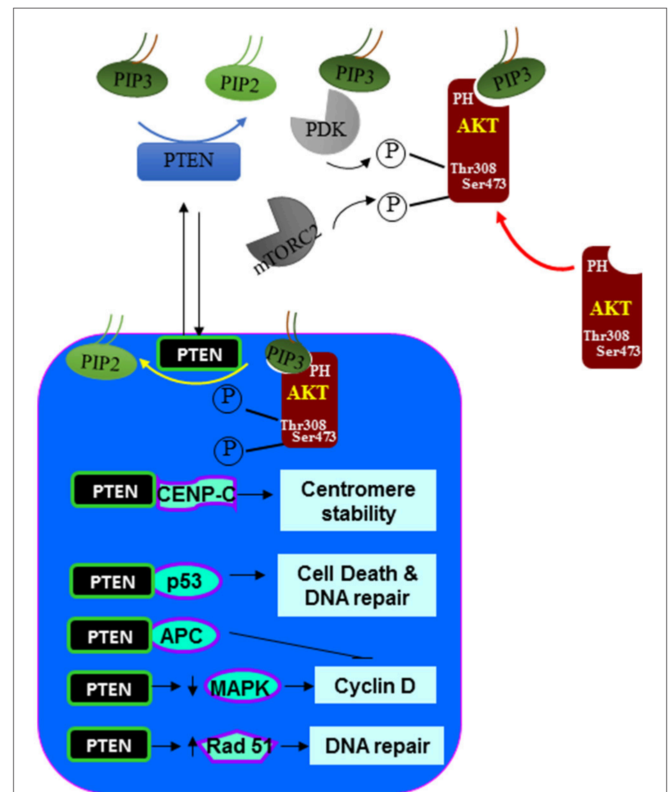

FIGURE 3 | Nuclear signals regulated by PTEN. In addition to the dephosphorylating PIP3 at the plasma membrane. PTEN is also found in the nucleus. In the nucleus, PTEN can act similarly as it does at the plasma membrane by inhibiting the function of AKT. In addition, PTEN also associate with a number of nuclear proteins and regulate other cellular functions such as centromere stability, DNA repair, cell death and proliferation.

PTEN AS A METABOLIC REGULATOR

As an important growth and survival regulatory gene, germline deletion of *Pten* in mice was shown to be embryonic (96–98). Heterozygous mice develop hamartomatous polyps in the colon and tumors in multiple other tissues (99). In human, germline PTEN mutation leads to a number of familial diseases that are characterized by multiple hamartomatous lesions and predisposition to cancer development (100). Conditional deletion of *Pten* in mouse models has been done in multiple organs. Collectively, these studies confirm the signaling studies verifying PTEN as a tumor suppressor that regulates cell growth and survival. These studies have been comprehensively reviewed previously (6, 101).

While PTEN loss promotes tumorigenesis in multiple organs, genetic studies also indicate that PTEN loss leads to a number of metabolic changes that collectively improve overall insulin sensitivity (**Figure 4**). PTEN, being a major negative regulator of the PI3K/AKT signaling, is found to play an important role in both lipid and glucose metabolism as well as regulation of mitochondrial functions. Studies in *C. elegans* and *Drosophila* have demonstrated a highly conserved signal regulated by PTEN for both growth control and metabolism. In these organisms, the insulin/PI3K pathway negatively regulated by PTEN is used to control dauer formation, metabolism, and life span in response to nutrient availability (102–104).

Regulation of Glucose Metabolism

Parallel signals for PTEN/PI3K have been reported for mammals as it was in *C. elegans* and *Drosophila*. Insulin and insulin-like growth factors (IGF) such as IGF-1 and IGF-2 binds to the insulin and IGF receptors. Binding of insulin and IGF to these receptors either directly induces the activation of PI3K or results in phosphorylation of insulin receptor substrate (IRS) as an adaptor protein to recruit and activate PI3K (10). Through this action and the downstream activation of AKT, adipocytes and myocytes sense the elevated insulin levels and initiate glucose uptake. The serine/threonine kinase AKT phosphorylates a 160-KDa substrate AS160 at Thr642 in adipocytes (105). Phosphorylation of this protein, identified as a GTPase-activating domain for Rab, is found to be responsible for membrane trafficking of GLUT4 induced by the insulin/PI3K signaling. In addition, AKT phosphorylates a variety of targets involved in the regulation of metabolism. Phosphorylation and inhibition of GSK3 not only contribute to regulation of β-catenin and the cell cycle, it also activates glycogen synthase (6). When PTEN is lost and GSK3 is phosphorylated, glycogen was found to accumulate in hepatocytes of the liver-specific *Pten* null mice (88). In hepatocytes, phosphorylation of FOXO by AKT blocks the transcription of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (74), two rate-limiting enzymes in the process of gluconeogenesis. In addition, AKT was also reported to directly phosphorylate proxisome proliferator-activated receptor gamma co-activator (PGC-1a) at S570 (106). This phosphorylation event was also found to mediate the transcriptional repression of G6Pase and PEPCK. These signals regulated by AKT and blocked by PTEN are important for how metabolic organs like the liver, muscle and adipose tissue respond to elevated insulin signals.

Consistent with these metabolic signals regulated by PTEN, deletion of *Pten* in the liver led to robust downregulation of

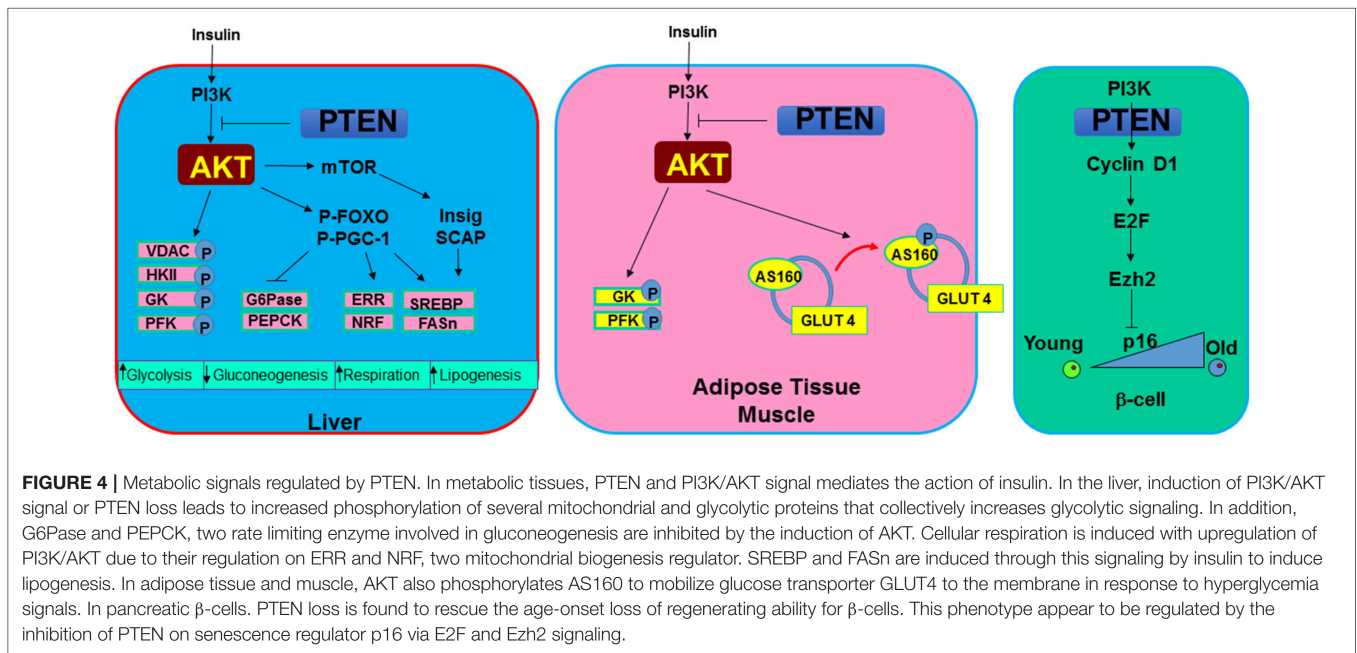

FIGURE 4 | Metabolic signals regulated by PTEN. In metabolic tissues, PTEN and PI3K/AKT signal mediates the action of insulin. In the liver, induction of PI3K/AKT signal or PTEN loss leads to increased phosphorylation of several mitochondrial and glycolytic proteins that collectively increases glycolytic signaling. In addition, G6Pase and PEPCK, two rate limiting enzyme involved in gluconeogenesis are inhibited by the induction of AKT. Cellular respiration is induced with upregulation of PI3K/AKT due to their regulation on ERR and NRF, two mitochondrial biogenesis regulator. SREBP and FASn are induced through this signaling by insulin to induce lipogenesis. In adipose tissue and muscle, AKT also phosphorylates AS160 to mobilize glucose transporter GLUT4 to the membrane in response to hyperglycemia signals. In pancreatic β-cells. PTEN loss is found to rescue the age-onset loss of regenerating ability for β-cells. This phenotype appear to be regulated by the inhibition of PTEN on senescence regulator p16 via E2F and Ezh2 signaling.

PEPCK (88). Moderate downregulation of G6Pase was also reported. In the adipose tissue, deletion of *Pten* resulted in increased insulin sensitivity and resistance to streptozotocin-induced diabetes (107). Increased GLUT4 membrane localization on adipocytes was observed in these mice.

Regulation of Lipid Metabolism

In addition to glucose metabolism, the PTEN-regulated PI3K signaling also controls lipid metabolism. The sterol receptor element binding protein, SREBP, serves as a key transcriptional factor for genes involved in the biosynthesis of fatty acids and their further incorporation into triglycerides and cholesterol. As a master transcriptional factor controlling the de novo lipogenesis process, SREBP binds to the promoters of many lipogenic enzyme genes, including fatty acid synthase (Fasn) and acetyl-CoA carboxylase (ACC), as well as those controlling the production of NADPH, a reducing equivalent needed for lipid biosynthesis. The PTEN/PI3K/AKT signaling-controlled SREBP expression is mediated through multiple levels including transcriptional and post-translational processing of SREBP. The downstream target of AKT, the forkhead transcriptional factor, FoxO1 regulates SREBP and lipogenesis by repressing SREBP transcription (108). Interestingly, the function of FOXO1 on Fasn expression is dependent on whether PI3K/AKT signal is induced (109). Using rapamycin and siRNA to inhibit mTORC1 and other signals involved in the AKT pathway, it was shown that transcription induction of SREBP1 and lipogenesis is also dependent on TORC1 activity (110). However, this effect was not supported by observations in the TSC1-deficient mice, defective in mTORC1 signaling, which are resistant, rather than sensitive, to high fat diet (HFD)-induced steatosis (111). The processing of SREBP is dependent on two proteins, SREBP cleavage-activating protein (SCAP) and insulin induced gene (Insig). In response to sterol demand, SCAP cleaves SREBP to produce the mature active form of transcriptional factor that moves to the nucleus. Binding of Insig to SCAP prevents this action and thus inhibits the processing of SREBP. While oxysterols suppresses the expression of Insig-1, inhibition of PI3K/AKT activity blocks this inhibition and allows the processing of SREBP (112), consistent with a role of AKT in SREBP processing. This processing is both mTORC1-dependent and mTORC1-independent (111). Thus, both PTEN/PI3K/AKT downstream signaling targets, TORC1 and FoxO1, play critical roles in controlling SREBP expression and lipogenesis. In addition, Maf-1, a central repressor of genes transcribed by RNA pol III is recently found to be regulated by PTEN through AKT2 and mTOR (113). While SREBP binds to the promoter of Fasn and positively regulates its expression, Maf-1 was shown to occupy the promoter and repress the expression of Fasn.

Consistent with these signaling analysis, loss of *Pten* in the liver led to elevated de novo lipogenesis through robust induction of SREBP and Fasn expression (88). The accumulation of lipid and elevated lipogenesis is a result of activation of AKT2 as deletion of *Akt2* completely reversed the phenotype (109, 114). This effect of AKT is both mTOR dependent and independent (111). In addition, FOXO1 gain of function has also been shown to induce lipid synthesis (115).

Regulation of Mitochondrial Metabolism

In recent years, studies attempt to elucidate the molecular signals underlying "Warburg effects" have led to the discoveries of novel roles for PI3K/AKT signaling in mitochondrial function (10). In addition to regulating pro- and anti-apoptotic factors (10), AKT promotes binding of hexokinase II to the mitochondrial voltage dependent anion channel (VDAC) (116). This event, occurring at

the mitochondrial outer membrane allows rapid phosphorylation of available glucose molecules and efficient conversion to ATP from glycolysis. AKT was also found to be localized in the inner membranes of the mitochondria (117, 118). In the mitochondria matrix, AKT phosphorylates mitochondrial pool of GSK3β and regulates mitochondrial respiration through phosphorylation of pyruvate dehydrogenase (PDH) (118). In addition, the mitochondrial localized AKT also plays a role in the transcription regulation of mitochondrial DNA. A FOXO3-response element has been found on the promoter of a mitochondrial encoded gene, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (119).

In the nucleus, the PI3K/AKT signaling controls mitochondrial gene transcription network through multiple different mechanisms. The forkhead transcriptional factor FOXO3 has been demonstrated to be a transcriptional regulator of mitochondrial genes. In colon cancer cells induced to express a constitutively active form of FOXO3a, a large number of mitochondrial genes are downregulated (120). These genes include Tfam and TFB1M&2M, the nucleus-encoded auxiliary factors for mitochondrial gene transcription. One of the global regulators of metabolism including lipid and glucose metabolism as well as mitochondrial metabolism is PGC-1 (121). As a transcriptional coactivator, members of PGC-1 family of coactivators have the ability to interact with a number of different transcriptional factors including PPARs for fatty acid oxidation, FOXO1 for lipogenesis, FOXO1 and glucocorticoid receptor (GR) for gluconeogenesis, and estrogen-related receptors (ERRs) for mitochondrial function.

The best characterized isoform of ERRs, ERRα is abundantly expressed in high oxidative organs and recognized as a key regulators of adaptive energy metabolism (122). ERRα, itself, is a weak transcriptional factor. Both the activity and expression of ERRα are significantly increased when physically bound by PGC-1α (123). AKT activation was found to control mitochondrial gene transcription by phosphorylating and activating CREB transcriptional factor independent of the cAMP mediated activation of PKA, the common signal that induces CREB phosphorylation (124). When phosphorylated, CREB induces the transcription of PGC-1. Thus, in addition to removing the inhibition of FoxO and phosphorylating PGC-1, activation of AKT also positively induces PGC-1 transcription by phosphorylating CREB. Being the coactivator, PGC-1 robustly increases the transcriptional activity of ERRα to promote transcription of genes encoding mitochondrial function, including TFAM, TFB1M&2M and medium-chain acyl-coA dehydrogenase (MCAD) (124). In hepatocytes, this induction of ERRα leads to increased oxygen consumption and elevated ROS production, likely contributed to the liver injury (and lipid accumulation) phenotypes observed with *Pten* loss in the liver.

In addition, the AKT substrate consensus sequence has been found on NRF1, another gene involved in mitochondrial gene transcription. In H4IIE hepatoma cells, phosphorylation of NRF1 by AKT is reported to mediate pro-oxidant t-BOOH induced Tfam expression (125). Thus, through directly phosphorylating FOXO and NRF1 or indirectly inducing ERRα expression, AKT controls the gene transcriptional networks of mitochondria. Consistently, over-expression of NRF1 and AKT has been shown to mimic the effect of TFAM to abrogate 1-methyl-4-phenyl-2, 3-dihydropyridinium ion induced mitochondrial damage (126), confirming a signaling relationship between PI3K/AKT/FOXO signal and mitochondrial gene transcription regulation.

PARADOXICAL ROLES OF PTEN REGULATED METABOLIC AND GROWTH SIGNALS ON TUMOR GROWTH AND METABOLISM

Metabolic Sensitivity Regulated by PTEN

In mammals, ectopic expression of PTEN by introduction of bacterial artificial chromosomes (BACs) into the mouse genome led to reduced body size, increased energy expenditure and low body fat content (127, 128). Consistent with the observations in *C. elegance* and *Drosophila*, these mice also have a longer tumor free lifespan. This enhanced metabolic phenotype however is paradoxical with the enhanced metabolic functions associated with PTEN loss observed with the tissue specific *Pten* deletion mice. In adipose tissue, liver, pancreatic β-cells and muscle, deletion of *Pten* consistently lead to enhanced insulin and metabolic sensitivity as well as resistance to HFD induced diabetes (88, 89, 107, 129–131). In addition to the enhanced ability to transport and metabolize glucose by adipocytes, myocytes and hepatocytes, PTEN loss was associated with enhanced energy expenditure in brown adipose tissue (128). In β-cells, deletion of *Pten* relieved the suppression of cell cycle re-entry by inhibiting the senescence regulatory gene p16^{Ink4a} through E2F/Ezh2 mechanism (132). This regulation led to the rescue of aging-induced loss of growth potential in β-cells. The enhanced ability of pancreatic islets to respond to hyperglycemic stress led to improved systemic metabolic health (89, 130). Overall, PTEN loss and activation of PI3K/AKT signal lead to the improved ability to handle metabolic stress in mice. The improved metabolic health phenotypes observed with overexpression of PTEN is likely contributed to metabolic adaptation.

Tumor Metabolism Regulated by PTEN

While loss of PTEN leads to improved insulin sensitivity, this metabolic effect has been credited for the tumor suppressing functions of PTEN (7). The metabolic signals regulated by this pathway, including the glycolytic signal such as localization of glucose transporters, activation of hexokinase and phosphofructokinase as well as induction of de novo lipogenesis are among the signals that are recognized as promoting factors for tumorigenesis. Indeed, a number of metabolic enzymes, particularly glycolytic genes have been found to have oncogenic or tumor suppressive functions as manipulation of these genes modulate tumor growth. Particularly, expression of isoform specific metabolic genes appears to be linked to tumorigenesis (133). Association of lipogenic and other lipid metabolic genes with tumors are recognized but more works are needed to understand their contributions to tumorigenesis.

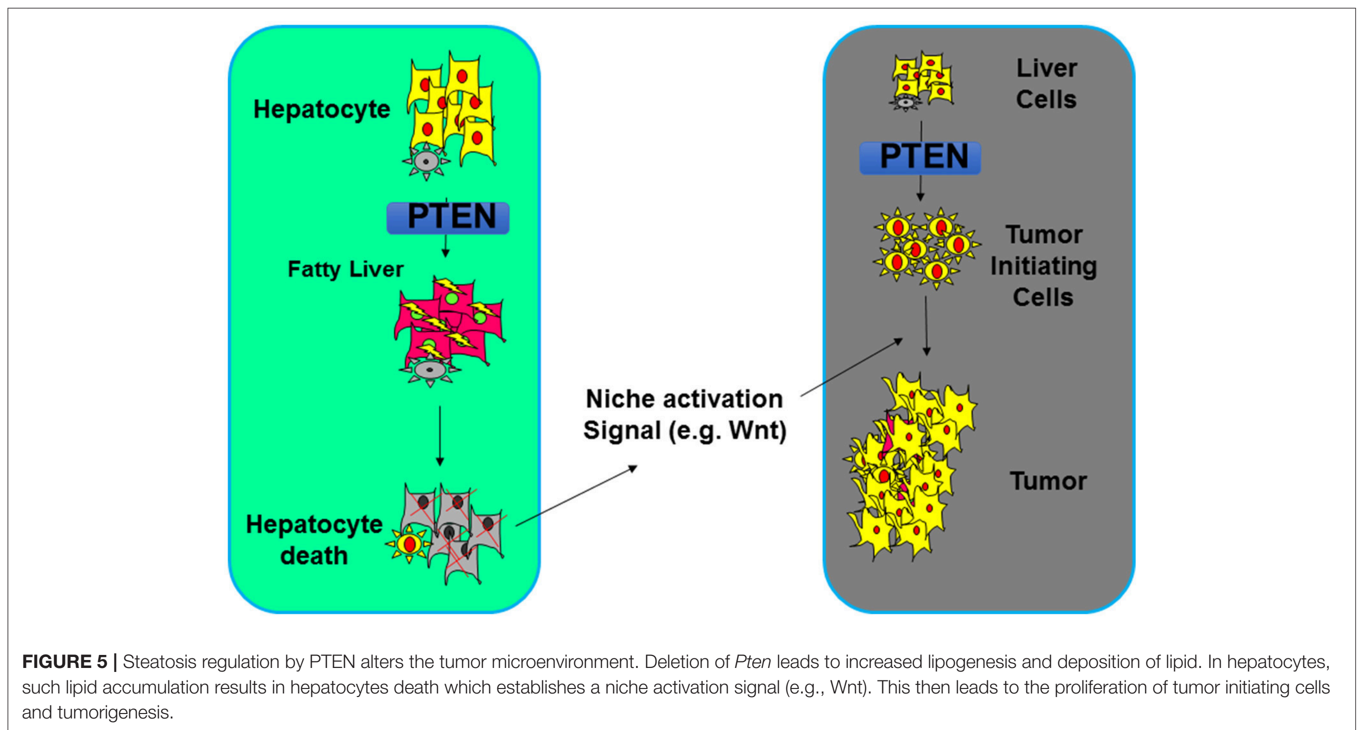

FIGURE 5 | Steatosis regulation by PTEN alters the tumor microenvironment. Deletion of *Pten* leads to increased lipogenesis and deposition of lipid. In hepatocytes, such lipid accumulation results in hepatocytes death which establishes a niche activation signal (e.g., Wnt). This then leads to the proliferation of tumor initiating cells and tumorigenesis.

Steatosis Due to PTEN Loss Establishes a Tumor Promoting Environment

During insulin resistance, suppression of hepatic glucose synthesis by insulin is blunted and the persistence of hepatic glucose output leads to postprandial hyperglycemia (134). At the same time, hyperinsulinemia signal in the liver induces lipogenesis, resulting in fatty liver disease that is a hallmark of insulin resistance syndrome. This differential response of gluconeogenesis and lipogenesis to insulin during insulin resistance has been termed "selective hepatic insulin resistance" (135). Mimicking insulin signal in the liver, loss of hepatic PTEN resulted in non-alcoholic steatohepatitis (NASH) while suppressing gluconeogenesis (89, 109, 136, 137). Unlike that observed with insulin resistance, NASH developed in the liver-specific *Pten* deletion mice is not due to hyperinsulinemia resulting from high circulating glucose levels. Locally enhanced hepatic PI3K/AKT signal actually led to improved ability for the liver to handle glucose, turning the liver into a glucose sink, leading to an improved ability to handle glycemic stress in these mice.

While the increased insulin/PI3K/AKT signal in the liver leads to improved systemic insulin sensitivity (88), the resulting NASH due to increased de novo lipogenesis however forms an environment that results in damage of the liver parenchymal (138–142). When NASH is inhibited via either dietary approach or genetic deletion of a metabolic AKT, *Akt2*, tumor development is inhibited (79, 114). The NASH thus serves as a tumor promoting event that promotes the development of tumors that arose from the PTEN loss transformed tumor-initiating cells (77–79, 114). How steatosis establishes a tumor environment is being explored currently. In the liver, inflammation as a result of damage to the liver parenchymal was shown to play an important role (**Figure 5**). Wnt signal produced by macrophages is one of the niche signals established by this NASH environment to promote tumorigenesis (79).

FUTURE CONSIDERATIONS

PTEN is a critical regulator of cell growth/survival as well as metabolism. As a metabolic regulator, PTEN controls the metabolism of both glucose and fatty acids. These effects of PTEN through targeting the PI3K/AKT dependent and independent pathways lead to suppressed insulin sensitivity and inhibited cell growth and survival. While the signals by which PTEN regulates growth and survival has been well elucidated, the mechanisms by which PTEN regulates metabolism, particularly lipid and mitochondrial metabolism is not well understood. Future studies to understand the molecular signals that PTEN controls to regulate these cellular functions are necessary for both the cancer and diabetes treatment.

AUTHOR CONTRIBUTIONS

C-YC and JC drafted the original manuscript. LH proofed the manuscript. BS edited the finalized the manuscript.

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REVIEW
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The Role for Myc in Coordinating Glycolysis, Oxidative Phosphorylation, Glutaminolysis, and Fatty Acid Metabolism in Normal and Neoplastic Tissues

*Eric S. Goetzman[1] and Edward V. Prochownik[2,3,4]**

[1] Division of Medical Genetics, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, United States, [2] Division of Hematology/Oncology, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, United States, [3] Department of Microbiology and Molecular Genetics, University of Pittsburgh Medical Center, Pittsburgh, PA, United States, [4] University of Pittsburgh Hillman Cancer Center, Pittsburgh, PA, United States

That cancer cells show patterns of metabolism different from normal cells has been known for over 50 years. Yet, it is only in the past decade or so that an appreciation of the benefits of these changes has begun to emerge. Altered cancer cell metabolism was initially attributed to defective mitochondria. However, we now realize that most cancers do not have mitochondrial mutations and that normal cells can transiently adopt cancer-like metabolism during periods of rapid proliferation. Indeed, an encompassing, albeit somewhat simplified, conceptual framework to explain both normal and cancer cell metabolism rests on several simple premises. First, the metabolic pathways used by cancer cells and their normal counterparts are the same. Second, normal quiescent cells use their metabolic pathways and the energy they generate largely to maintain cellular health and organelle turnover and, in some cases, to provide secreted products necessary for the survival of the intact organism. By contrast, undifferentiated cancer cells minimize the latter functions and devote their energy to producing the anabolic substrates necessary to maintain high rates of unremitting cellular proliferation. Third, as a result of the uncontrolled proliferation of cancer cells, a larger fraction of the metabolic intermediates normally used by quiescent cells purely as a source of energy are instead channeled into competing proliferation-focused and energy-consuming anabolic pathways. Fourth, cancer cell clones with the most plastic and rapidly adaptable metabolism will eventually outcompete their less well-adapted brethren during tumor progression and evolution. This attribute becomes increasingly important as tumors grow and as their individual cells compete in a constantly changing and inimical environment marked by nutrient, oxygen, and growth factor deficits. Here, we review some of the metabolic pathways whose importance has gained center stage for tumor growth, particularly those under the control of the c-Myc (Myc) oncoprotein. We discuss how these pathways differ functionally between quiescent and proliferating normal cells, how they are kidnapped and corrupted during the course of transformation, and consider potential therapeutic strategies that take advantage of common features of neoplastic and metabolic disorders.

Keywords: fatty acid oxidation, glutaminolysis, glycolysis, mitochondria, oxidative phosphorylation, Randle cycle, Warburg effect

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****Correspondence:***
Edward V. Prochownik
procev@chp.edu

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THE ABNORMAL METABOLISM OF CANCER CELLS: GLYCOLYSIS VERSUS OXIDATIVE PHOSPHORYLATION (OXPHOS) AND BEYOND

The distinct metabolic behaviors of cancer cells have been appreciated since the 1950s when Otto Warburg first observed their high rates of glycolysis even when there was sufficient oxygen present to support OXPHOS (1–4). Such "aerobic glycolysis," now termed the Warburg effect, was initially attributed to defective mitochondria but is now known to occur in rapidly growing normal cells and in cancers with no identifiable mutations in genes encoding mitochondrial proteins. More recently, the reprogramming of glutamine and fatty acid metabolism has also been identified in cancer cells (5–10). The still evolving consensus formulated over the past several years is that the altered metabolism of cancer cells is one of their so-called "hallmark" characteristics (11) and is both a direct and indirect consequence of oncogene and tumor suppressor gene mis-expression and/or mutation. Much less commonly does reprogramming occur as the result of metabolic gene mutation (12, 13). The two main advantages that metabolic re-wiring imparts to cancer cells are the ability to ensure sustained supplies of anabolic building blocks and to generate the energy needed for their assembly into macromolecules. This supports several other cancer hallmarks, including survival, sustained proliferation, tissue invasion and metastasis, and the participation in tumor-initiated angiogenesis (11).

Several general themes have begun to emerge from the study and cataloging of tumor-specific metabolic changes. One of these is that normal and malignant cells typically use the same basic metabolic pathways, which are deregulated in the latter and thus run at markedly different rates and/or are utilized to achieve different ends. For example, normal quiescent cells utilize glycolysis predominantly to generate small amounts of ATP (2 molecules/molecule of glucose) and pyruvate. Pyruvate is then completely oxidized by the TCA cycle within mitochondria to generate the reducing equivalents needed to power the electron transport chain (ETC) and to generate considerably more ATP (~36 additional molecules). By contrast, cancer cells often utilize glycolysis at an exaggerated pace for the same energy-generating purpose but also as a source of anabolic precursors to support rapid proliferation. For example, pyruvate is the initial substrate for the biosynthesis of alanine, aspartate, and threonine, and pyruvate's immediate upstream precursor, phosphoenol pyruvate (PEP), is the starting substrate for tyrosine, tryptophan, and phenylalanine. The even more proximal glycolytic intermediate 3-phosphoglycerate can be directed into the synthesis of glycine and serine as well as purine nucleotides and the initial product of glucose catabolism, glucose-6-phosphate, can be diverted into the anabolic pentose phosphate pathway (PPP). TCA cycle intermediates such as citrate, succinyl coenzyme A (CoA), and oxaloacetate may also be used in non-mitochondrial biosynthetic pathways to furnish additional anabolic substrates for lipid, amino acid, and nucleotide biosynthesis, respectively. Any resulting depletion of these substrates from their mitochondrial stores may then be addressed by mobilizing the so-called anaplerotic (or "filling in") reactions such as the conversion of glutamine to α-ketoglutarate, the β-oxidation of odd-chain fatty acids to succinyl-CoA, and the carboxylation of pyruvate to oxaloacetate.

Another theme is that these metabolic pathways are highly flexible and responsive in ways that ultimately benefit the growth and survival of the transformed cell. Indeed, cells with the most adaptable pathways will eventually outcompete their more metabolically rigid peers and be favored to survive and clonally expand over the course of tumor evolution. Such metabolic plasticity is particularly advantageous given the rapidity and extent to which the tumor microenvironment can change and the relatively small distances over which these changes can occur (14–17). The consequences of an inimical metabolic environment, which normally might promote cell cycle arrest or death, might be further assuaged by virtue of the loss of proapoptotic pathways mediated by TP53 and other tumor suppressors. In some cases, these losses not only delay or inhibit the apoptotic response to nutrient deprivation or the reactive oxygen species (ROS) associated with them but can themselves further alter metabolic pathways in favor of survival (18, 19). Maximizing survival and proliferation as a consequence of metabolic adaptability can also allow for the acquisition of additional mutations that further contribute to tumor evolution and adaptability (20).

Finally, a third theme is that some cancer-related metabolic reprogramming generates metabolites that can dramatically impact tumor behavior and even alter gene expression profiles. These effects can be direct or indirect, and the metabolites can either be the normal products of cellular respiration or the so-called "onco-metabolites," which possess neomorphic properties and are generated as a consequence of mutations in mitochondrial enzymes. Examples of the first type include the excessive lactate generated by high rates of Warburg-type glycolysis. Lactate excretion lowers extracellular pH, thereby potentiating certain extracellular proteases and thus facilitating tumor invasiveness and metastatic spread (21). Lactate also upregulates vascular endothelial growth factor and hypoxia-inducible factor 1 alpha (HIF-1α), an oxygen-sensitive transcription factor that positively regulates glycolysis, particularly in collaboration with c-Myc (Myc), which is deregulated in the majority of human cancers (22–26). Furthermore, lactate, as well as Myc, can impart radioresistance in some tumors and contribute to the escape from immune surveillance (27–30). Tumor-generated lactate can also stimulate neighboring fibroblasts to increase their synthesis and release of hyaluronan, an extracellular high-molecular weight glycosaminoglycan, which increases motility and facilitates tumor cell spread (31). Point mutations in the TCA cycle enzymes isocitrate dehydrogenase (IDH) 1 or IDH2, most of which have been described in myeloid leukemias and gliomas (32–35), can cause the enzymes to generate the novel onco-metabolite D-R-2-hydroxyglutarate (D-R-2HG) rather than the normal TCA cycle intermediate α-ketoglutarate. D-R-2HG is a potent inhibitor of the Ten–Eleven Translocation 2 protein that normally converts 5-methylcytosine to 5-hydroxymethylcytosine, a reaction that serves as an intermediate step in DNA de-methylation (32, 36, 37). In a variation of this theme, both hypoxia- and lactate-mediated intracellular acidification can impart new catalytic properties to the enzymes lactate dehydrogenase (LDH) and

malate dehydrogenase, allowing them to switch their normal substrate preferences and instead convert α-ketoglutarate to a 2HG enantiomer, L-S-2HG, which is also a potent epigenetic regulator (38–40). Finally, in an example that combines each of the above mechanisms, the accumulation of succinate, due to inactivating mutations in any one of the four subunits of the heterotetrameric succinate dehydrogenase (SDH) complex, has been linked to paragangliomas, pheocytochromocytomas, and gastrointestinal stromal cell tumors. While the exact mechanism by which excess succinate leads to transformation and why it is only associated with these rare tumor types are currently unknown, suspected culprits include excess ROS, HIF-1α stabilization, aberrant genome methylation, and tumor-promoting inflammatory changes (41–43).

Here, we review some of the major metabolic pathways that go awry in cancer, particularly those under the purview of Myc, and attempt to relate these to normal metabolic functions. It is important to emphasize that our focus on Myc arises from the fact that it is among the most frequently deregulated oncoproteins across all cancer types, is virtually never mutated, and regulates numerous metabolic functions (5, 22, 24, 44–46). Thus, metabolic alterations attributable to Myc are due to quantitative and not qualitative differences in its behavior, thus making it somewhat easier to understand its role in normal metabolic processes. Myc's wide-spread overexpression in cancer can most likely be attributed to the fact that it is a major transcriptional integrator of most, if not all, normal and oncogenic growth factor pathways (22, 24, 44–47). Understanding how Myc reprograms metabolic pathways can explain much of how they are altered by upstream mutant oncoproteins that constitutively upregulate Myc expression. Moreover, the most prominent transcript families under Myc's control tend to encode proteins that supervise energy production, anabolic pathways, protein synthesis, and cell cycle progression, all of which intimately impact both tumor and normal cell growth and survival and likely explain why many tumors are "addicted" to Myc (22, 45, 47–50). The differential regulation of these pathways by Myc permits unique glimpses into how they respond to different levels of this central transcriptional regulator while providing a basis for understanding why pharmacologic inhibition of Myc is considered a "Holy Grail" in cancer therapy and why it may also be useful in the treatment of non-malignant diseases of excessive cell proliferation (51, 52). We also summarize how certain pathways under Myc's influence differ functionally in quiescent and proliferating normal cells and how they are altered in tumors by Myc's deregulated expression (22, 24, 44).

THE EARLY DAYS: HINTS THAT MYC (AND OTHER ONCOGENES) REGULATE CELLULAR METABOLISM

In the aftermath of the initial discovery that Myc is the cellular homolog of the retroviral v-Myc oncogene (53), little more than a year elapsed before recognizing that the former was commonly rearranged, amplified, and/or otherwise deregulated in human cancers, most notably Burkitt's lymphoma (54–62). Shortly thereafter, endogenous Myc was found to be responsive to various mitogenic and differentiation-promoting stimuli, with the first type tending to upregulate and the second tending to downregulate its expression. Deliberately overriding these behaviors tended to reverse these tendencies, thereby demonstrating that Myc served as an active participant rather than a passive bystander. It was also shown that elevated and deregulated Myc expression frequently accompanies tumor progression and that the overexpression of Myc, either alone or in combination with other oncoproteins, was potently transforming both *in vitro* and *in vivo* (63–88).

It was not until the mid-1980s, however, that the relationships between protooncogene expression, normal and neoplastic proliferation, and altered metabolism began to truly take shape and mold our current outlook. For example, the eventual classification of Myc as a so-called "immediate-early" gene in response to growth factor stimulation in fibroblasts (65, 78, 80, 81) led to the finding that the ectopic conditional expression of Myc alone was sufficient to promote an abortive G0 → S-phase transition (63). Shortly thereafter, studies in quiescent thymocytes and fibroblasts additionally showed that Myc induction following mitogenic stimulation was preceded by rapid and sequential changes in phosphoinositide metabolism, Ca^{2+} release, the activation of phospholipid-dependent kinase C and altered Na^+/H^+ exchange (89, 90). Enforced Myc or Ras expression in log-phase Rat1 fibroblasts was also then found to stimulate glycolysis, which was further enhanced by the addition of the growth factor TGF-β (91). Subsequently, differential screening of cDNA libraries prepared from quiescent and serum-stimulated Balb/3T3 murine fibroblasts identified a small number of transcripts that were induced within 12 h of applying this mitogenic stimulus (92). In addition to Myc, these encoded LDH and enolase, thus hinting at the idea that Myc might be involved in the regulation of metabolism, that specific genes within the glycolytic pathway might be important for initiating the biomass accretion necessary for growth and division, and that Myc might somehow be involved in the regulation of these genes. Being mindful of proper historical context, it is important to note that these studies preceded by over a year the initial reports that Myc was a DNA-binding transcription factor (93–96). Thus, the relationship between Myc and transcripts encoding metabolic enzymes remained enigmatic until this critical Myc function was unmasked. It is now known that nearly all genes encoding glycolytic enzymes are direct Myc targets and that the Warburg effect is at least partially under Myc control (97–99). Together, these findings underscore two of the three major themes mentioned in the preceding section: first, that the metabolic changes accompanying rapid normal and malignant proliferation utilize the same pathways as normal quiescent cells, although not always for the same reasons; and second, that malignant cells maintain or corrupt these pathways for the singular purpose of gaining a proliferative and/or survival advantage over their normal counterparts, or even their transformed but less metabolically adaptable relatives.

Over the next several years, it gradually emerged that one of Myc's principle functions, both in normal and cancer cells, was to regulate cell mass and, in doing so, to directly modulate the expression of genes involved in ribosomal biogenesis including ribosomal structural genes, tRNAs, rRNAs, and all three eukaryotic RNA polymerases that control the expression of these

genes (100–109). The identification of these novel Myc targets was a satisfying observation as it began to shed light on how an oncoprotein could promote proliferation on the one hand while coordinating this with protein synthetic rates (or at least the protein synthetic machinery) and the doubling of cell mass that must precede division on the other (110). It also complemented earlier observations that transformed cells increase their uptake of both amino acids and the glucose analog 2-deoxy-glucose (2-DG) (91, 110). Yet even by this time and with these observations in hand, little attention was paid to the other metabolic changes needed to support the growth and proliferation of cancer cells. Not that they had been entirely ignored; indeed, there were hints as early as the mid-1950s that such changes were intimately associated with the increased proliferation of cancer cells including the eponymous Warburg effect mentioned earlier (3, 111, 112).

MYC AND THE REGULATION OF GLYCOLYSIS, OXPHOS, AND ENERGY BALANCE

As emphasized above, Myc's pivotal role in the control of carbohydrate metabolism emerged gradually in the mid-late 1980s and early 1990s. However, it first required demonstrating that Myc is a sequence-specific DNA-binding transcription factor (92–95, 113, 114), together with the subsequent development of high-throughput and unbiased methodologies to identify Myc-target genes (115–119). From among the earliest such attempts emerged one of the first direct Myc-target genes (120), namely, the "A" isoform of LDH (LDH-A), which, as noted above, had been previously identified, along with Myc itself, as being induced by serum in fibroblasts (92). Shim et al. and Lewis et al. subsequently demonstrated that Myc-mediated fibroblast transformation was attenuated following the genetic suppression of LDH-A, that these fibroblasts were highly susceptible to apoptotic death in the face of glucose deprivation and that LDH-A could cooperate with another Myc target, rcl, to transform fibroblasts (120–122). Rather than being directly transforming, however, it seems more likely that LDH's role is more related to the fact that the LDH-mediated generation of lactate from pyruvate requires NADH as an electron donor and that the product of this reaction, NAD^+, may then function as an electron acceptor to support more proximal glycolytic reactions (**Figure 1**). This co-dependency between glycolysis and lactate generation ensures that the former pathway can be efficiently maintained regardless of its rate, particularly when oxygen concentrations are low, the generation of lactate is high and glycolysis is the major ATP source. Further supporting the importance of this positive feedback loop was the subsequent observation that most glycolytic enzyme-encoding genes are regulated at some level by Myc (120, 123–127). Thus, the production and excretion of lactate, an otherwise energetically wasteful activity, is actually necessary to sustain the Warburg effect as it ensures the continuous generation of NAD^+ to serve as an electron acceptor during glucose oxidation. In the presence of oxygen, cytoplasmic NAD^+ may also be supplied *via* the glycerol-3-phosphate shuttle and the malate–aspartate shuttle in which reducing equivalents are transferred to the mitochondria in exchange for generating an oxidized cytoplasm to maintain glycolysis (128, 129). While these shuttles are likely to be less important under the hypoxic conditions that often prevail during tumor growth, they nonetheless allow for cross talk, cooperation, and coordination between glycolysis and OXPHOS in well-oxygenated environments (130–132).

Warburg-type glycolysis, in conjunction with the abovementioned shuttles, couples the generation of reducing equivalents in the form of NADH with their indirect transfer into the mitochondrial matrix to drive the ETC and maintain membrane potential ($\Delta\Psi M$) and the protomotive force that generates ATP *via* Complex V. The glycerol-3-phosphate and malate–aspartate shuttles thus provide sources of mitochondrial reducing equivalents independent of those generated by the TCA cycle when the supply of pyruvate-derived acetyl-CoA (AcCoA) might be compromised due to the diversion of glycolytic intermediates for anabolic purposes and/or the production of lactate. These shuttles also sustain mitochondrial NADH levels when TCA-generated substrates are consumed by other anabolic reactions, such as the synthesis of lipids and certain amino acids. Interestingly, rather than being transferred to Complex I or Complex II as occurs with TCA cycle-derived reducing equivalents, the NADH supplied by these shuttles is confined to the inner mitochondrial membrane and surrenders its electrons directly to Coenzyme Q and then to Complex III of the ETC (133). Such reducing equivalents, derived from glycolysis, thus provide an electron source that bypasses Complex I and Complex II and is under different regulatory supervision.

Glycolysis also indirectly generates reducing equivalents in the form of NADPH during the proximal steps of the PPP. NADPH is a source of cytoplasmic reducing equivalents that supports the reductive *de novo* synthesis of sterols and fatty acids, regenerating $NADP^+$ in the process and providing an energetic and self-sustaining link between the biosynthesis of pentose sugars and lipids (134, 135). Thus, high rates of glycolysis and the lactate generation made in response to oncogenic stimuli such as Myc ensure a well-balanced and oxygen-independent supply of cytoplasmically generated ATP, reducing equivalents, and critical anabolic precursors (**Figure 1**).

Increased glycolytic flux ensures a rich supply of substrates that can be redirected into energy-consuming anabolic pathways with sufficient amounts remaining to generate AcCoA and drive the TCA cycle. But precisely how this occurs and whether any control is exerted remains somewhat enigmatic. Do the glycolysis-linked pathways simply "drink from the fire hose" of glycolytic intermediates as they rush down the pathway, or are there mechanisms that specifically direct glycolytic intermediates into anabolic pathways at the expense of mitochondrial OXPHOS, aided by the law of mass action? Support for the latter notion comes with the observation that the expression of the two isoforms of pyruvate kinase (PK), PKM1, and PK2, is altered in some cancers. PKM1, which tends to predominate in normal tissues, is replaced by PKM2, which has a higher K_m for its substrate, PEP; the K_m is even further increased by posttranslational modification. The overall lessened PK activity serves to redirect PEP and other upstream glycolytic substrates away from energy-generating OXPHOS and into energy-consuming anabolic pathways (136–140).

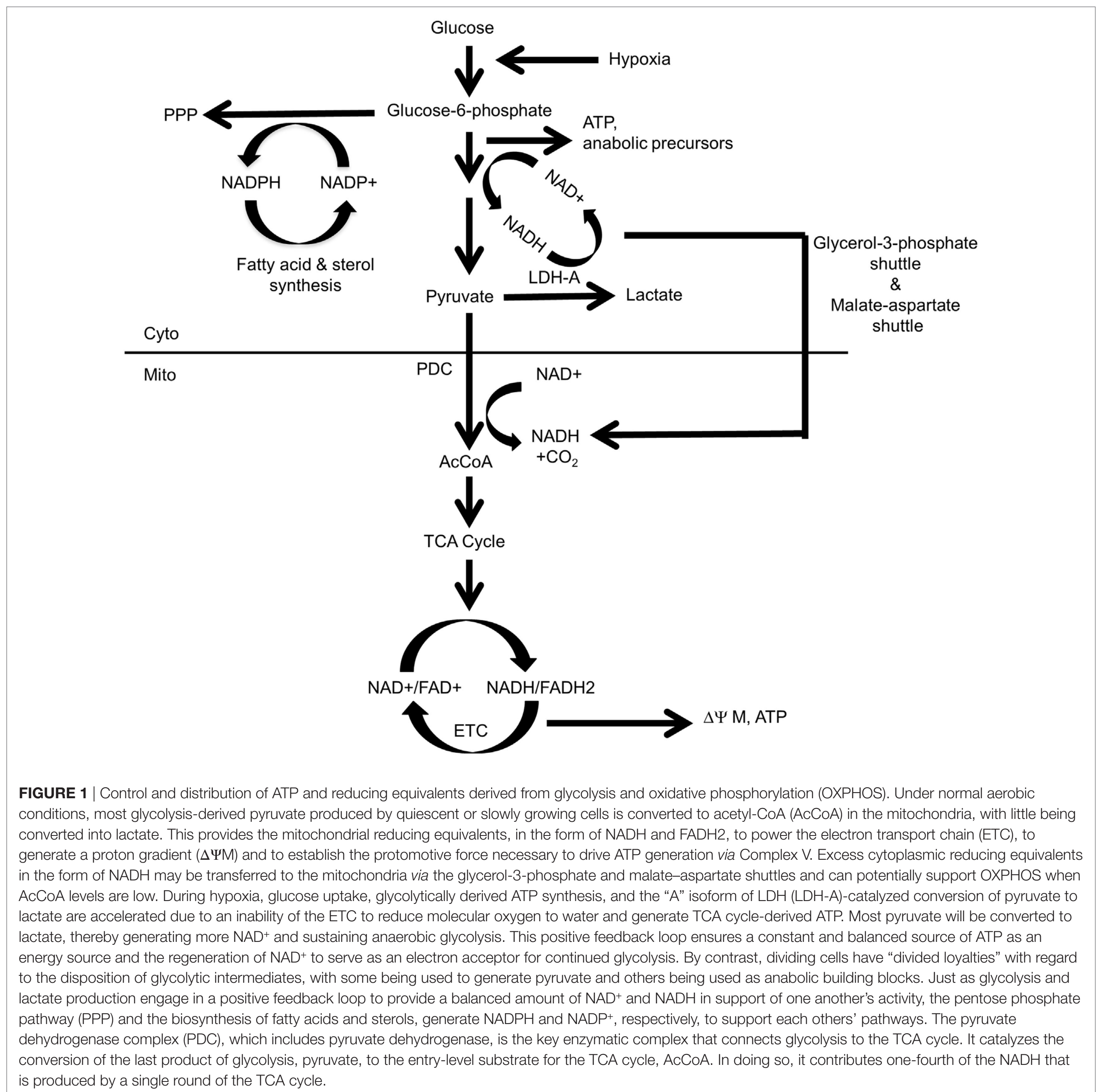

FIGURE 1 | Control and distribution of ATP and reducing equivalents derived from glycolysis and oxidative phosphorylation (OXPHOS). Under normal aerobic conditions, most glycolysis-derived pyruvate produced by quiescent or slowly growing cells is converted to acetyl-CoA (AcCoA) in the mitochondria, with little being converted into lactate. This provides the mitochondrial reducing equivalents, in the form of NADH and FADH2, to power the electron transport chain (ETC), to generate a proton gradient (ΔΨM) and to establish the protomotive force necessary to drive ATP generation *via* Complex V. Excess cytoplasmic reducing equivalents in the form of NADH may be transferred to the mitochondria *via* the glycerol-3-phosphate and malate–aspartate shuttles and can potentially support OXPHOS when AcCoA levels are low. During hypoxia, glucose uptake, glycolytically derived ATP synthesis, and the "A" isoform of LDH (LDH-A)-catalyzed conversion of pyruvate to lactate are accelerated due to an inability of the ETC to reduce molecular oxygen to water and generate TCA cycle-derived ATP. Most pyruvate will be converted to lactate, thereby generating more NAD^+ and sustaining anaerobic glycolysis. This positive feedback loop ensures a constant and balanced source of ATP as an energy source and the regeneration of NAD^+ to serve as an electron acceptor for continued glycolysis. By contrast, dividing cells have "divided loyalties" with regard to the disposition of glycolytic intermediates, with some being used to generate pyruvate and others being used as anabolic building blocks. Just as glycolysis and lactate production engage in a positive feedback loop to provide a balanced amount of NAD^+ and NADH in support of one another's activity, the pentose phosphate pathway (PPP) and the biosynthesis of fatty acids and sterols, generate NADPH and $NADP^+$, respectively, to support each others' pathways. The pyruvate dehydrogenase complex (PDC), which includes pyruvate dehydrogenase, is the key enzymatic complex that connects glycolysis to the TCA cycle. It catalyzes the conversion of the last product of glycolysis, pyruvate, to the entry-level substrate for the TCA cycle, AcCoA. In doing so, it contributes one-fourth of the NADH that is produced by a single round of the TCA cycle.

The PKM1 and PKM2 isoforms differ from one another as a result of mutually exclusive alternate mRNA splicing such that the PKM1 transcript encodes exon 9 but not exon 10 and PKM2 encodes exon 10 but not exon 9. The splicing decision is governed by at least three heterogeneous nuclear ribonucleoproteins, hnRNP1, hnRNP2, and hnRNP, which bind to intron regions flanking exon 9 and negatively regulate its splicing. Interestingly, hnRNP1 and hnRNP2 are positively regulated by Myc (141, 142). However, the switch to aerobic glycolysis, by whatever means, hardly signals an irreversible commitment; indeed, not only is the Warburg effect plastic but many tumors-even those of similar types are more dependent upon OXPHOS than glycolysis for their energy requirements and/or can rapidly balance these two processes to suit their needs (143). In addition to regulating glycolysis, Myc also exerts significant influence over mitochondrial structure and function. Initially relegated to a metabolic backwater following the realization that many cancer cells rely on the Warburg effect at the expense of OXPHOS, mitochondria do in fact play important roles in cancer metabolism (144, 145). Indeed, certain cancers remain highly reliant on OXPHOS as a means of energy production and show little predilection for Warburg-type metabolism (146). Analogous to glycolysis,

however, both mitochondria and the energy producing pathways they encompass undergo significant structural and functional revisions in response to Myc deregulation.

Among the first studies to examine Myc's effect on mitochondrial structure and function were those of Li et al. (147). Utilizing P493-6 human B cells transfected with a tetracycline-inducible human Myc transgene, they showed that mitochondrial mass increased approximately twofold within 72 h of Myc induction, as measured by nonyl acridine orange staining, MitoTracker staining and mitochondrial DNA (mtDNA) content. Oxygen consumption increased to a similar extent thereby indicating a close relationship between structure and function. Transcriptional profiling also identified approximately 200 Myc-responsive genes encoding mitochondrial proteins, most of which were upregulated. Most notable among these was the transcript encoding Tfam, a nuclear-encoded mitochondrial transcription factor that also participates in mtDNA replication (148–150). The *TFAM* gene proximal promoter was also shown to contain a Myc-binding E-Box element. In serum-stimulated fibroblasts, endogenous Myc was shown to bind the same site (147). Although no functional studies were performed, it was assumed that this positive effect of Myc on mitochondrial biogenesis was accompanied by a parallel increase in TCA cycle activity and OXPHOS.

Elucidating the role of endogenous Myc in the regulation of metabolic pathways was hampered for quite some time by the lack of a suitable knockout model. This is because even short-term Myc depletion in virtually all cell lines is accompanied by cell cycle arrest, apoptosis, or differentiation, thereby severely compromising long-term studies (50, 151, 152). Similarly, whole body inactivation of the *myc* gene is an embryonic lethal (153). Thus, the generation of a *myc*$^{-/-}$ cell line from rat fibroblasts by Mateyak et al. in 1997 (154) provided a tremendous technical advance despite the fact that it remains unresolved as to how these cells survive and replicate.

Although viable, the *myc*$^{-/-}$ fibroblasts described by Mateyak et al. (154) are extremely abnormal. For example, they divide only once every 2–3 days versus every 18–24 h for the *myc*$^{+/+}$ parental line, display an extremely flattened morphology and are highly contact inhibited. Cell cycle regulation is severely compromised at multiple points, with *myc*$^{-/-}$ cells showing a *ca.* 12-fold reduction in the expression of CyclinD–Cdk4 and CyclinD–Cdk6 complexes during the G_0/G_1 transition and delayed activation of CyclinE–Cdk2 and CyclinA–Cdk2 complexes (145, 155). That most of these abnormalities can be rescued at least partially with retrovirally expressed Myc, the Myc homologs N-Myc and L-Myc, or the Myc-target genes MYCT1/MT-MC1, HMG-IY and SHMT (156, 157) indicates that the various phenotypes of *myc*$^{-/-}$ fibroblasts are directly related to *myc* gene inactivation and do not represent compensatory and Myc-independent growth-enhancing adaptations.

The absence of Myc was also reflected in the mitochondrial structure and metabolism of these cells. Graves et al. (158) showed that, compared with *myc*$^{+/+}$ fibroblasts, the mitochondria of *myc*$^{-/-}$ fibroblasts were smaller, fewer in number, deficient in cristae and poorly interconnected. The activation of a Myc-estrogen receptor fusion protein (MycER) by 4-hydroxytamoxifen (4OHT) complemented these defects, although, surprisingly, it required 4–5 weeks before maximal mitochondrial mass was restored. The extremely low ΔΨM of these cells (*myc*$^{-/-}$ MycER) cells also increased in parallel over this same time period. This provided indirect evidence that Myc was likely controlling some aspect of the TCA cycle and/or the availability of reducing equivalents needed to drive the ETC. Following 4OHT's removal and the silencing of Myc, mitochondrial mass, ΔΨM and interconnectivity returned to near the baseline levels of *myc*$^{-/-}$ cells over another prolonged period exceeding 10 days (158).

In addition to the above major structural defects, which confirmed and extended those of Li et al. (147), mitochondrial function was also severely compromised in *myc*$^{-/-}$ cells in a manner that mirrored their structural defects. For example, the basal oxygen consumption rate (OCR) of *myc*$^{-/-}$ cells was about half that of *myc*$^{+/+}$ cells and only 15% that of *myc*$^{-/-}$ cells stably reconstituted with a lentiviral vector that drove high level, constitutive Myc expression (*myc*$^{-/-}$ Myc cells). The most prominent effect of Myc on OCR was seen in *myc*$^{-/-}$ Myc cells where the maximum respiratory capacity in response to the de-polarizing agent FCCP was >20 times higher than that of *myc*$^{-/-}$ cells and 6 times higher than that of *myc*$^{+/+}$ cells. Because Myc positively regulates virtually all glycolytic genes (96, 123, 124, 159), the basal rate of glycolysis in *myc*$^{-/-}$ cells was also about half that of *myc*$^{+/+}$ cells and one-third that of *myc*$^{-/-}$ Myc cells (158).

Consistent with their markedly impaired OXPHOS and glycolysis, *myc*$^{-/-}$ cells showed a nearly 70% reduction in basal ATP levels, which normalized following Myc re-expression. In all three cell lines (*myc*$^{-/-}$, *myc*$^{+/+}$, and *myc*$^{-/-}$ Myc), exposure to 2-DG caused a more pronounced ATP depletion than did the inhibition of OXPHOS with rotenone. Taken together, these results suggested that at least half the energy in these cells was derived from glycolysis. Consistent with their ATP deficient state, *myc*$^{-/-}$ cells expressed high levels of activated (phosphorylated) AMP-activated protein kinase (AMPK), a serine/threonine kinase that responds to ATP depletion (or more precisely to a high AMP:ATP ratio) by upregulating ATP-generating pathways and downregulating ATP-consuming pathways (160–162). However, since many of the energy-sparing and energy-generating effects of AMPK rely on the upregulation of Myc (160, 163, 164), AMPK seems to be unable to achieve a state of true energy equilibrium in *myc*$^{-/-}$ cells, thereby leading to its constitutive activation in the face of a chronic energy deficit. The restoration of Myc in *myc*$^{-/-}$ Myc cells did lead to AMPK dephosphorylation that correlated with the normalization of ATP levels (160).

The observation that *myc*$^{+/+}$ and *myc*$^{-/-}$ Myc cells contained identical ATP levels could not initially be reconciled with the finding that the latter cells had significantly higher rates of glycolysis and OXPHOS. This discrepancy was resolved by showing that the ATP half-life in the latter cells was nearly 50% shorter (2.6 versus 3.6 min) (158). This was consistent with the previous finding that *myc*$^{-/-}$ WT cells had significantly faster growth rates than *myc*$^{+/+}$ cells and thus likely utilized more ATP (165).

To further understand the role of endogenous Myc in maintaining basal rates of glycolysis and OXPHOS in transformed cells, Graves et al. (158) utilized the conditional, doxycycline-regulatable expression of a short hairpin RNA directed against Myc to silence the oncoprotein's expression in A549 human small

cell lung cancer cells, which normally express high levels of Myc. Conforming to the findings in the above-discussed rat fibroblast studies, the knockdown of Myc was associated with marked growth inhibition, a flattened cellular morphology, reduced mitochondrial mass, and the collapse of ΔΨM.

Relative to *myc*$^{+/+}$ and *myc*$^{-/-}$ Myc cells, *myc*$^{-/-}$ cells demonstrated abnormalities in overall structure and function of the ETC (158). Among these were reduced amounts of the so-called "supercomplexes" (SCs) between Complexes I, III, and IV, which allow for more efficient electron transfer (166, 167). Consistent with their atrophic mitochondrial cristae, which are believed to serve as a platform for the formation and accretion of SCs (168, 169), *myc*$^{-/-}$ mitochondria also contained lower levels of Complex I, II, and III as well as both the monomeric and dimeric forms of Complex V ATPase (V_m and V_d, respectively) relative to *myc*$^{+/+}$ cells. In general, SC function in *myc*$^{-/-}$ cells, as measured by *in situ* enzymatic activity of individual complexes separated by non-denaturing blue native gel electrophoresis (BNGE), closely matched Coomassie Blue staining patterns. *myc*$^{-/-}$ fibroblast mitochondria also contained significant levels of an enzymatically inert complex (Complex "X") that was shown by mass spectroscopy to be comprised of multiple subunits from Complexes II–V. It was speculated that during periods of relative oxidative quiescence, Complex X functions as a reservoir for certain mitochondria proteins, which can be rapidly summoned and assembled into their respective ETC complexes in response to increased metabolic needs. This seems like a logical cellular strategy in that cells with depleted energy levels as a result of ETC dysfunction might be better served by utilizing preexisting ETC components for rapid assembly and resumption of ETC function rather than expending even more energy by synthesizing them anew. On the other hand, the surprisingly long time it takes to restore normal mitochondrial structure and function in *myc*$^{-/-}$ cells (158) raises questions as to whether this is the true function of this complex.

Interestingly, *myc*$^{-/-}$ Myc cells showed only a partial normalization of ETC structure and function as measured by the above methods, despite their high-level Myc expression. Relative to *myc*$^{+/+}$ cells, *myc*$^{-/-}$ Myc cells contained only about half the levels of SCs, two-thirds the level Complex V monomers (V_m) and ~15% the level of Complex V dimers (V_D) as measured by both enzymatic activity and BNGE. While both V_D and V_M possess ATP synthase activity, the dimer appears to be more important for dictating the shape of mitochondrial cristae (170). Because the high-level re-expression of Myc also greatly increased glycolysis, it was surmised that this failure to entirely normalize ETC structure and function was due to a combination of factors including structural changes to the mitochondria and their cristae, differences in the relative contribution of glycolysis and OXPHOS to the energy landscape, subtle nuances relating to the control of Myc protein expression, and differential cellular growth rates and their resulting anabolic requirements (158).

The normalization of mitochondrial morphology by the enforced re-expression of Myc in *myc*$^{-/-}$ Myc cells (158) suggested that Myc might influence mitochondrial fusion and/or fission. The former process is regulated by the so-called "mitofusin" proteins such as Mfn1, Mfn2, and Opa1 whereas the latter process is regulated by the proteins Fis1 and Drp/Dlp (171–175). Fusion is believed to maximize mitochondrial energy production by allowing old and/or damaged organelles to be "rejuvenated" by combining their contents with those of younger ones, thereby extending their life span and functional integrity and capacity (176, 177). By contrast, fission provides a mechanism by which mitochondrial mass can be reduced during periods of relative metabolic quiescence or Warburg-type respiration or by which defective and/or aged mitochondria can be eliminated. Both fusion and fission can exert significant influence upon mitochondrial energy production and cell survival (176, 178, 179).

Virtually, all the above mitochondrial fission and fusion proteins were expressed at higher levels *in* Myc replete cells relative to *myc*$^{-/-}$ cells making it unclear how, as an integrated group, they affected mitochondrial biogenesis and, if so, which of the processes this favored. The question was answered by experiments in which 4OHT-treated *myc*$^{-/-}$ MycER cells were separately transfected with mitochondrially targeted green or red fluorescent proteins (GFP or RFP, respectively). Cells from the two populations were then mixed and fused by exposure to polyethylene glycol and the rate of GFP$^+$ and RFP$^+$ mitochondrial fusion into "yellow" merged organelles was quantified either in the continued presence of 4OHT or following its removal. Cells actively expressing Myc showed nearly twofold higher rates of mitochondrial fusion compared with cells in which Myc had been silenced for 2 days by removing 4OHT. The faster rate of mitochondrial turnover in the former cells suggested that they were under constant pressure to maintain only the youngest and healthiest mitochondria to meet the increased metabolic needs of this energetically more demanding and faster growing population. Thus, along with affecting the levels of key mitochondrial transcription factors such as Tfam (123, 147), Myc also influences mitochondrial biogenesis and lifespan by modulating the levels of fission/fusion proteins.

Subsequent work demonstrated that alterations in mitochondrial structure and function can reciprocally impact the function of both endogenous and overexpressed Myc. In these studies, Sarin et al. (180) enforced the expression of the mitochondrial fission protein Drp/Dlp in Rat1a-MycER cells, leading to a state of non-stop, fission-induced mitochondrial fragmentation and a pronounced reduction in overall mitochondrial size, mass and interconnectivity. Accompanying this was a nearly 15-fold higher rate of mitochondrial fusion relative to control cells suggesting that Drp1/Dlp1-overexpressing cells constantly upregulate fusion in a futile compensatory attempt to offset their excessive Drp1/Dlp1-driven fission. Despite the fact that these cells expressed normal levels of Myc, their mitochondria were both structurally and functionally reminiscent of those from *myc*$^{-/-}$ cells. Structurally, their ETC complexes were defective; BNGE revealed a 28% reduction Complex I, a 45% reduction in Complex V and a 38% reduction in SCs. Furthermore, Complex "X," the proposed repository for certain ETC subunits in *myc*$^{-/-}$ cells (158), now appeared. Thus, enforced and uncorrected mitochondrial hyper fission leads to a loss of mitochondrial structural integrity resembling that of *myc*$^{-/-}$ cells in the face of otherwise normal Myc levels.

Further characterization of Drp1/Dlp1-overexpressing cells showed that, like *myc*$^{-/-}$ cells, they too had a critical energy shortage, with a >80% reduction in ATP levels and impaired glycolysis and OXPHOS. This energy-depleted state, coupled with the failure to adequately compensate for it, was evidenced by a *ca*. 30% decrease in mean cell volume and a >10-fold increase in phosphoAMPK (180). Collectively, these findings suggested that the price for such energy-conserving processes was a reduction in energetically demanding biomass accumulation. Most likely as a result of their abnormal ETC structure and/or their ATP deficit, Drp1/Dlp1 overexpressing cells, like *myc*$^{-/-}$ cells, expressed higher levels of ROS than control cells (181, 182). No obvious growth differences between control and Drp1/Dlp1-overexpressing cells were observed under standard conditions although the latter were significantly more resistant to apoptosis in response to Myc overexpression or serum deprivation and this was supported by the less pronounced release of cytochrome c from mitochondria. Treatment with 5-amino-1-b-D-ribofuranosyl-imidazole-4-carboxamide, an AMP analog that activates AMPK and increases ATP pools (161, 162, 183) doubled the ATP content of Drp1/Dlp1 overexpressing cells, normalized their size and increased their sensitivity to apoptotic stimuli (180). The prolonged survival of Drp1/Dlp1-overexpressing cells may reflect the fact that ATP depletion tends to protect against apoptosis, perhaps by inhibiting caspases 3 and 8 and Apaf-1, and that, in some circumstances AMPK activation can restore or promote apoptosis (184–188). Collectively, these results show that enforced mitochondrial fission driven by Drp1/Dlp1 can override Myc's role in maintaining normal mitochondrial integrity and adequate ATP levels. Whether this is due to a lack of response of mitochondria as a result of their inability to fuse in response to Myc (as seems likely) or another effect of Drp1/Dlp1 remains to be determined.

The above studies showed that, at least in proliferating fibroblasts propagated *in vitro*, where nutrient supplies and oxygen concentrations are high and non-rate-limiting, both glycolysis and OXPHOS are subject to positive regulation by endogenous Myc. Moreover, they establish that the overexpression of Myc, to the levels required to drive proliferation and transformation, continues to exert a simultaneous positive effect on both glycolysis and OXPHOS. The effects are quite heterogeneous, are both direct and indirect and involve changes in transcripts encoding glycolytic enzymes and mitochondrial structural and functional components. These studies show that the Warburg effect and OXPHOS are by no means mutually exclusive. Rather they are better viewed as being complementary, with neither one being entirely dispensable (**Figure 2**).

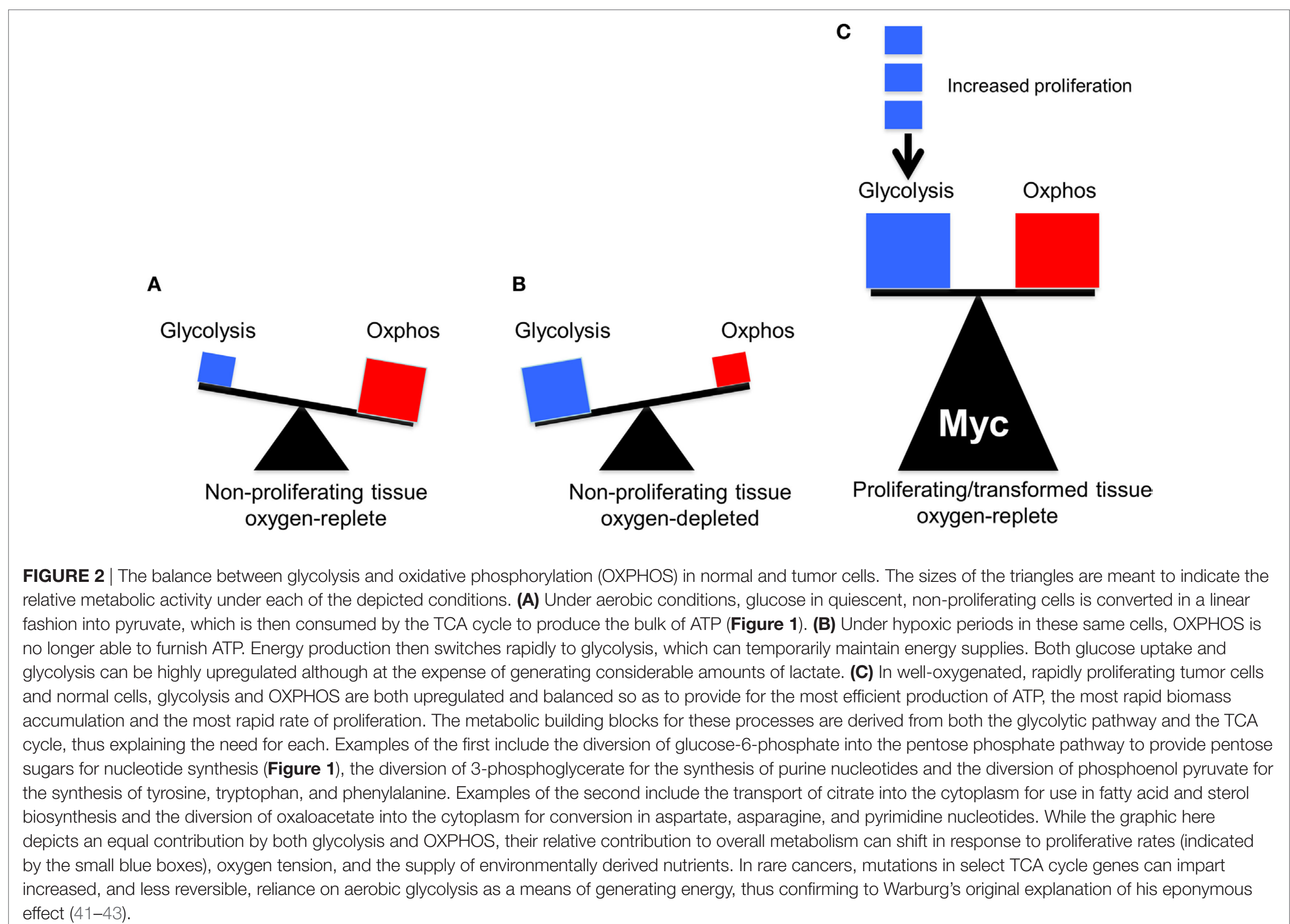

FIGURE 2 | The balance between glycolysis and oxidative phosphorylation (OXPHOS) in normal and tumor cells. The sizes of the triangles are meant to indicate the relative metabolic activity under each of the depicted conditions. **(A)** Under aerobic conditions, glucose in quiescent, non-proliferating cells is converted in a linear fashion into pyruvate, which is then consumed by the TCA cycle to produce the bulk of ATP (**Figure 1**). **(B)** Under hypoxic periods in these same cells, OXPHOS is no longer able to furnish ATP. Energy production then switches rapidly to glycolysis, which can temporarily maintain energy supplies. Both glucose uptake and glycolysis can be highly upregulated although at the expense of generating considerable amounts of lactate. **(C)** In well-oxygenated, rapidly proliferating tumor cells and normal cells, glycolysis and OXPHOS are both upregulated and balanced so as to provide for the most efficient production of ATP, the most rapid biomass accumulation and the most rapid rate of proliferation. The metabolic building blocks for these processes are derived from both the glycolytic pathway and the TCA cycle, thus explaining the need for each. Examples of the first include the diversion of glucose-6-phosphate into the pentose phosphate pathway to provide pentose sugars for nucleotide synthesis (**Figure 1**), the diversion of 3-phosphoglycerate for the synthesis of purine nucleotides and the diversion of phosphoenol pyruvate for the synthesis of tyrosine, tryptophan, and phenylalanine. Examples of the second include the transport of citrate into the cytoplasm for use in fatty acid and sterol biosynthesis and the diversion of oxaloacetate into the cytoplasm for conversion in aspartate, asparagine, and pyrimidine nucleotides. While the graphic here depicts an equal contribution by both glycolysis and OXPHOS, their relative contribution to overall metabolism can shift in response to proliferative rates (indicated by the small blue boxes), oxygen tension, and the supply of environmentally derived nutrients. In rare cancers, mutations in select TCA cycle genes can impart increased, and less reversible, reliance on aerobic glycolysis as a means of generating energy, thus confirming to Warburg's original explanation of his eponymous effect (41–43).

The role of endogenous Myc in sustaining glycolysis and mitochondrial function has received additional support from studies in other cell types with several structurally and mechanistically distinct small molecule Myc inhibitors (189–191). HL60 promyelocytic cells exposed for only 2–5 days to these compounds dramatically reduced their ATP content, activated AMPK, accumulated neutral lipids and downregulated Myc as they underwent terminal myeloid differentiation. Because the manipulation of Myc had long been know to exert profound effects on the differentiation of hematopoietic and other cell types (68, 72, 77, 87, 192), Wang et al. repeated these experiments using two mechanistically distinct inhibitors of Complex I, metformin and rotenone (191). Decreased ATP, AMPK activation and myeloid differentiation were again noted, but Myc levels were unaffected. These studies supported the idea that ATP levels are a strong and Myc-independent determinant of differentiation, at least in myeloid cells (193). They further implied that a major role of Myc in differentiation is to maintain ATP levels, most likely with the purpose of allowing for the continued accumulation of biomass. This is also likely aided by Myc's ability to induce the expression of many genes involved in cell cycle progression (101, 194, 195). Differentiation may therefore represent one possible means of maintaining viability in response to energy-depleted states.

In other studies using a transgenic mouse model of neuroblastoma driven by the highly related Myc homolog N-Myc, Zirath et al. (196) showed that the treatment of tumor-bearing animals with the small molecule Myc inhibitor 10058-F4 (197), which also binds to and distorts the structure of N-Myc (198), inhibited tumor growth and promoted tumor differentiation as evidenced by neurite outgrowth. It also caused the accumulation of high levels of intracellular neutral lipid (196).

Activated T-cells are among the most rapidly dividing metazoan cells. Following antigen stimulation, they accumulate biomass for approximately 24 h and then enter a phase of rapid proliferation and clonal expansion, with cell division occurring as frequently as every 4 h (199). It has long been known that this replicative phase is associated with markedly increased glycolysis and glutaminolysis although the precise pathways needed to effect this metabolic reprogramming remain ill defined (200–202). Wang et al. (203) investigated Myc's contribution to the biomass accretion and proliferative expansion following *ex vivo* stimulation of murine T-cells with anti-CD3$^+$ anti-CD28. Immediately following their initial 24 h growth period, control cells entered the expected rapid proliferative phase during which time they were subject to metabolomic profiling using mass spectroscopy. Wang et al. (203) found that these cells accumulated metabolites during the initial growth phase and then activated glycolysis and directed glucose into the PPP. Concurrently, FAO declined as did the delivery of pyruvate into the TCA cycle. By contrast, OXPHOS and glutaminolysis increased, with a significant amount of the glutamine-associated carbon and nitrogen ultimately being incorporated into α-ketoglutarate and nucleotides, respectively. This latter finding indicated that exogenous glutamine was directed along two distinct pathways, the first being the TCA cycle in which glutamine was converted to glutamate and then to α-ketoglutarate and the second being the purine synthesis pathway in which both the N3 and N6 positions of the purine ring are derived from the glutamine amide moiety. Thus, not unlike the case of Myc-overexpressing fibroblasts discussed earlier (158), T-cell activation was accompanied by increases in both glycolysis and OXPHOS although the source of substrates for the latter pathway shifted from fatty acids and glucose to glutamine. Impaired proliferation was observed when the cells were deprived of either glucose or glutamine or when glycolysis and glutaminolysis, but not FAO, were blocked pharmacologically.

To investigate the molecular basis for the above-described metabolic reprogramming, Wang et al. (203) excised Myc from *myc*$^{flox/flox}$ T cells following conditional activation of a 4OHT-inducible CreER transgene and compared their *ex vivo* response to anti-CD3$^+$ anti-CD28 activation to the above control *myc*$^{flox/flox}$ cells. They noted a severe impairment of both the initial mass accretion (growth) phase and the subsequent expansion phase. *In vivo* testing of these cells following their stimulation with staphylococcal enterotoxin B revealed a blunted response similar to that observed *ex vivo*, with a decrease in both the growth and activation phases. Metabolomic profiling showed that the accumulation of amino acids, nucleotides and lipids in these cells was lower than that measured in similarly activated *myc*$^{flox/flox}$ cells. Of note was that *myc*$^{-/-}$ cells still activated both ERK and AKT pathways at levels commensurate with those seen in control *myc*$^{flox/flox}$ cells. This supported the idea that the observed effects were not a consequence of an inability to respond to signals upstream from Myc but rather to the lack of Myc itself. Further metabolomic inquiry showed that *myc*$^{-/-}$ T cells had impaired FAO and glucose flux during both the growth and proliferative phase, accumulated less lactate and directed less glucose-derived carbon into the PPP. Consistent with these findings, the induction of several glycolytic enzymes and glucose transporters were also suppressed as was glutaminolysis. The PKM2 isoform was also less highly induced in *myc*$^{-/-}$ T-cells.

THE ROLE OF GLUTAMINOLYSIS IN METABOLIC REPROGRAMMING

It has been known since the mid-1950s that both normal and transformed cells share a particular predilection for exogenous glutamine and sometimes even prefer it to glucose as an energy-generating substrate (204–207). Indeed, some tumors have such exaggerated demands for this amino acid that they can deplete host plasma glutamine levels despite its being the most abundant amino acid (208, 209). Recent evidence supports the idea that glycolysis and glutaminolysis cooperatively support high rates of cell proliferation (210, 211).

Glutaminolysis offers several advantages that explain its ability to complement and/or replace glucose as an energy source (**Figure 3**). First, it can be used directly for *de novo* protein synthesis as can the amino acids derived from it including glutamate, proline, histidine, alanine, aspartic acid, and arginine. Second, it may facilitate the uptake of other amino acids, thereby regulating and coordinating their availability for protein synthesis as well (212–214). Third, it serves as the starting point for the biosynthesis of purine nucleosides, thereby linking protein and nucleic acid synthesis. It is noteworthy that

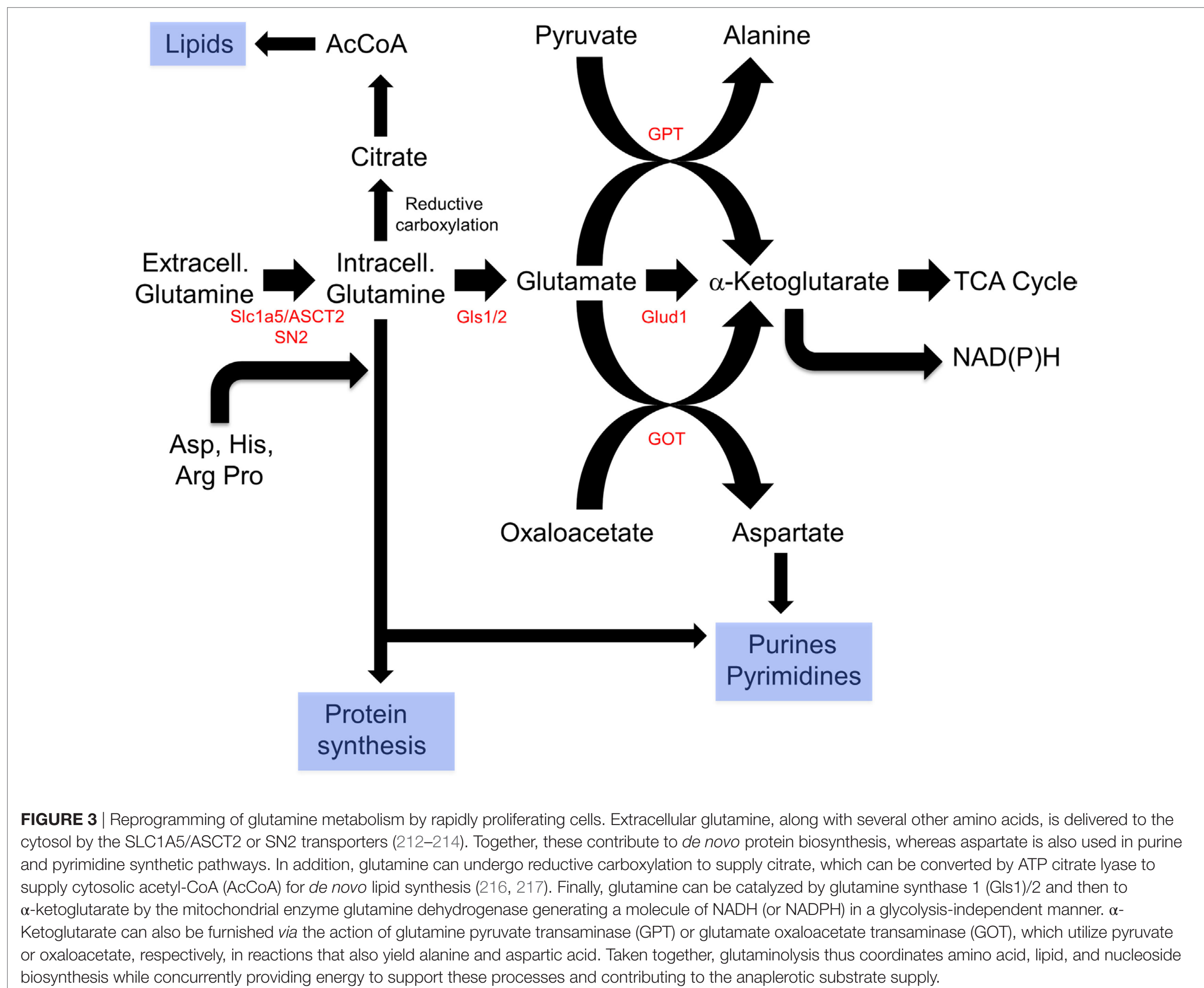

FIGURE 3 | Reprogramming of glutamine metabolism by rapidly proliferating cells. Extracellular glutamine, along with several other amino acids, is delivered to the cytosol by the SLC1A5/ASCT2 or SN2 transporters (212–214). Together, these contribute to *de novo* protein biosynthesis, whereas aspartate is also used in purine and pyrimidine synthetic pathways. In addition, glutamine can undergo reductive carboxylation to supply citrate, which can be converted by ATP citrate lyase to supply cytosolic acetyl-CoA (AcCoA) for *de novo* lipid synthesis (216, 217). Finally, glutamine can be catalyzed by glutamine synthase 1 (Gls1)/2 and then to α-ketoglutarate by the mitochondrial enzyme glutamine dehydrogenase generating a molecule of NADH (or NADPH) in a glycolysis-independent manner. α-Ketoglutarate can also be furnished *via* the action of glutamine pyruvate transaminase (GPT) or glutamate oxaloacetate transaminase (GOT), which utilize pyruvate or oxaloacetate, respectively, in reactions that also yield alanine and aspartic acid. Taken together, glutaminolysis thus coordinates amino acid, lipid, and nucleoside biosynthesis while concurrently providing energy to support these processes and contributing to the anaplerotic substrate supply.

the NADPH derived from the diversion of glycolytic substrates into the PPP can also positively impact glutamine uptake and sustain reactions involved in lipid synthesis (215, 216). Fourth, the glutamate dehydrogenase-mediated conversion of glutamate to α-ketoglutarate generates an additional molecule of NADH. This, together with the NADH generated by the α-ketoglutarate dehydrogenase and malate dehydrogenase reactions and the FADH2 generated by the SDH reaction, ensures that nearly normal levels of reducing equivalents can be supplied in a manner that is independent of AcCoA, glycolysis and FAO. Fifth, glutamine-derived α-ketoglutarate can also participate in a reverse carboxylation reaction to furnish citrate for the generation of cytoplasmic AcCoA for use in *de novo* fatty acid and cholesterol biosynthesis (216, 217). Finally, during periods of oxidative stress as commonly occur in many tumors, high levels of ROS can inhibit the aconitase-catalyzed conversion of citrate to isocitrate, thus limiting the supply of glycolytically derived α-ketoglutarate. Glutaminolysis provides a means of overcoming metabolic roadblocks such as this and thereby ensuring a more stable and consistent supply of α-ketoglutarate and the downstream reducing equivalents derived from it (215, 218).

Given the central role of glutaminolysis for sustaining cellular proliferation, coupled with a previous finding that the apoptosis mediated by the absence of glutamine is Myc dependent (219), it was not surprising that Myc regulates this process at several levels. Wise et al. (220) first reported that glioma cells consumed large amounts of glutamine to drive OXPHOS and that the shRNA-mediated suppression of Myc reduced glutamine consumption. They further observed that glioma cells were unable to survive in glutamine-deficient medium, even when supplied with glucose, and that the cell-permeable α-ketoglutarate analog dimethyl-α-ketoglutarate could substitute for glutamine, thereby providing strong evidence that the shunting of exogenous glutamine into the TCA cycle was directly responsible its effect on survival. Moreover, they showed that glycolysis and glutaminolysis, while both Myc regulated, were under distinct forms of control by virtue of the fact that PI3K/Akt signaling regulated the former but not the latter. Myc was subsequently found to bind selectively to

E-box-containing promoter regions of the glutamine transporter genes *SLC1A5/ASCT2* and *SN2*. shRNA-mediated suppression of Myc resulted in lowered expression of both transporters' transcripts and reduced glutamine consumption. Finally, the enforced transient expression of high levels of Myc in MEFs upregulated SLCA15/ASCT2 as well as glutamine synthase 1 (Gls1) and diverted glucose away from its oxidative metabolism by the TCA cycle and into the Warburg-type aerobic glycolysis instead. The mechanism by which Myc upregulated Gls1 appeared to involve increased transcription and/or stabilization of its mRNA.

Gao et al. (221) extended these results by showing that the levels of Gls1 protein in P-493 B cells and PC3 prostate cancer cells varied in direct proportion to the degree of Myc expression. However, and in contrast to Wise et al. (220), this was not true for Gls1 transcript levels leading the investigators to eventually determine that Myc regulated Gls1 at the posttranscriptional level by inhibiting the expression of two microRNAs, miR23a and miR23b (miR23a/b). Both miRNAs were noted to have homology to potential "seed" binding sequences in the Gls1 mRNA 3′-untranslated (3′ UT) region. A luciferase reporter vector containing the Gls1 3′ UTR was shown to be responsive to these miRNAs. Thus, the Myc-mediated upregulation of Gls1is indirect by virtue of its inhibition of at least two miRNAs, which inhibit Gls1 mRNA translation.

In a variation of the above theme, Qing et al. (222), extended these findings to include several human neuroblastoma cell lines and 80 primary human neuroblastomas with varying degrees of N-Myc overexpression. As with the studies of Wise et al. and Gao et al. (221), they found that the cell lines underwent apoptosis in an N-Myc-dependent manner when deprived of glutamine. They also found higher levels of expression of Gls2 (but not Gls1), glutamate oxaloacetate transaminase (GOT2), SLCA15/ASCT2 and several other amino acid transporters that correlated with N-Myc expression levels. Apoptosis in cell lines in response to glutamine deprivation could also be inhibited or delayed by providing the cell-permeable TCA substrate dimethyl α-ketoglutarate in place of glutamine.

Pérez-Escuredo et al. (211) showed that the uptake of ^{3}H-glutamine and its utilization by highly oxidative cervical cancer cells was enhanced by lactate, which also accelerated tumor growth. They also showed that Matrigel-embedded tumor cells grown subcutaneously in immunocompromised nu/nu mice in the presence of high local concentrations of extracellular lactate upregulated Slca1A5/ASCT2 and Gls1 at the protein level. shRNA-mediated knockdown of the lactate transporter MCT1 abolished these effects indicating that actual transport of extracellular lactate was mediating the effects on glutaminolysis. The upregulation of Slca1A5/ASCT2 and Gls1 appeared to be mediated by Myc, whose levels were significantly increased by as little as a 6-h exposure to lactate. Further investigation found that the conversion of lactate to pyruvate blocked the activity of prolyl hydroxylases, which are negative regulators of hypoxia-inducible factors (HIFs) 1α and 2α (223). It was suggested that HIF-2α stabilizes Myc *via* its intranuclear binding to Myc-Max heterodimers (224) and indeed, this tripartite interaction was observed in co-immunoprecipitation experiments. Furthermore, the silencing of HIF-2α abolished the upregulation of Myc, Slc1A5/ASCT2 and Gls1. Although HIF-1α was not shown to interact with Myc or Max, it is known to collaborate with Myc to induce the expression of glycolytic genes, thus potentially contributing to the intracellular lactate burden and further stabilizing Myc (224). These effects may have been further aided by the stabilization of HIF-1α by Myc itself (225).

Wang et al. (203) also examined glutamine dependency in the previously mentioned model of normal T-cell activation discussed earlier. They determined that glutamine deprivation resulted in impaired T-cell activation as well as decreased lipid and protein biosynthesis and led to an eventual G_0/G_1 arrest without affecting viability.

Although the Warburg effect and glutaminolysis are typically associated with high levels of proliferation (1, 2, 5, 226), they have also been observed in response to hypertrophy in otherwise non-dividing cells. Piao et al. (227), showed that the heart, which relies primarily on glycolysis and FAO for energy, reverts to using glutminolysis and also increases glucose utilization when subject to conditions that induce hypertrophy. Using two different models of right ventricular hypertrophy, they found variable degrees of Myc induction and increased expression of glutamine receptors SLC1A5/ASCT2 and Slc7A5 as well as increases in the mRNAs encoding the Glut1 glucose transporter and hexokinase (HK) 1. Consistent with the former of these findings, the investigators also noted increased $^{14}CO_2$ production derived from ^{14}C-labeled glutamine. In response to the glutamine antagonist 6-diazo-5-oxo-L-norleucine a decrease in glutaminolysis was noted and was associated with a compensatory increase in glucose oxidation and elevated cardiac output. These studies strongly implicate glutaminolysis as being directly involved in the biomass accumulation that accompanies active proliferation but not in proliferation *per se*.

DISTINCT *IN VIVO* METABOLIC ROLES FOR MYC: NOT ALWAYS THE SAME FUNCTION IN NORMAL AND NEOPLASTIC TISSUES

Myc's unequivocal role in integrating normal mass accretion and proliferative signals with altered metabolism in fibroblasts, myeloid cells, T-cells, and other cell types *in vitro* as discussed earlier contrasts sharply with recent studies in hepatocytes where Myc was found to be entirely dispensable for the long-term regeneration of normal liver parenchyma (228). Several previous studies had indicated that mice with a conditional, hepatocyte-specific knockout of the *myc* gene could regenerate hepatic mass following two-thirds partial hepatectomy (PH) (229–232). Less clear was whether this was achieved as rapidly as occurred in control livers. To some extent, this uncertainty was the consequence of different groups having used different and mostly indirect techniques to measure hepatocyte proliferation and liver regeneration. Further compounding this was the fact that the PH model is a relatively crude and suboptimal way to measure long-term regenerative potential given that the average hepatocyte must divide only about 1.6 times to replace the resected liver mass and that the entire regenerative process is complete within

7–10 days (233). Moreover, as many as 30–40% of the hepatocytes in the regenerating liver remnant remain quiescent following PH, and about the same amount of "regeneration" can be attributed to hypertrophy rather than actual cell division (234, 235). Thus, none of these reports actually addressed the question of whether Myc was necessary to support sustained, long-term hepatocyte proliferation as might occur during the course of normal hepatocyte turnover or repair from chronic injury, both of which are processes of much longer duration (234). It further left open the questions of whether subtle but nonetheless significant differences in regeneration rates might have escaped detection using the PH model and what, in fact, was actually being measured in these other reports.

Edmunds et al. (228) addressed all of these issues by capitalizing on an elegant, robust, and sensitive murine model of Type I hereditary tyrosinemia (236, 237). In these mice, as in humans, inactivation of the fumarylacetoacetate hydrolase (FAH) gene, which encodes the final enzyme in the pathway for tyrosine catabolism, leads to the accumulation of toxic levels of the upstream tyrosine catabolites maleylacetoacetate and fumarylacetoacetate, eventually causing hepatocyte death, fibrosis, and hepatic failure (238). This ultimately fatal outcome can be blocked with the drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (Nitisinone or NTBC), a reversible inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) (239). HPPD is a more proximal enzyme in the pathway and converts the first tyrosine catabolite 4-hydroxyphenylpyruvate to homogenistate. In this way, *fah*$^{-/-}$ mice can be maintained in a healthy state simply by providing NTBC in their drinking water and thereby blocking tyrosine catabolism and the accumulation of the deleterious metabolites.

Fah$^{-/-}$ mice can also be cured by the intrasplenic injection of as few as 10^5 *fah*$^{+/+}$ hepatocytes followed by the intermittent discontinuation and resumption of NTBC (236, 237). Animals initially lose weight as they accumulate the toxic tyrosine catabolites. However, as endogenous *fah*$^{-/-}$ hepatocytes are gradually replaced by the *fah*$^{+/+}$ donor population over 4–5 months, the recipient mice are eventually rendered NTBC-free and their livers are comprised of 50–80% donor hepatocytes (228, 236, 237, 240). As a way of monitoring hepatocyte proliferative potential, this model offers several advantages over PH. First, because the donor cells must divide 50- to 100-fold during recipient liver repopulation, they undergo many more population doublings than do post-PH hepatocytes, thereby providing a more demanding and long-term replicative challenge. Second, donor hepatocytes from different sources can be used in "competitive" repopulation assays, analogous to those used for decades in bone marrow transplantation studies (241, 242). Provided that the recipient and donor populations can be distinguished, the ultimate contribution of each to the steady-state transplanted liver can be assessed with exquisite precision and even quite small deviations from the input donor ratios can be easily quantified (228, 240). Third, if so desired, the input ratio of the competing donor populations can be varied to reveal even more dramatic differences in regenerative potential. Finally, because the competing donor populations replicate in identical environments, differences in regeneration rates can be ascertained with many fewer animals than are required with PH-based experiments.

Edmunds et al. (228) exploited the FAH model to assess the regenerative capacities of mixed *fah*$^{+/+}$ populations of *myc*$^{+/+}$ and *myc*$^{-/-}$ hepatocytes. Surprisingly, the ratio of the two donor hepatocyte populations recovered from the fully reconstituted recipient livers more than 4 months after their co-transplantation was identical to that of the input donor populations. Thus, even under the most demanding of circumstances, Myc's absence did not impair the long-term regenerative potential of hepatocytes in this particular model.

In addition to being at odds with the above-discussed role for Myc in the proliferation of fibroblasts and T-cells and numerous other cell types (154, 158, 191, 196, 203), these results also differ from studies in *Drosophila* and some cancer lines showing that cells expressing higher levels of Myc tend to outcompete those with low levels (243–245). Similarly, the conditional deletion of *myc* or its dominant-negative inhibition in intestinal crypt cells or bone marrow cells is associated with severe proliferative defects although these may be ameliorated over time (49, 246–248). Thus, the elimination of endogenous Myc seems to have highly variable and tissue-specific effects, with liver representing an atypical although perhaps not unique example.

Ultimately, while the metabolic consequences of endogenous Myc loss are tissue specific and variable, it seems reasonable to conclude that, in most cases, Myc is responsible for maintaining context-appropriate levels of ATP and anabolic substrates by regulating the uptake and oxidation of nutrients that furnish glycolysis and the TCA cycle. In Myc's absence, as noted above, many tissue types appear to adapt to the associated nutrient and energy deficits *via* various strategies that include variable reductions in cell mass, proliferative rate and anabolic activity (49, 158, 180, 228, 249). Interestingly, while Edmunds et al. (228) did not observe any significant differences in cell size, ATP levels or AMPK phosphorylation in the livers of mice following transplantation with *myc*$^{+/+}$ or *myc*$^{-/-}$ hepatocytes, these studies were performed on hepatocytes that had already re-populated the liver and reached a non-proliferating equilibrium state. It is certainly possible that more profound energy deficits might have been observed had the actual proliferating population been assessed at an earlier time following transplant. Nonetheless, these studies clearly demonstrated that *myc*$^{-/-}$ hepatocytes remain as fully capable as their wild-type counterparts at contributing to the long-term repopulation of the liver irrespective of whatever defects they may harbor.

Although *myc*$^{-/-}$ hepatocytes demonstrated no obvious proliferative impairment in the above-described repopulation assay, they nevertheless showed several abnormalities that were evident even prior to transplantation (228). First, despite body weights identical to those of *myc*$^{+/+}$ mice, juvenile mice with hepatocyte-specific deletion of *myc* had smaller livers, consistent with a previous observation that *myc* hypomorph mice tend to have smaller numbers of otherwise normal-sized cells in some organs, including liver (249, 250). At first glance, this would seem to be inconsistent with fact that *myc*$^{+/+}$ and *myc*$^{-/-}$ hepatocytes competed equally in repopulation studies (228). However, it is possible that the requirements for Myc in the developing liver versus the fully developed liver are different. There may thus exist a phase early in development, but not beyond, during which Myc is required for hepatocyte expansion. Alternatively, the smaller

size of *myc*$^{-/-}$ livers may more reflect an unappreciated role of Myc in regulating organ size (251, 252) than in limiting the proliferative potential of its individual constituent cells, which is what is measured in hepatocyte transplant studies.

In contrast to the above findings, adult *myc*$^{-/-}$ livers actually weighed *more* than *myc*$^{+/+}$ livers. The former possessed a significantly higher neutral lipid and triglyceride content, which likely accounted for their increased mass (228). This implied either that *myc*$^{-/-}$ livers take up and store greater amounts of these lipids and/or utilize less of them. Arguing against the latter point was the finding that *myc*$^{-/-}$ livers showed variable but significant increases in FAO. This suggested that, in the absence of Myc, hepatocytes both take up and utilize more fatty acids, with the former process outpacing the latter, eventually culminating in an increased storage pool, not unlike that seen in *myc*$^{-/-}$ fibroblasts or following short-term pharmacologic Myc or N-Myc inhibition in other cell types (191, 196, 228).

Edmunds et al. (228) studied the structure and function of isolated mitochondria from *myc*$^{+/+}$ and *myc*$^{-/-}$ livers using BNGE and noted no obvious differences in the stoichiometries of the protein subunits of ETC Complexes I–IV or the ATP synthase (Complex V). Mass spectroscopic quantification of over 400 mitochondrial proteins, including all 93 subunits of the ETC, also showed no significant quantitative differences between the two groups. However, Complex I and Complex II activities were modestly but significantly reduced in mitochondria from *myc*$^{-/-}$ livers in response to ADP and succinate whereas the activity of Complex V was increased by about the same amount. Coupled with the finding that ATP levels in *myc*$^{+/+}$ and *myc*$^{-/-}$ livers were comparable, this suggested that the loss in ETC function in *myc*$^{-/-}$ livers was compensated for by a more efficient generation of ATP *via* Complex V and/or by the more inherently efficient process of FAO (253).

Collectively, these findings suggest a form of metabolic reprogramming by the *myc*$^{-/-}$ liver only partially resembling that seen in *myc*$^{-/-}$ fibroblasts, which may reflect the different tissues under consideration as well as their different proliferative rates. For example, ATP and AcCoA levels were markedly diminished in *myc*$^{-/-}$ fibroblasts but were maintained at normal levels in *myc*$^{-/-}$ livers (158, 228) (**Figure 4**). This appears to have been due to the employment of FAO as an alternative energy source by hepatocytes as well as to an increase in neutral lipid accumulation that has been previously reported in *myc*$^{-/-}$ fibroblasts, in hematopoietic cells following short-term inhibition of Myc, and in neuroblastomas following treatment with small molecule Myc inhibitors (158, 191, 196). The neutral lipid accumulation by *myc*$^{-/-}$ hepatocytes was even more striking following their transplantation into *fah*$^{-/-}$ recipient mice. Oil Red O staining of fully reconstituted livers confirmed their significantly higher triglyceride content as well as larger and more numerous neutral lipid droplets (228). Indeed, the lipid within these livers was so abundant that much of it was extracellular. This was likely responsible for the significant inflammatory cell infiltrate that was observed as well as for the upregulation of numerous transcripts involved in acute and chronic inflammation, leukocyte signaling and fibrosis. Immunohistochemical staining for 4-hydroxynonenal, a by-product of ROS-mediated lipid peroxidation, was also detected as was evidence for dysregulated mitochondrial structure and function as previously reported in mice maintained on high fat diets (254–256). Other findings included the downregulation of 19 of the 44 transcripts encoding subunits of Complex I and seven of the 20 transcripts encoding subunits of Complex V. In the latter group, 10 of the remaining 13 transcripts were upregulated. Taken together, these studies suggested that the loss of Myc expression in resting hepatocytes is initially associated with the gradual accumulation of neutral lipid, not unlike that seen in non-alcoholic fatty liver disease (NAFLD) in association with ETC dysfunction (257, 258). When coupled with the metabolic stress imposed by regeneration-associated proliferation and chronic inflammation, this progressed to a phenotype closely resembling non-alcoholic steatohepatitis (NASH), a long-term consequence of NAFLD that is associated with high levels of oxidative stress, inflammation, fibrosis and eventual hepatic failure (257, 259). It is tempting to speculate that the loss of Myc in these hepatocytes, particularly during times of proliferation and high energy demand, leads to an attenuated glycolytic response and the dysregulation of mitochondrial structure and function. The resulting energy depletion and mitochondrial stress is accompanied by an increased reliance on FAO coupled with unbalanced fatty acid uptake and storage, intracellular ROS generation, inflammation, and long-term parenchymal damage that mimics NAFLD and NASH (228).

The above findings do not provide simple explanations for how endogenous Myc affects metabolism during normal growth. They do suggest, however, that these changes occur at multiple levels and that the compensation by *myc*$^{-/-}$ hepatocytes ultimately affects neither ATP generation, AcCoA levels nor proliferation. It remains to be determined precisely how ATP levels in *myc*$^{-/-}$ livers are maintained but it appears to be dependent on several different pathways including an increased efficiency of ATP production as suggested by the Complex V assays and the provision of alternate sources of AcCoA mainly from enhanced FAO. An alternative source might originate with increased glutaminolysis, which could conceivable circumvent the need to rely on glycolysis- or FAO-derived AcCoA by providing reducing equivalents in the form of both NADH and FADH2. Whatever the cumulative corrective mechanisms are that help to normalize hepatocyte energy generation and proliferative function, they come at considerable metabolic cost in the form of increasingly severe lipid storage and utilization defects.

Metabolic changes have also been studied in *myc*$^{-/-}$ hepatocytes in response to oncogenic signaling following the induction of hepatoblastoma (HB). HB is the most common pediatric liver cancer, almost invariably arising in children under the age of 3 years and is associated with somatic mutations of the β-catenin gene in >80% of cases (260, 261). A useful mouse model of HB has recently been described in which tumors arise with nearly 100% penetrance following the hydrodynamic tail vein injection-mediated delivery of "Sleeping Beauty" plasmids (262, 263) encoding patient-derived mutant forms of β-catenin and yes-associated protein (YAP) (264–266). A major transcriptional target for β-catenin is Myc (267) and, not unexpectedly, Myc is among the most highly upregulated genes in HBs (Monga and Prochownik, unpublished data).

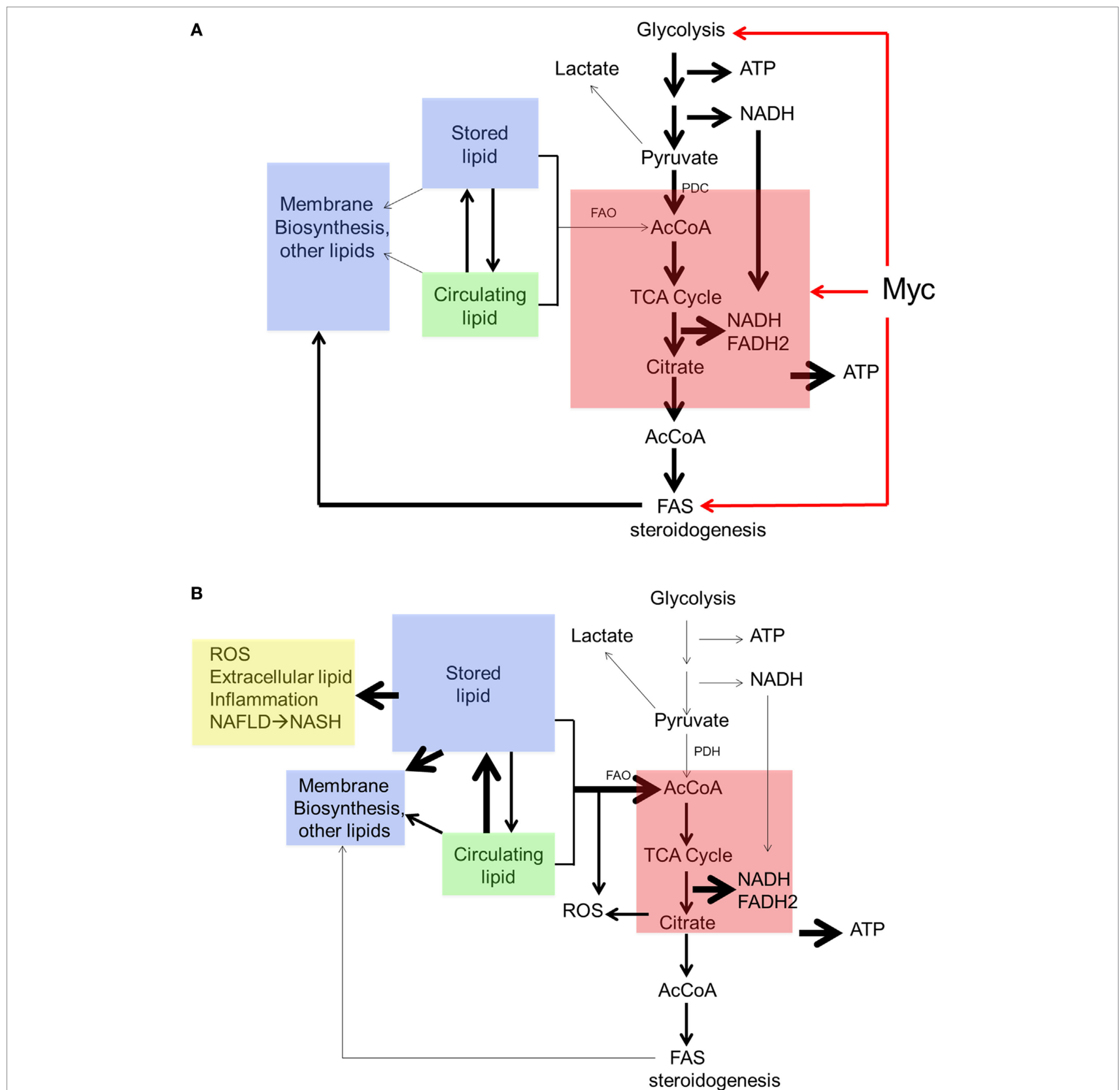

FIGURE 4 | Regulation of neutral lipid accumulation in *myc*$^{-/-}$ hepatocytes is both Myc dependent and Myc independent. **(A)** In *myc*$^{+/+}$ hepatocytes, the pathways indicated by red arrows are regulated both by Myc and Myc-independent factors. Stored lipid and circulating lipid are maintained in equilibrium. Stored lipid contributes minimally to energy generation (*via* FAO) or the synthesis of new membranes or other lipids when ATP and acetyl-CoA (AcCoA) are derived *via* glycolysis. Under these conditions, lipids such as fatty acids and sterols are largely synthesized from cytoplasmic AcCoA that originates from TCA cycle-derived citrate. **(B)** In *myc*$^{-/-}$ hepatocytes, basal rates of glycolysis, oxidative phosphorylation, and fatty acid synthesis are depressed thereby allowing for a compensatory increase in FAO. This allows basal levels of ATP and AcCoA to be sustained to meet energetic needs. New membrane synthesis thus becomes more reliant upon the uptake of exogenous lipids, which accumulate in the form of neutral lipids, leading to mitochondrial dysfunction and reactive oxygen species (ROS) generation (254–256). Additional ROS are generated as a result of the accumulation of intracellular and extracellular neutral lipid (257–259).

Given that Myc plays no role in normal hepatocyte repopulation as discussed earlier (228), it was of interest to determine whether Myc is needed to support the more rapid growth and increased metabolic demands of HBs. Indeed, Wang et al. (125) found that, although tumors arose in *myc*$^{-/-}$ livers at the same frequency as they did in *myc*$^{+/+}$ livers, their growth rates were significantly impaired. Thus, while Myc was dispensable for normal hepatocyte proliferation and for the induction of

HBs, it was clearly required for determining the rate of tumor growth (125, 228).

To understand the basis for Myc's selective role in HB proliferation (228), the metabolic properties of *myc*$^{+/+}$ and *myc*$^{-/-}$ HBs were compared. An initial assessment of mitochondrial function showed both types of HBs had lower OCRs and Complex II activity compared with corresponding livers. However, less suppression of these activities was seen in *myc*$^{-/-}$ HBs. It is likely that this reflected their slower growth rates and thus a reduced tendency to rely on Warburg-type aerobic glycolysis for energy generation. Consistent with this, glycolytic transcripts as a group were upregulated in *myc*$^{+/+}$ HBs to a significantly greater extent than they were in *myc*$^{-/-}$ HBs (11.2-fold versus 8.9-fold, $P < 0.0004$). The presumptive lower glycolytic rate of the latter likely accounted for their lower AcCoA levels. Despite these differences, both tumor types showed elevated levels of ATP and a downregulation of phosphoAMPK relative to their respective livers. This suggested that, despite differences in their metabolic activities, the slower growth rates of *myc*$^{-/-}$ HBs were not the result of any obvious energy deficit. On the other hand, it is conceivable that *myc*$^{-/-}$ HBs were energetically constrained and that they maintained a rate of growth that was compatible with these limitations while still allowing normal levels of ATP to be maintained. The presumptive reduced rate of glycolysis by *myc*$^{-/-}$ HBs may also have restrained the supply of anabolic precursors necessary for sustaining rapid tumor growth. In addition, these tumors were also less able than *myc*$^{+/+}$ tumors to upregulate the group of transcripts encoding the ~80 ribosomal protein genes (3.6-fold versus 5.2-fold, $P < 10^{-4}$) (125). Thus, rather than being severely deprived of energy, as in the case for *myc*$^{-/-}$ fibroblasts, *myc*$^{-/-}$ HBs may instead be deprived of anabolic precursors and the ability to increase protein synthesis rates, despite adequate energy supplies. Protein synthesis rates and ribosomal protein content are known to be rate-limiting factors in the growth of many cancers (108, 268).

In further pursuit of an explanation for the overall downregulation of OXPHOS by tumors, Edmunds et al. (228) quantified mtDNA using qPCR to amplify two distinct regions of the mitochondrial genome. They documented a ~60–80% reduced mitochondrial mass in both *myc*$^{+/+}$ and *myc*$^{-/-}$ tumors, thus providing a structural explanation for the Warburg effect. Results published at about the same time by Reznick et al. (269) showed a similar loss of mtDNA from a wide variety of human cancers whose genomic data had been compiled in *The Cancer Genome Atlas* (https://cancergenome.nih.gov).

In HBs arising in *myc*$^{+/+}$ and *myc*$^{-/-}$ livers, eight of the 14 most deregulated pathways identified by Ingenuity Pathway Analysis involved lipid biosynthesis. Importantly, transcripts encoding several key enzymes involved in fatty acid synthesis (FAS) such as ATP citrate lyase (ACLY), fatty acid synthase, and AcCoA carboxylase were markedly upregulated, although not in a Myc-dependent manner. By contrast, transcripts encoding enzymes involved in FAO such as trifunctional protein, carnitine palmitoyltransferase-2 and very long-chain acyl-CoA dehydrogenase were markedly downregulated. Indeed, even transcripts whose encoded enzymes participate in peroxisomal FAO such as the fatty acid transporter ATP-binding cassette-D3, peroxisomal biofunctionalized protein, and acyl-CoA oxidase-1 were downregulated. Functional assays of the FAO pathway were consistent with these findings and showed marked downregulation of FAO activity in both *myc*$^{+/+}$ and *myc*$^{-/-}$ tumors (125). Taken together, these studies imply that, unlike the case in fibroblasts, where the loss of Myc leads to a near cessation of both glycolysis and OXPHOS and an upregulation of FAO as an alternate energy source, in HBs, glycolysis and OXPHOS are much less affected and rely less on FAO to supply energy.

METABOLIC LINKS BETWEEN NORMAL AND NEOPLASTIC STATES

As discussed at length above, proliferating tumor cells require a continual supply of the basic cellular building blocks consisting of amino acids, nucleic acids, and lipids. In this regard, tumor cells are not very different from the progenitor cells that give rise to our various organs during embryogenesis, or the regenerative stem cells now known to reside within many of our adult tissues. Furthermore, differentiated cells may dedifferentiate and then proliferate to repair an injury, followed by re-differentiation. The metabolic similarities between these different types of proliferating cells are striking: all rely heavily upon glucose and glutamine to meet their steep anabolic needs (270–272). Not coincidentally, Myc maintains stem cell pluripotency and self-renewal, perhaps through its effects on driving glucose/glutamine uptake and metabolism (273). On the other end of the spectrum are non-proliferative, differentiated cells that are often metabolically reliant upon FAO. Several fetal tissues that rely upon glucose during growth and development undergo a metabolic switch to FAO after birth. The best-known example of this is the heart (274) and we have made similar observations in human kidney (Goetzman, unpublished data). Senescence is also characterized by a switch to FAO while lipid synthesis is turned off (275, 276). With some notable exceptions (i.e., prostate cancer) most cancers are glucose/glutamine dependent and not FAO dependent (277, 278).

The glucose/glutamine versus FAO dichotomy is maintained in both normal and neoplastic tissues by a key metabolic phenomenon called the Randle cycle, sometimes simply referred to as the glucose–fatty acid cycle. The Randle cycle refers to the reciprocal nature of mitochondrial rates of pyruvate oxidation and FAO (279, 280). The Randle cycle centers on pyruvate dehydrogenase (PDH) and carnitine palmitoyltransferase 1 (CPT1), which are the rate-limiting enzymes of pyruvate oxidation and FAO, respectively. When glucose is abundant and glycolysis is high, pyruvate concentrations rise and inhibit pyruvate dehydrogenase kinase (PDK1), the kinase responsible for phosphorylating and silencing PDH. PDK1 inhibition activates PDH and increases mitochondrial pyruvate oxidation to generate AcCoA and NADH. Increases in intramitochondrial AcCoA and NADH have two consequences for FAO. First, the intramitochondrial FAO machinery is directly inhibited by high NADH/NAD$^+$ and AcCoA/CoA ratios (281). Second, the entry of pyruvate-derived AcCoA into the TCA cycle produces citrate, which is exported from mitochondria to the cytosol where it is reconverted to AcCoA *via* the action of ACYL.

Cytoplasmic AcCoA is then converted to malonyl-CoA by AcCoA carboxylase (ACC). Malonyl-CoA in turn is a potent inhibitor of CPT1. CPT1 is the gatekeeper for FAO *via* its role in regulating fatty acid transport across the mitochondrial membrane; thus, the result of sustained PDH flux is the sequestration of fatty acids in the cytosol, away from the degradative FAO machinery. Cytosolic fatty acids are then either converted to triglyceride droplets to fuel the cell should glucose become unavailable, or in the case of proliferating cells, incorporated into phospholipid acyl-chains for synthesizing new membranes. The same malonyl-CoA which silences CPT1 also feeds into the *de novo* FAS pathway to further support the anabolic needs of the cell (282). Because of this cycle, the rate of PDH flux has been shown to highly correlate with lipogenesis in many cell types. In the liver, for example, insulin increases PDH activity and lipogenesis from glucose-derived AcCoA, while starvation rapidly inhibits PDH activity and thus limits glucose-derived lipogenesis (283).

The Randle cycle also works in the opposite direction, i.e., high rates of FAO suppress glucose utilization. In the same way that glucose-derived AcCoA and NADH inhibits the FAO machinery, FAO-derived AcCoA and NADH potently inhibit PDH *via* activation of PDK1. There is also some evidence that fatty acid-derived citrate exported to the cytosol can suppress glycolysis at the level of phosphofructokinase-1 (284–286). The resulting accumulation of glucose-6-phosphate feeds back to inhibit hexokinase (HK) and glucose uptake (**Figure 5**) (286). Overall, the net result of these various points of negative feedback control are a downregulation of glucose utilization by FAO. A key theme of the Randle cycle is that lipogenesis sides with glucose oxidation, and that cells cannot simultaneously conduct FAO and FAS. This is because activation of FAS, by virtue of cytosolic conversion of citrate to AcCoA to malonyl-CoA, would inhibit FAO at the level of CPT1. This maybe the reason why proliferating cells, be they cancerous or not, suppress FAO such that they can drive their absolute requirement for FAS and membrane synthesis. Likewise, it explains how terminally differentiated and senescent cells are able to sustain high rates of FAO for their energetic needs. In short, because of FAO suppression of glucose-derived FAS, it is difficult for cells to proliferate while conducting a high rate of FAO. Exogenous fatty acids can be incorporated into membranes directly, but in FAO-dependent cells the phospholipid synthesis pathways would be in continual competition with mitochondria for incoming exogenous fatty acids.

The Randle cycle and connection between glucose utilization and FAS would suggest that tumors would tend to have low FAO and high FAS. Indeed, this has been observed in many cancers, and inhibition of the FAS pathway has been shown to slow cancer growth in many instances (287, 288). In the HB mouse model discussed at length above, FAO was found to be markedly lower than in normal control liver while PDH activity was increased

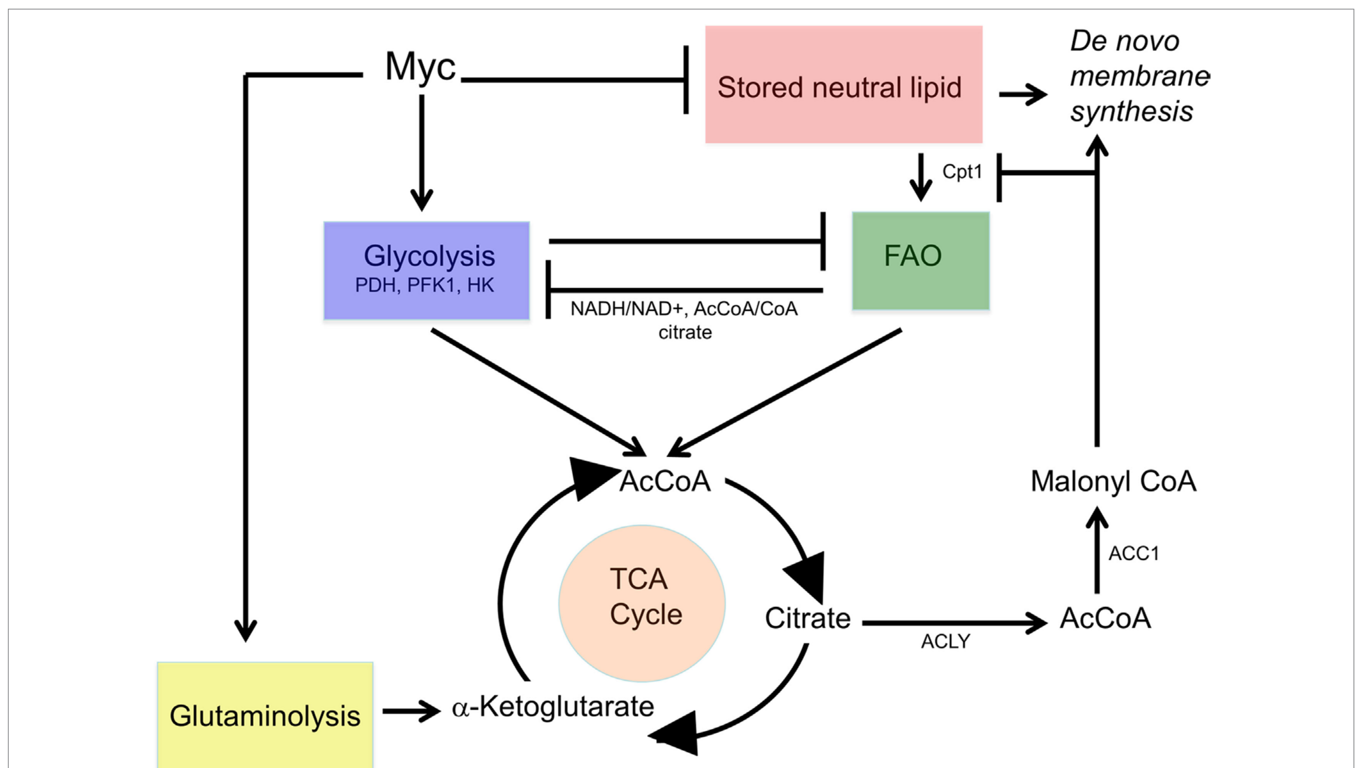

FIGURE 5 | Co-regulation of glycolysis and FAO. Starvation or glucose deprivation mobilizes lipolytic pathways and upregulates FAO to provide an alternate source of acetyl-CoA (AcCoA) and maintain oxidative phosphorylation. More so than glycolysis, FAO increases NADH/NAD$^+$ and AcCoA/coenzyme A ratios as well as cytoplasmic citrate thereby inhibiting pyruvate dehydrogenase (PDH), phosphofructokinase, hexokinase (HK), and glucose uptake (284–286). By contrast, glucose utilization by the TCA cycle tends to produce a greater buildup of citrate-derived, cytoplasmic coenzyme A (CoA) and malonyl-CoA, which exerts potent negative control over Cpt1.

several-fold (240). This occurred both in the presence and absence of Myc suggesting that there are multiple mechanisms responsible for driving the glucose–fatty acid cycle in tumors. A similar reciprocal relationship between FAO and PDH activity was also seen in a model of hepatocellular carcinoma induced by the deregulated, doxycycline-regulated induction of Myc (126). Of course, as is true with all aspects of cancer metabolism, there have also been multiple studies demonstrating cancers with the opposite metabolic phenotype, i.e., high FAO and low PDH activities (289–294). It is interesting to note that prostate cancer, the best-characterized example of a cancer with high FAO, is well known for its typically slow growth rate. Some studies have shown that maximizing pyruvate flux through PDH by inhibiting PDK with dichloroacetate can sensitize cells to other chemotherapies, presumably by promoting mitochondrially driven apoptosis (295). As with many metabolic pathways, too much of any one thing may lead to cell death. In the case of PDH, it has been shown in normal liver that most PDH capacity is not being used, even under fed, insulin-stimulated conditions (283). Only about 10% of the PDH in fed rodent liver is in the active (unphosphorylated) state; acute insulin exposure doubles this to 20%. Dichloroacetate increased the active state to 100%. Therefore, it remains possible that either supraphysiological activation or inhibition of PDH may be beneficial in crippling cancer growth, with the usual caveat that it depends upon the cancer being treated.

With regard to liver cancers, the commonality of high PDH activity in both HBs and HCC (125, 240) led to the hypothesis that eliminating PDH may slow tumor growth. First, Jackson et al. examined the consequences of conditional inactivation of the catalytic PDHA1 subunit on normal hepatocyte proliferation in murine liver (240). Using the above-described competitive hepatocyte re-population assay, it was determined that wild-type and PDH knockout hepatocytes contributed equally to the long-term reconstitution of *fah*$^{-/-}$ recipient livers. Thus, despite the severing of the direct line of communication between glycolysis and the TCA cycle, and between glucose utilization and FAS, proliferation remained unaffected. That PDH is profoundly linked to lipogenesis was demonstrated by the striking loss of lipid stores in PDH KO liver (**Figure 6**). Based on this observation it is tempting to speculate that PDH inhibition could represent a novel therapeutic approach for the treatment of NAFLD. What remains to be determined is the magnitude of PDH suppression needed to achieve this outcome, the length of time PDH suppression must continue, and how rapidly lipid re-accumulates following its reactivation.

Two approaches were next taken to ascertain how inactivation of PDH altered the properties of HBs induced by mutant β-catenin + YAP (125, 240). In the first approach, both the resultant tumors and the adjacent, non-transformed parenchyma were rendered PDH-negative ("pan KO"), whereas in the second, only tumors and not the adjacent non-transformed liver tissue were rendered PDH-negative ("restricted KO"). Survival of the pan KO tumor group did not differ significantly from that of the control group with normal levels of PDH expression in all tissues whereas survival of restricted KO tumor group was significantly prolonged (mean survival ~125 versus 90 days). Further investigation showed that pan KO HBs tended to be smaller than control tumors despite the similar survival of the two groups. By contrast, HBs from the long-surviving restricted KO group were about the same size as control tumors. These apparent discrepancies were ultimately resolved by showing that mice in the pan KO group had a severe lactic acidosis at the time of their demise. It was concluded that, in the face of the absence of PDH activity in pan KO hepatocytes, HB-derived lactate could not be metabolized back to pyruvate by the untransformed hepatocytes as it could by the PDH-expressing hepatocytes of restricted KO mice. The demise of the pan KO animals was thus due to a combination of tumor burden and lactic acidosis. The normal respiratory compensation that attempts to correct metabolic acidosis was almost certainly compromised in these mice as well due to the restricted diaphragmatic mobility imposed by the tumor burden.

The most notable metabolic defect in PDH KO tumors was a *ca.* 80% reduction in total AcCoA levels. Surprisingly, only a slight, statistically non-significant increase in compensatory FAO was observed (240). Importantly, while the Randle cycle shows that driving one pathway will limit the other, these studies suggest that directly limiting one pathway does not necessarily stimulate the other (i.e., eliminating PDH may not necessarily stimulate high FAO). Interestingly and unexpectedly, the PDH KO studies indicated that despite the profound inability to maintain normal levels of AcCoA, KO hepatocytes were able to sustain normal or near-normal rates of growth even following their transformation into highly proliferative tumor cells. It is possible that reductive carboxylation of glutamine, which was not examined, could supply just enough cytosolic AcCoA to drive the fatty acid synthesis needed to supply lipids for new membranes. In this scenario, the minimal induction of compensatory FAO might be explained by glutamine-derived malonyl-CoA, which would limit the capacity for FAO by inhibition of CPT1. In short, this series of studies with PDH KO mice highlight the amazing metabolic plasticity of both normal and neoplastic livers, and stress the difficulty of targeting metabolic pathways to treat cancers.

THERAPEUTIC STRATEGIES AIMED AT TARGETING MYC AND ITS EFFECTORS

Myc has long been viewed as an exciting but challenging oncologic target (51, 52, 296). The enthusiasm stems from the fact that Myc is among the most commonly deregulated oncoproteins in all of cancer (24, 51, 52, 97). Even in those neoplasms where Myc deregulation is not immediately appreciable, other oncogenic signaling pathways that *are* deregulated invariably converge upon Myc which then carries out their transcriptional bidding. In these cases, inhibiting Myc still leads to cell cycle arrest, the collapse of ATP production and apoptosis (50, 191). Unfortunately, Myc's lack of enzymatic activity or prominent structural features makes it extremely difficult to target with small molecules (51, 52, 296). The means by which Myc inhibition has been attempted and descriptions of the successes and (mostly) failures along the way have been recently reviewed (297). These attempts included strategies such as altering the structure of Myc DNA-binding sites, inhibiting the structure and/or function of Myc-Max heterodimers, and inhibiting Myc's engagement with and activation

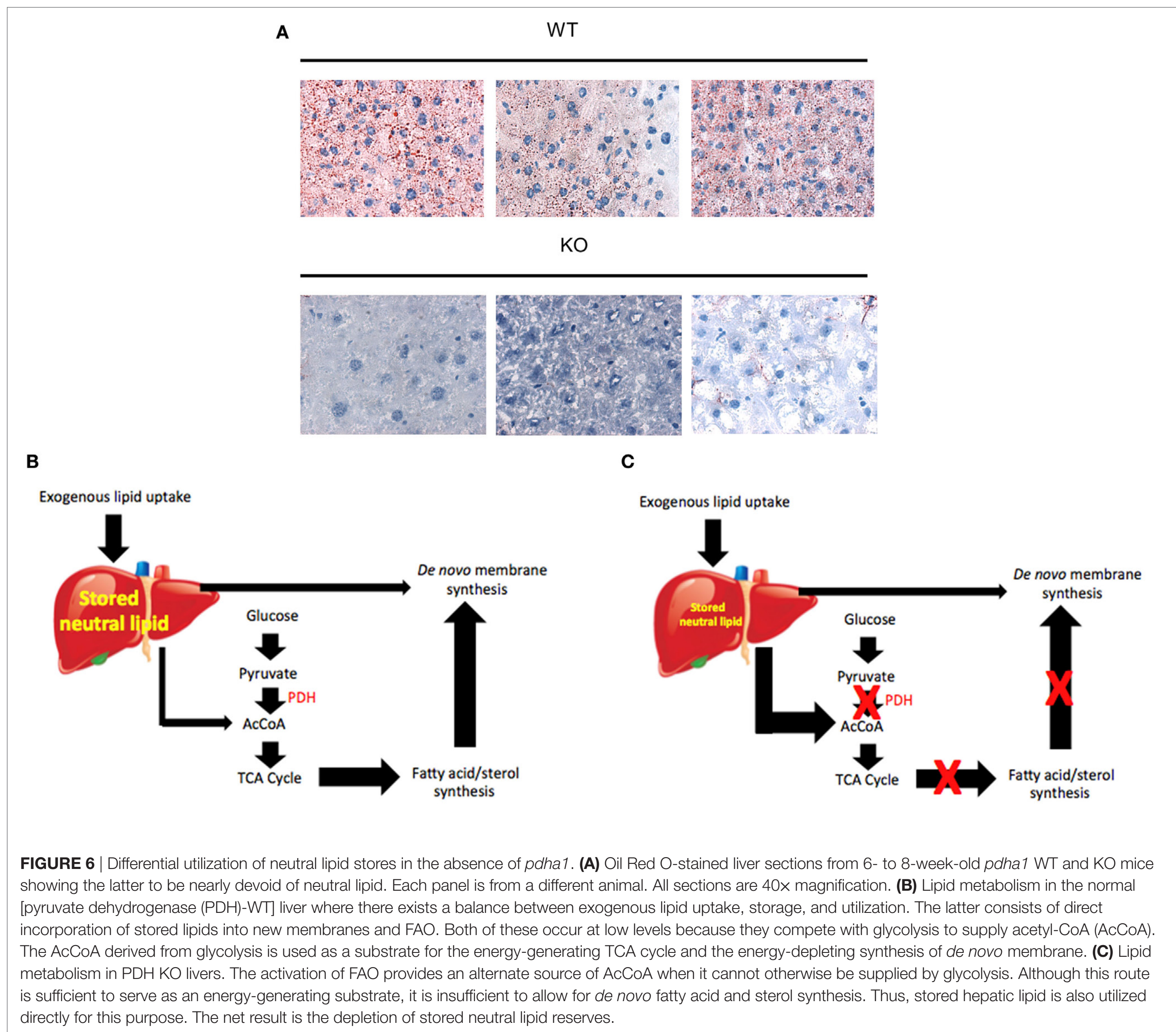

FIGURE 6 | Differential utilization of neutral lipid stores in the absence of *pdha1*. **(A)** Oil Red O-stained liver sections from 6- to 8-week-old *pdha1* WT and KO mice showing the latter to be nearly devoid of neutral lipid. Each panel is from a different animal. All sections are 40× magnification. **(B)** Lipid metabolism in the normal [pyruvate dehydrogenase (PDH)-WT] liver where there exists a balance between exogenous lipid uptake, storage, and utilization. The latter consists of direct incorporation of stored lipids into new membranes and FAO. Both of these occur at low levels because they compete with glycolysis to supply acetyl-CoA (AcCoA). The AcCoA derived from glycolysis is used as a substrate for the energy-generating TCA cycle and the energy-depleting synthesis of *de novo* membrane. **(C)** Lipid metabolism in PDH KO livers. The activation of FAO provides an alternate source of AcCoA when it cannot otherwise be supplied by glycolysis. Although this route is sufficient to serve as an energy-generating substrate, it is insufficient to allow for *de novo* fatty acid and sterol synthesis. Thus, stored hepatic lipid is also utilized directly for this purpose. The net result is the depletion of stored neutral lipid reserves.

of the transcriptional activation machinery (297). A second challenge to inhibiting Myc is that the plethora of target genes whose expression is altered, either directly or indirectly, in response to Myc deregulation includes many that are transforming in their own right. Thus, an obvious alternate strategy, such as targeting one of these rather than the intimidating Myc itself, is likely to fail because of their sheer number and oncogenic functional redundancy. A final challenge is that many of the early Myc inhibitors that have been described to date, even those which function well *in vitro*, have demonstrated disappointing therapeutic efficacy *in vivo* due to poor tissue penetration, low potency, or rapid metabolism (51, 52, 296, 297).

While these issues are clearly important when considering the targeting of oncogenic pathways, some become less so when contemplating the employment of inhibitors in non-neoplastic settings where Myc is a potential target that may not require systemic inhibition. For example, the intimal hyperplasia that accompanies atherosclerotic stenosis has long been known to be accompanied by and to be dependent upon Myc overexpression (52, 298, 299). We have proposed that drug-eluting stents, which released Myc inhibitors continuously and over short distances, would provide a means by which high-local compound concentrations could be achieved thus overcoming some of the pharmacologic shortcomings that plague their systemic delivery (52). Myc antisense oligonucleotides have shown efficacy is this regard even though their ability to reduce Myc levels has been somewhat modest (300, 301).

Non-neoplastic settings also now permit the consideration of downstream Myc effectors that would not otherwise represent tractable targets due to their neoplastic redundancy as mentioned earlier. An excellent example of this is PDH, which is neither an oncoprotein nor even a direct Myc target although it is dramatically upregulated and activated in response to activation of the Myc pathway (**Figure 6**) (125, 126, 240). Inhibiting PDH

via more traditional approaches than are available for Myc could prove to be of enormous therapeutic benefit for the treatment of NAFLD, a common condition associated with considerable non-neoplastic morbidity and mortality as well being a significant predisposing factor for the development of cancer (257). Time will tell whether the inhibition of Myc and its partners in crime will be of greater therapeutic benefit for cancer, for metabolic disorders, or for those diseases harboring overlapping features of both.

AUTHOR CONTRIBUTIONS

This review manuscript was written and edited by EP and EG. EP designed and prepared the figures.

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REVIEW
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ANRIL: A lncRNA at the CDKN2A/B Locus With Roles in Cancer and Metabolic Disease

Yahui Kong, Chih-Heng Hsieh and Laura C. Alonso*

Department of Medicine, Diabetes Center of Excellence, University of Massachusetts Medical School, Worcester, MA, United States

The *CDKN2A/B* genomic locus is associated with risk of human cancers and metabolic disease. Although the locus contains several important protein-coding genes, studies suggest disease roles for a lesser-known antisense lncRNA encoded at this locus, called *ANRIL*. *ANRIL* is a complex gene containing at least 21 exons in simians, with many reported linear and circular isoforms. Like other genes, abundance of ANRIL is regulated by epigenetics, classic transcription regulation, splicing, and post-transcriptional influences such as RNA stability and microRNAs. Known molecular functions of *ANRIL* include *in cis* and *in trans* gene regulation through chromatin modification complexes, and influence over microRNA signaling networks. Polymorphisms at the *ANRIL* gene are linked to risk for many different cancers, as well as risk of atherosclerotic cardiovascular disease, bone mass, obesity and type 2 diabetes. A broad array of variable reported impacts of polymorphisms on *ANRIL* abundance, splicing and function suggests that *ANRIL* has cell-type and context-dependent regulation and actions. In cancer cells, *ANRIL* gain of function increases proliferation, metastasis, cell survival and epithelial-mesenchymal transformation, whereas *ANRIL* loss of function decreases tumor size and growth, invasion and metastasis, and increases apoptosis and senescence. In metabolic disease, polymorphisms at the *ANRIL* gene are linked to risk of type 2 diabetes, coronary artery disease, coronary artery calcium score, myocardial infarction, and stroke. Intriguingly, with the exception of one polymorphism in exon 2 of *ANRIL*, the single nucleotide polymorphisms (SNPs) associated with atherosclerosis and diabetes are non-overlapping. Evidence suggests that *ANRIL* gain of function increases atherosclerosis; in diabetes, a risk-SNP reduced the pancreatic beta cell proliferation index. Studies are limited by the uncertain relevance of rodent models to *ANRIL* studies, since most *ANRIL* exons do not exist in mouse. Diverse cell-type-dependent results suggest it is necessary to perform studies in the relevant primary human tissue for each disease. Much remains to be learned about the biology of *ANRIL* in human health and disease; this research area may lead to insight into disease mechanisms and therapeutic approaches.

Keywords: *ANRIL*, *CDKN2A*, *CDKN2B*, long noncoding RNA, diabetes, pancreatic islet, cancer, metabolic disease

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Reviewed by:
Subrata Chakrabarti, University of Western Ontario, Canada
Tania Lee Slatter, University of Otago, New Zealand

Correspondence:
Laura C. Alonso
Laura.Alonso@umassmed.edu

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INTRODUCTION

The discovery of functional noncoding RNAs has opened a kaleidoscopic world of unanticipated mechanisms extending far beyond the DNA-RNA-protein paradigm; noncoding RNAs may in fact outnumber coding RNAs (1). Long noncoding RNAs (lncRNAs) have been discovered throughout the genome; scientists are working to explore their functions in health and disease. The *ANRIL* lncRNA was first identified in a melanoma kindred with a large (403 kb) deletion at the *CDKN2A/B* locus (2). *ANRIL* has attracted broad attention because it is located at a genomic hotspot for disease heritability, the *CDKN2A/B* locus. Although protein coding genes at this locus have important well-studied roles in cell cycle regulation, data suggest that some locus disease-associated single nucleotide polymorphisms (SNPs) act through effects on *ANRIL* itself. Intriguingly, studies suggest *ANRIL* not only impacts the biology of cancer, but also has cell-type-specific roles in metabolic disease. Although *ANRIL* has been reviewed in the past (3, 4), knowledge has exponentially increased in recent years. Here we review advances in ANRIL SNPs, gene regulation, cell biology, and disease roles of *ANRIL*.

THE *CDKN2A/B* LOCUS

ANRIL, or *CDKN2B-AS1*, is located at the human *CDKN2A/B* locus at 9p21.3. This gene cluster, extending over a nearly 350 kb genomic region housed within a single topologically associated domain (TAD) (5), contains three protein coding genes and, antisense to them, the *ANRIL* lncRNA (**Figure 1**). The protein coding genes include *S-methyl-5′-thioadenosine phosphorylase* (*MTAP*), *CDKN2A*, which encodes splice variants p16^{INK4A} and p14^{ARF}, and *CDKN2B*, which encodes p15^{INK4B} (9, 10). *MTAP* lies at one end of the locus, 192 kb telomeric to the 5′ start of *ANRIL*. At the centromeric end of the locus, the *ANRIL* gene contains 19–21 reported exons over a 126 kb region. *CDKN2A* lies between *MTAP* and *ANRIL*, near the first exon of *ANRIL*; *CDKN2B* is located within the first intron of *ANRIL*, in an antisense direction. The proteins encoded by *CDKN2A* and *CDKN2B* are tumor suppressors with well-established roles in cell proliferation, apoptosis, senescence and aging (11, 12). p16^{INK4A} and p15^{INK4B} are cyclin dependent kinase (CDK) inhibitors, inhibiting retinoblastoma phosphorylation by CDK4/6. The p14^{ARF} protein, a splice variant of *CDKN2A* which due to a frame shift has no amino acid homology to the principal other *CDKN2A* splice variant, p16^{INK4A}, modulates p53 activity. *ANRIL* is transcribed by RNA polymerase II and spliced into multiple linear and circular isoforms in a tissue-specific manner. In general, *ANRIL* roles, explored in detail below, include gene regulation in *cis* and in *trans* through interaction with polycomb repressive complex (PRC) histone modifiers, as well as RNA-RNA interactions such as microRNA (miRNA) sponge activity (3, 13). Known biological impact of *ANRIL* activities include modulation of proliferation, apoptosis and cellular adhesion pathways (14).

Abbreviations: CAD, coronary artery disease; CAC, coronary artery calcium; CDK, cyclin dependent kinase; circ*ANRIL*, circular *ANRIL;* circRNA, circular RNA; GWAS, genome-wide association study; lncRNA, long noncoding RNA; miRNA, microRNA; MI, myocardial infarction; MTAP, S-methyl-5′-thioadenosine phosphorylase; PBMC, peripheral blood mononuclear cell; PBTL, peripheral blood T-lymphocytes; PRC, polycomb repressive complex; SNP, single nucleotide polymorphism; VSMC, vascular smooth muscle cells.

EVOLUTION OF THE *ANRIL* GENE

The evolutionary development of the human *ANRIL* gene has been studied by comparative analysis of the genomes of 27 organisms including non-mammalian vertebrates, non-placental mammals, non-primate placental mammals, and primates (15). *ANRIL* originated in ancestors of the Eutherian (placental) mammalian clade. Initially the gene contained only a few exons; over time, *ANRIL* underwent clade-specific evolution, adding exons in many mammals but losing exons in rodents. The full 21 exon gene is present only in simians. *ANRIL* genes contain many repeat elements, both intronic, and exonic; evidence suggests that transposon activity has mediated many of the observed evolutionary changes in exon presence or absence, location, sequence, conservation, and structure, as well as introduction of splice sites (15).

Early *ANRIL* variants were likely not transcribed or functional (15). One hallmark of functional RNAs is splice signals at intron/exon boundaries. In simians, 191 intron/exon boundaries contained canonical splice signals, while 20 did not. In lower mammals, however, only about half of intron/exon junctions contained identifiable splice signals (15). This finding suggests that as *ANRIL* gained exons, and exon sequences became more conserved across species, it also increased the number of splice signals and gained functionality. Taken together, data suggest that *ANRIL* may be functional only in simians, and that functionality may have been introduced by transposon activity (15).

ANRIL ISOFORMS AND STRUCTURE

With at least 21 exons (new exons discovered as recently as 2017 (7)), the *ANRIL* gene can potentially generate a large number of splice variants. In fact, many *ANRIL* isoforms have been reported (**Figure 1**) (16). Exon numbering has changed over time as new exons were discovered. Studies observe multiple isoforms in any given cell type, mostly at low abundance. A different range of isoforms may be identified from one cell type to another, but tissue-dependent isoform expression in primary cells or tissues has not yet been comprehensively quantified using the same reagents and techniques. Intriguingly, many studies have now identified both linear and circular *ANRIL* isoforms (6–8). The longest open reading frame identified in any *ANRIL* variant is 86 codons, supporting the concept that functionality of this gene is through RNA activity (16).

Linear and Circular Isoforms

Many conventional linear polyadenylated *ANRIL* isoforms are detected in different cell types. Circular *ANRIL* (circ*ANRIL*) isoforms, without polyadenylation, have also been described. Circular RNAs, which are formed by "back-splicing" in which a downstream splice donor site is joined to an upstream splice

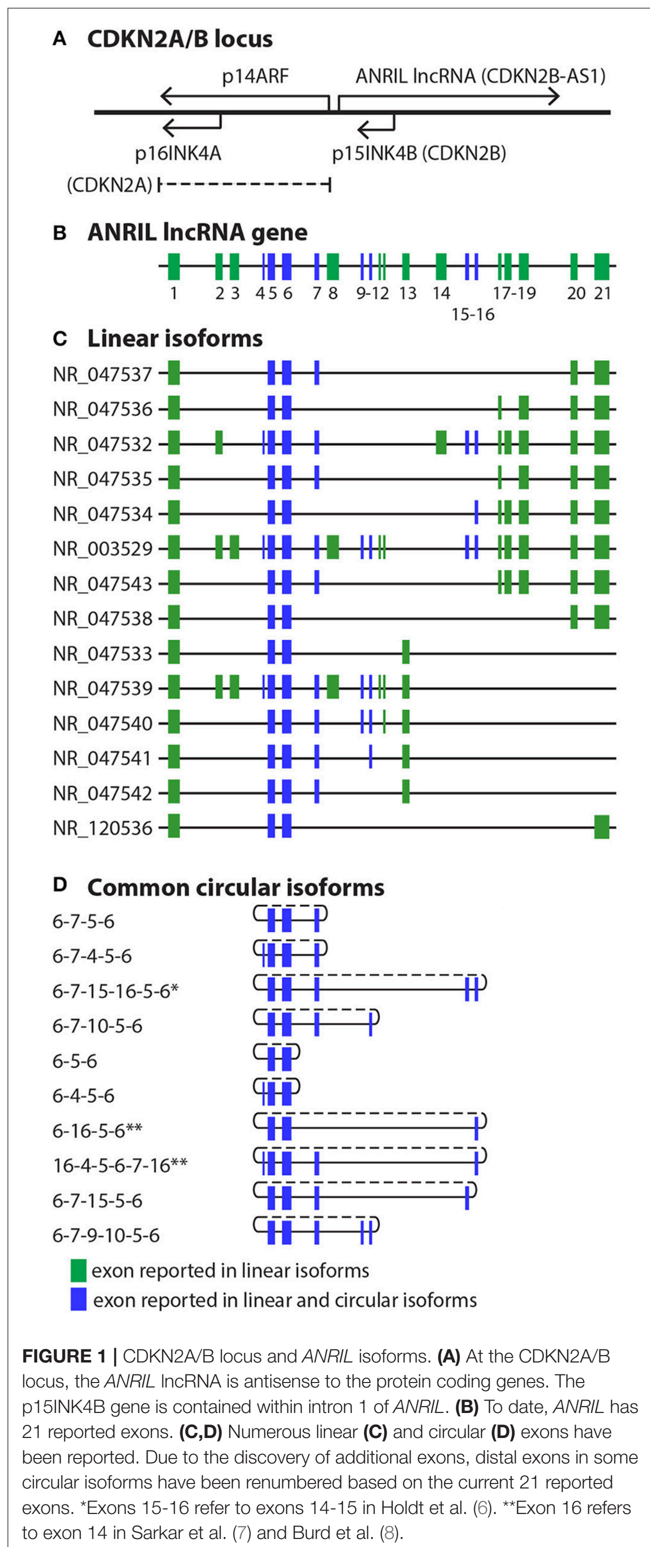

FIGURE 1 | CDKN2A/B locus and *ANRIL* isoforms. **(A)** At the CDKN2A/B locus, the *ANRIL* lncRNA is antisense to the protein coding genes. The p15INK4B gene is contained within intron 1 of *ANRIL*. **(B)** To date, *ANRIL* has 21 reported exons. **(C,D)** Numerous linear **(C)** and circular **(D)** exons have been reported. Due to the discovery of additional exons, distal exons in some circular isoforms have been renumbered based on the current 21 reported exons. *Exons 15-16 refer to exons 14-15 in Holdt et al. (6). **Exon 16 refers to exon 14 in Sarkar et al. (7) and Burd et al. (8).

acceptor site, were discovered in 2012 to be a broadly occurring phenomenon across developmental stages and tissues, arising from at least 14% of human transcribed genes (17, 18). CircRNAs enjoy distinct properties from linear RNAs, including, in general, enhanced stability and longevity, cytoplasmic localization, and lack of translation (although if an IRES is engineered, circRNAs can support translation) (19). Traditional PCR using antisense-oriented primers cannot distinguish between linear and circular isoforms; other methodology, such as PCR using "outward-facing" primers directed away from each other, detection of specific exon-exon junctions, or protection from RNAse R digestion can quantify circular RNAs.

Careful examination of melanoma cell lines showed that the abundance of individual *ANRIL* exons is non-uniform, supporting the presence of different isoforms (7). In both transformed cell lines and in human brain derived cells, abundance of *ANRIL* exons was lower than exons from locus protein-coding genes *CDKN2A* and *CDKN2B* (8). Linear isoforms tend to include proximal exons (1-2), whereas isoforms with only central exons (4-16) are more likely to be circular (8). In melanoma cells, proximal exons (exon 1 and exon 5-6) were more highly expressed than distal exons. This suggests that short isoforms of *ANRIL*, which tend to include proximal exons, are more abundant than longer isoforms in this cell type (7). In human peripheral blood mononuclear cells and a monocyte cell line, four major groups of *ANRIL* transcripts were found, all with common proximal exons including exons 1, 5, and 6 but with different distal exons, of various lengths (8). Multiple circular *ANRIL* isoforms have been detected. A circ*ANRIL* isoform with an exon 14-5 head-to-tail junction was reported to be the predominant form in both an immortalized fibroblast cell line (8) and in a majority of melanoma cell lines (7). Other non-canonical back-spliced junctions observed in melanoma samples included exon 14-5, 7-4, 10-5, and 14-4 (7). The exons most commonly observed in circ*ANRIL* in melanoma cells were 4, 5, 6, 7, 10, 13, and 14; in varied human cell types, the majority of circ*ANRIL* species were exon 5-6-7 containing (6). *ANRIL* exons 1, 2, 3, 8, 9, 11, and 12 were rarely included in circular RNA products (8). In melanoma lines, no correlation was observed between abundance of linear and circ*ANRIL* (7). However, circ*ANRIL* expression was inversely correlated with linear *ANRIL* expression in peripheral blood mononuclear cells in a cardiovascular cohort (6). Circ*ANRIL* was found to be resistant to RNAse R digestion compared with linear *ANRIL*, and an actinomycin D time course confirmed enhanced stability of the 14-5 circ*ANRIL* isoform compared with linear isoforms (7, 8).

Secondary Structure

Structure and function of lncRNAs is of high interest in the scientific community, given the increasing recognition of lncRNA roles in cancer and the normal biology of higher organisms. As such, prediction of lncRNA structures is an important computational challenge. One approach is to identify structural elements through comparison of related lncRNAs. The MONSTER tool was used to compare *ANRIL* to two lncRNAs with similar biological function: *HOTAIR* and *COLDAIR* (20). MONSTER identifies sequence-predicted secondary structure, such as regions likely to be single stranded RNA, double stranded RNA, hairpin loops, interior loops and bulges. Comparing predictions of two lncRNAs with similar functions is proposed as a mechanism to identify structural motifs.

When *HOTAIR*, *COLDAIR,* and *ANRIL* were compared, several common structures were identified, putative structural motifs related to their common function in epigenetic regulation, which could lead to a molecular understanding of mechanism of action in future studies (20). Another study identified the region of ANRIL that interacts with CBX7, a polycomb repressor component; secondary structure analysis revealed hairpin structural motifs with significant binding affinity to CBX7. Fluorescence anisotropy suggested a ternary complex between a particular loop of ANRIL, CBX7 and a H3K27me3 methylated histone peptide (21).

Cellular Localization

RNA localization impacts function. In melanoma cells, linear *ANRIL* species containing proximal (exon 1) and distal (exons 13b, 19) exons were predominantly found in the nucleus. However, middle exons (exons 5, 6, and 7), which are found in both linear and circ*ANRIL*, were observed in cytoplasmic fractions, suggesting that *circANRIL* species may be predominantly cytoplasmic (7). Nuclear localization suggests linear isoforms may be responsible for the known *ANRIL* function of regulating gene transcription via chromatin modulation (see below). Conversely, cytoplasmic localization suggests *circANRIL* forms may participate in post-transcriptional functions. In gastric (22), prostate (21), and urothelial (23) cancer cells, ANRIL was predominantly nuclear. In a beautiful high-resolution analysis of single-molecule lncRNA localization, ANRIL was found to be mostly localized to cell nuclei, in one or several bright foci. Like other lncRNAs analyzed, ANRIL nuclei foci were lost in mitotic cells (24). Physiological stimuli that change ANRIL localization may provide clues as to ANRIL functions. Intriguingly, in a retinal cell line *ANRIL* isoforms were observed by fluorescence in situ hybridization to localize to the peri-nuclear cytoplasmic space. *ANRIL* abundance was induced by glucose, but *ANRIL* localization did not change with high glucose exposure (25). On the other hand, a study in HUVEC cells, using primers predicted to detect both linear and circular isoforms, found *ANRIL* to be mostly nuclear; nuclear *ANRIL* was increased after exposure to TNF-α (26). At least one study has used *ANRIL* as a nuclear positive control to test localization of other transcripts (27). Future cell type specific studies of ANRIL localization under basal, stimulated, and stress conditions may lead to clues as to ANRIL roles in tissue health and disease.

REGULATION OF *ANRIL* ABUNDANCE

Abundance of *ANRIL* species is determined by promoter transcriptional activity, splicing decisions, and RNA stability (**Figure 2**). Like other genes, *ANRIL* promoter activity is influenced by epigenetic control and transcription factor occupancy. Intriguingly, epidemiological findings suggest that epigenetic regulation of *ANRIL*, through promoter methylation, has important long-lasting consequences for tissue function (29–31). As such, *ANRIL* regulation is one mediator of the impact of early life environmental signals on adult human health.

ANRIL Promoter Methylation

Epidemiological and experimental findings demonstrate that methylation of the *ANRIL* promoter region regulates *ANRIL* gene expression and has functional importance. The first exons of *ANRIL* and *p14ARF* are separated by only 300 bp, in head-to-head antisense orientation; the intergenic region between them is a bidirectional promoter (4, 32, 33). *In silico* analysis of ENCODE ChromHMM data (34) revealed that this region is enriched for both promoter and enhancer activity, and DNAse I hypersensitivity, across multiple cell types, suggesting this is a regulatory region. This region is bound by CTCF, usually considered to be a transcriptional repressor, insulating promoters from enhancer activity. Oddly, CTCF binding at the *ANRIL* promoter was associated with active-chromatin mark histone H3K4 trimethylation (35). CTCF binding, and *ANRIL* and p14ARF expression, were inhibited by methylation of local CpG islands and increased by demethylation. Knockdown of CTCF prevented the demethylation-induced expression of *ANRIL* and p14ARF, confirming that CTCF is a methylation-sensitive positive regulator of *ANRIL* promoter activity (35).

Additional evidence supports the functional importance of CpG sites for *ANRIL* promoter activity, locus gene expression and transcription factor binding. Mutagenesis of the CpG sites affects both *ANRIL* and p14ARF promoter activity (29, 30). Methylation status of several CpG differentially methylated regions at *ANRIL* in umbilical cord tissues was positively associated with abundance of linear but not circular *ANRIL*, but inversely associated with p14ARF and p16INK4a expression (29). CpG methylation also affects other transcription factors binding at the *ANRIL* promoter to regulate downstream gene expression, such as interferon gamma, SMAD3/4 and ERα (29, 30). Methylation status of CpG islands around the *p16INK4A* transcription start site was also shown to coordinate transcription of *ANRIL* and p16INK4A in human cells (36). Given the multiple protein complexes binding across different CpG sites, and variable quantitative impact of individual CpG region mutagenesis on *ANRIL* isoforms and locus gene expression, regulation appears to be complex.

Transcription Factors Regulate *ANRIL* Production

ANRIL expression is influenced by cellular processes such as genotoxic stress, tumorigenesis, senescence, and inflammation. Activity at the bidirectional promoter region upstream of both *ANRIL* and *p14ARF* genes is influenced by the critical cell cycle regulator E2F1 (32, 33). In response to the genotoxic stress of DNA damage, E2F1 transcriptionally activates *ANRIL* in an ATM-dependent manner (33, 37). In this case, *ANRIL* is thought to promote cell growth by suppressing locus INK-family inhibitors after DNA repair is complete, allowing re-entry into cell cycling (33). The *ANRIL* promoter was also responsive to E2F1 in cancer cells (32). In addition to E2F1, several known potent oncogenes regulate *ANRIL* expression in various cancers. In lung cancer, c-MYC binds to an E-box in the *ANRIL* promoter and induces *ANRIL* expression (38). In nasopharyngeal carcinoma, transcription factor SOX2 was

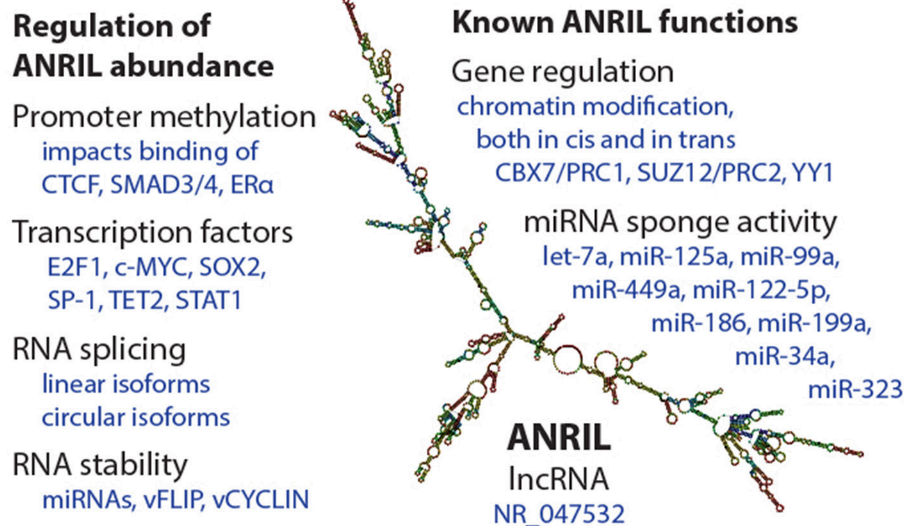

FIGURE 2 | Summary of regulation and functions of the ANRIL lncRNA. **(Left)** Some of the known mechanisms by which ANRIL abundance is regulated, at the transcriptional and post-transcriptional levels. On the **(Right),** a selection of known ANRIL cellular functions are depicted. We apologize for observations not included in this summary image. The ANRIL structural prediction in the center is of a common long-isoform of ANRIL, and was generated from Gruber et al. (28).

shown to bind directly to the *ANRIL* promoter and activate transcription of *ANRIL* and its downstream effector β-catenin (39). In liver cancer, SP1 binds the *ANRIL* promoter and positively regulates *ANRIL* transcription (40). On the other hand, TET2, a tumor suppressor in human gastric cancer, binds to the promoter region of *ANRIL* and regulates expression of *ANRIL* as well as p16INK4a, p15INK4b, and p14ARF (41). Transcription regulation of *ANRIL* is involved not only in cell DNA damage and oncogenesis, but also in disparate processes such as cell senescence and inflammation. In senescence, oncogenic Ras was found to reduce expression of *ANRIL* (13, 21, 42). In inflammation, STAT1 activates the *ANRIL* locus in vascular endothelial cells has been reported; CAD-associated *ANRIL* SNP rs10757278, located in a known downstream enhancer region, disrupts the STAT1 binding site and modulates IFN-γ induced *ANRIL* expression via stimulation (43). Intriguingly, the binding of STAT1 at this enhancer exerts cell-type specific regulation of *ANRIL* expression: repression in lymphoblastoid cells lines, but activation in HUVEC cells (43). In sum, data support an important role for cell-type specific transcriptional regulation of the *ANRIL* lncRNA in a range of cellular processes and outcomes.

Regulation of *ANRIL* Splicing

Cell type dependent variation in abundance of different *ANRIL* isoforms suggests that splicing may be a point of regulation (8, 44). Almost nothing is known about *ANRIL* splicing decisions. Disease-associated *ANRIL* gene polymorphisms have shed light on this process. In lymphocytes, the coronary artery disease (CAD) associated SNP rs10757278 (intron 12) correlates with abundance of certain circular (14-5 and 4-6) and linear (exon 1-2, but not 18-19, containing) isoforms (8). The rs10757278 A allele was found to inhibit skipping of exon 15, promoting circ*ANRIL* species ending in exon 14 (8). Mechanisms regulating *ANRIL* splicing require further study.

Post-Transcriptional Regulation

Determinants of *ANRIL* transcript longevity and stability remain uncertain, but miRNAs can participate. *ANRIL*, downregulated following Kaposi's sarcoma associated herpesvirus (KSHV) infection, contains multiple seed matches for KSHV miRNAs. Forced miRNA expression decreased *ANRIL* abundance, and miRNA pull-down experiments confirmed a direct interaction. In addition, KSHV latency associated proteins vFLIP and vCyclin also decreased *ANRIL* abundance, suggesting post-transcriptional miRNA-dependent and independent regulation (45).

FUNCTIONS OF THE *ANRIL* LNCRNA

Transcription Regulation via Chromatin Modifying Complexes

Many studies show that ANRIL functions in cells to regulate gene expression via chromatin modification. Acting *in cis*, *ANRIL* interacts with both PRC-1 and−2 to mediate epigenetic transcriptional repression of neighboring genes *CDKN2A* and *CDKN2B*, through mechanisms involving histone modification and chromatin remodeling (13, 21, 33). *ANRIL* interacts with PRC1 component CBX7 to recruit PRC1 to the p14ARF and p16INK4A loci, silencing the *CDKN2A* locus by H3K27-trimethylation (21). At *CDKN2B*, *ANRIL* was shown to recruit SUZ12, a subunit of the PRC2 (13). *ANRIL* also interacts with PRC-associated protein YY1 (46). Intriguingly, the structural conformation of the methyl-lysine binding pocket

in the chromodomain of CBX7, which interacts with H3K27-trimethylation to cause chromatin compaction, is influenced by allosteric RNA-protein binding with *ANRIL* (47). However, despite this well-documented repression of other locus genes by *ANRIL*, a positive correlation between *ANRIL* (both short and long isoforms), *CDKN2A* and *CDKN2B* RNA abundance has been frequently reported, suggesting transcriptional co-regulation of these genes predominates in many tissues (8, 10, 14, 16, 44, 48–51).

ANRIL also acts in a PRC1/2 dependent mechanism to repress distant genes *in trans* (32, 46). Trans regulation by *ANRIL* may be dependent on Alu motifs, which are found both in *ANRIL* transcripts and in the promoters of *ANRIL* target genes (46). This mechanism was shown to regulate the CARD8 gene in endothelial cells (52). Polycomb group proteins, which are highly enriched near Alu motifs across the genome, are recruited to target gene promoters upon *ANRIL* over-expression. In support of this concept, silencing *ANRIL* impacts expression of a large number of genes across the genome (14). Separate from chromatin modification, *ANRIL* is reported to regulate Wnt signaling by binding to SOX2, increasing transcriptional activity of the WNT/β-catenin pathway (39).

miRNA Abundance and Activity

ANRIL also influences gene expression via miRNA networks. *ANRIL* regulates miRNAs both at the epigenetic level, through regulation of miRNA transcription, and through direct binding to miRNAs, acting as a miRNA "sponge." In gastric cancer cells, *ANRIL* epigenetically silences miR-99a/miR-449a through a PRC2 mechanism (22). In general, expression of *ANRIL* and its target miRNAs are negatively correlated in tissues and cell lines (22, 53–57). *ANRIL* has been described as having pro-oncogenic effects by sponging miRNAs (see below for more details). On the other hand, circ*ANRIL* containing exons 5-6-7 was found to lack miRNA sponge activity (6). Inhibition of miRNAs can reverse the effects of *ANRIL* knockdown.

Cellular Outcomes of *ANRIL* Activity

ANRIL has broad impacts on cell biology, including influence over proliferation, senescence, apoptosis, extracellular matrix remodeling, and inflammation (14). In cancer, *ANRIL*-miRNA interactions regulate networks of downstream targets of miRNAs, promoting an oncogenic role for *ANRIL* in cell proliferation, metastasis, invasion, radio-resistance, drug-induced cytotoxicity and apoptosis, involving many different signaling pathways (22, 53–57). Specifically, repression of cell cycle inhibitors p14ARF, p15INK4B, and p16INK4A increases proliferation, decreases senescence, and contributes to the DNA damage response (13, 21, 33). PRC-mediated epigenetic repression of Kruppel-like factor 2 (KLF2) influences proliferation and apoptosis (40, 58). Cooperation between *ANRIL* and PRC-associated YY1 increases TNF-alpha dependent inflammatory mediators (IL-6, IL-8) through NF-kB (26). *ANRIL* influences the cellular response to oxidative stress through a miR-125a regulation of MCL-1 (59). Circular *ANRIL* species were found to regulate ribosome biogenesis in vascular smooth muscle cells (6).

THE *ANRIL* GENE IS ASSOCIATED WITH HUMAN DISEASE

A primary driver of interest in *ANRIL* is the large body of genomic data linking the *ANRIL* gene with risk of human disease. Genome-wide association studies (GWAS) have identified many disease-associated SNPs in or near the *ANRIL* gene (60). The *CDKN2A/B* locus is remarkable for the large number of associated diseases, ranging from aging and frailty to cancer to metabolic disease. Perhaps surprisingly given the validated importance of the products of the *CDKN2A* and *CDKN2B* genes in cell biology, in some cases *ANRIL* expression shows stronger phenotype association than protein-coding *CDKN2A/B* locus genes (4, 48), linking *ANRIL* itself to a range of important human diseases.

ANRIL SNPs and Disease Risk

Studies indicate that SNPs in the ANRIL gene can impact ANRIL expression and function. The *CDKN2A/B* locus is associated with risk of cancer, atherosclerotic disease, type 2 diabetes, stroke, aneurysm, periodontitis, Alzheimer's disease, aging, frailty, glaucoma, endometriosis, multiple sclerosis, hypertension (10, 61). Reviewed here are only SNPs within or downstream of the *ANRIL* gene; broader *CDKN2A/B* locus disease associations have been reviewed previously (10, 62). Integrating information from published observations and the NCBI linkage disequilibrium database (63), we find that disease-associated SNPs in the *ANRIL* gene that modulate locus gene expression fall into approximately six groups (**Table 1**) defined loosely by linkage block and reported effects. Exceptions outnumber the rules, however; for nearly all groups there are reports of SNPs with different or even opposite effects. Summarized here is a generalized synopsis of the majority of reports. Group A SNPs, while located in *ANRIL* introns, generally impact *CDKN2A/B* but not *ANRIL* biology (48, 50, 64). All other SNP groups have reported impacts on *ANRIL* itself, but reports often describe conflicting direction of change. Some SNPs are reported to fall in enhancer regions (43, 49, 64) or to impact *ANRIL* splicing (8) or secondary structure (69, 74). The data are incomplete. A particular weakness of the field is that although tissue-specific effects are likely to determine how polymorphisms impact disease risk, in many cases the relevant primary tissue has not been tested.

Disease-Associated SNPs May Influence *ANRIL* Abundance

There is no consistent global pattern with respect to SNP impact on *ANRIL* abundance. For most SNP groups, risk-SNPs are reported that both increase and decrease *ANRIL* levels in different studies. Variability may be related to differences in technique used to detect *ANRIL* that favor one isoform over others, cell type studied, acute and chronic biology and genetic origin of the cellular material studied, and of course the individual biology of each polymorphism. Most *ANRIL* SNPs fall in large linkage blocks, which are variable among different human genetic groups; in many cases the SNP tested may not be the causative SNP in the linkage block, and published linkage blocks may not apply to the material tested if not carefully matched by

TABLE 1 | Disease associated SNPs in/near the *ANRIL* gene that modulate locus gene expression.

SNP group	Location in *ANRIL*	SNPs	Diseases associated	Cell type tested	Impact	References
A	Intron 1	rs2811712 rs598664 rs3218018 rs3218005	Frailty, cancers, diabetes, MI, CAC	Blood, leukocytes	Altered *CDKN2A* and *CDKN2B* expression, but no change in *ANRIL* expression or not reported	(14, 48, 50, 64, 65)
	Intron 2	rs662463				
B	Intron 1	rs3217992 rs3218020	CAD, glaucoma, cancer	Blood	Risk SNPs decrease *ANRIL* expression	(14, 48)
C	Intron 1	rs1063192	CAD, glaucoma, stroke, MI, diabetes, cancers	Blood, lympho-blastoid cells, HUVEC, lymphocytes, islets	Increase/decrease *ANRIL* expression. Possible enhancer. Disrupt miRNA binding site. Reduce beta cell proliferation index	(14, 48, 66–68)
	Exon 2	rs564398				
	Intron 2	rs7865618				
D	Intron 1	rs7044859 rs496892	Cancers, CAD, Stroke, MI, CAC, glaucoma, cancers	Blood, PBMC, lymphoblastoid cells, HUVEC, leukocytes	Exonic SNPs change predicted *ANRIL* free energy calculation, may impact secondary structure. Most intronic SNPs decrease *ANRIL* expression; possible predicted enhancers	(4, 14, 43, 48, 50, 69, 70)
	Exon 2	rs10965215				
	Intron 3	rs2151280				
	Exon 6	rs10738605				
	Intron 6	rs944799				
E	Intron 13	rs10116277 rs6475606 rs10738607 rs10757274	CAD, stroke, intracranial aneurysm, MI, endo-metriosis, hypertension, cancers	Blood, PBMC, PBTL, VSMC, atherosclerotic plaque, primary vascular tissue, lympho-blastoid, HUVEC	Isoform-specific *ANRIL* up/downregulation. Experimentally tested enhancer regions. rs10757278 may impact *ANRIL* splicing, promoting circ*ANRIL* production	(8, 14, 16, 43, 44, 48, 49, 51, 71–73)
	Intron 14	rs10757278				
	Intron 18	rs2383206 rs2383207				
	Intron 19	rs1333045				
	Distal to exon 21	rs10811656 rs1333049				
F	Distal to exon 21	rs2383208 rs10811661	Type 2 diabetes	Blood, islets	Decrease/increase *ANRIL* expression, Predicted enhancer region	(48, 68)

Groups A–F are defined loosely based on linkage disequilibrium (defined as LD>0.8 in Caucasian population in LDHap) and by predicted or tested impact on ANRIL expression or structure. Intron and exon numbers are based on 21 exons.

origin. It is entirely possible that all conflicting results are correct; for example, a CAD risk-SNP could increase pro-proliferative *ANRIL* isoforms in endothelial, macrophage or vascular smooth muscle cells to drive atherosclerosis, whereas a diabetes risk-SNP at the same position could decrease proliferative *ANRIL* isoforms in beta cells to limit beta cell mass. The complexity of the human system necessitates testing the relevant *ANRIL* isoforms in the relevant cell type, preferably in primary cells, in tissue- and disease-specific manner.

A comprehensive review of all SNP effects is beyond the scope of this review. Some *ANRIL* located disease-associated SNPs impact both *ANRIL* expression and *CDKN2A/CDKN2B* expression (14, 48); others impact *ANRIL* but not *CDKN2A* or *CDKN2B* (14, 48, 60), and still others impact *CDKN2A/CDKN2B* but not *ANRIL* (48, 50, 64). Some SNPs are located within predicted or proven enhancer regions (10, 43, 48, 49, 64, 75) or miRNA binding sites (65, 66), providing possible mechanisms of cell type specific gene regulation.

Disease-Associated SNPs May Influence *ANRIL* Structure or Function

Beyond regulation of *ANRIL* transcription, polymorphisms could impact *ANRIL* function by influencing relative abundance of different isoforms through RNA splicing or stability, or through

altering the secondary structure or interactions of any given isoform. Several studies have identified *ANRIL* isoform-specific effects (50, 53, 67, 71, 72); for example, four SNPs forming an atherosclerosis risk haplotype were associated with increased expression of some, but not all, *ANRIL* isoforms (44). SNPs may influence the relative abundance of linear compared to circular isoforms (8). Several SNPs are reported to impact *ANRIL* free energy of folding, resulting in a predicted change in secondary structure, with implications for function and stability (48, 69, 74).

ANRIL IN CANCER

ANRIL was initially identified in a kindred of familial melanoma-neural system tumor with a germ-line deletion of the entire *CDKN2A/B* locus (2). Although the *CDKN2A/B* locus is deleted or silenced in approximately 40% of human cancers, related to the tumor suppressive actions of *CDKN2A* and *CDKN2B* (76), *ANRIL* itself has pro-oncogenic properties. *ANRIL* is implicated in many malignancies, including cancers of the bladder (77), ovary (78, 79), lung (38, 58, 80–82), liver (40, 54, 83), stomach (22), breast (57, 84, 85), esophagus (86), nasopharyngeal cavity (39, 87, 88), thyroid (89), bone (90), cervix (91), colon (92), prostate (21, 56), glioma (55), and others (76). High tissue abundance of *ANRIL* in cancers is associated with aggressive clinicopathologic features such as high histological grade tumor size, advanced tumor-node-metastasis stage, and poor overall survival (22, 38, 40, 58, 78, 79, 83, 87, 89, 91–93). Certain SNPs within the *ANRIL* gene are associated with *ANRIL* and *CDKN2A/B* locus gene expression and clinical parameters (4, 48, 70, 94–96). *ANRIL* may be useful as a prognostic biomarker and a therapeutic target for clinical cancer management.

Molecular Mechanisms of *ANRIL* in Cancer

Accumulating evidence suggests that *ANRIL* participates in tumorigenesis by influencing cell proliferation, apoptosis and metastasis. Depletion or overexpression of *ANRIL* changes expression levels of many genes involved in proliferation, cellular adhesion and apoptosis (14, 32, 46). *ANRIL* overexpression promotes proliferation, migration, invasion, and epithelial-mesenchymal transformation but inhibits cell apoptosis; *ANRIL* loss-of-function represses tumor size and growth rate, cell proliferation, migration, invasion, metastasis, and enhances apoptosis and senescence (22, 38, 55, 56, 58, 77, 80, 81, 84, 89–92). Suppression of *ANRIL* is required for Ras-induced senescence (13, 21, 42). High *ANRIL* levels are associated with resistance to chemotherapy, and *ANRIL* knockdown may promote chemosensitivity (37, 79, 88, 97–99). On the other hand, *ANRIL* mediated anti-oncogenic effects of phospholipase D in lung cancers (82).

ANRIL may promote carcinogenesis through a number of mechanisms. Canonical *ANRIL* transcriptional mechanisms may play a role, such as by *in cis* suppression of the *CDKN2A/CDKN2B* tumor suppressor genes (80, 81, 100), or through PRC-mediated *in trans* gene regulation (40, 58, 80). *ANRIL* miRNA regulation has been implicated in cancers as well, including mechanisms involving let-7a and miR-125a in nasopharyngeal and oral carcinoma (56, 88, 101), miR-99a/miR-449a in gastric cancer (22), miR-122-5p in hepatocellular carcinoma (54), miR-186 in cervical cancer (91), and miR-199a in breast cancer (57), miR-34a in glioma (55), and miR-323 in pediatric medulloblastoma (102). Transcription factors affected by *ANRIL* in cancers include KLF2 (40, 58), SMAD (56, 86, 89) and β-catenin. *ANRIL* interacts with signal transduction pathways in cancers such as PI3K/AKT, p38 MAPK, TGF-β, ATM-E2F1, and MTOR (33, 55, 56, 86, 89, 99, 103). *ANRIL* can also drive cancer progression by increasing glucose uptake for glycolysis (87), through lymphangiogenesis via LYVE-1, VEFG-C, and VEGFR-3 (92), and through invasion and metastasis via MET and MMP3 (78). An intriguing but mostly unexplored phenomenon is breakpoint fusion transcripts including exons from *ANRIL* fused with exons from *MTAP*, a neighboring protein-coding gene, which were identified in 20% of screened melanoma cell lines (104).

ANRIL IN METABOLIC DISEASE

In addition to cancer, genome-wide association studies have repeatedly and confidently identified links between the genomic region containing *ANRIL* and risk of developing cardiometabolic disease, including type 2 diabetes and manifestations of atherosclerosis such as CAD and stroke (10, 62). This locus influences risk not only of classic type 2 (obesity-related) diabetes, but also with related syndromes such as gestational diabetes, transplant-associated diabetes, and cystic fibrosis related diabetes, but not risk of type 1 (autoimmune) diabetes (10). Although diabetes is a clinical risk factor for atherosclerosis, the genetic influence for these conditions at the *ANRIL* locus is mostly non-overlapping, with atherosclerosis SNPs located throughout the *ANRIL* gene, and T2D SNPs located distal to the last *ANRIL* exon (7). One exception is a SNP located in *ANRIL* exon 2, rs564398, which is associated with both T2D and CAD (105). Since *CDKN2A/B* locus genes are known for their roles in cell cycle regulation and cancer, and not metabolism, many questions remain as to how this locus impacts metabolic disease.

ANRIL and Atherosclerotic Disease

Since *ANRIL* locus SNPs influence risk of atherosclerosis, many studies have now tested whether *ANRIL* gene expression is related to atherosclerosis-associated diseases. In subjects with angiographically confirmed CAD in the Leipzig heart study, specific *ANRIL* isoforms were positively correlated with CAD risk SNP haplotype in PBMCs, whole blood, and atherosclerotic plaque tissue (44). In the Framingham heart study, *ANRIL* SNPs were associated with multiple CAD-related outcomes, and showed isoform-specific *ANRIL* correlation in leukocytes, with short isoforms predicted to contribute to CAD pathogenesis (50). CAD risk-SNPs may regulate the relative abundance of linear and circular *ANRIL* isoforms (8). Intriguingly, abundance of *ANRIL* in circulating plasma was positively correlated with in-stent restenosis (53), but in PBMCs harvested at the time of angioplasty/reperfusion, *ANRIL* levels were lower in subjects with myocardial infarction, but higher in subjects with older age, diabetes, hypertension. In this cohort, *ANRIL* levels in PBMCs improved model prediction of subsequent left ventricular dysfunction (106). *ANRIL* promoter methylation may mediate an epigenetic influence on future cardiac risk; higher CpG

methylation at birth was associated with higher pulse wave velocity, a marker for increased arterial stiffness indicating greater cardiovascular risk, at 9 years of age (31).

Mechanisms by which *ANRIL* impacts atherosclerotic disease remain debated. In aortic smooth muscle cells, knockdown of *ANRIL* using siRNA targeting exon 1 or exon 19 revealed altered gene expression networks impacting cell proliferation, apoptosis, extracellular matrix, and inflammation (14). Atherogenic gene expression networks were regulated by *ANRIL* via the Alu mechanism, in which Alu motifs target *ANRIL* to particular gene locations, recruiting PRC complexes and altering gene methylation status (46). *ANRIL* may impact risk of ischemic stroke by regulating the Caspase recruitment domain 8 (CARD8) gene in endothelial cells (52). A known CAD-associated miRNA, miR-92a, may mediate some *ANRIL* effects; *ANRIL* targets GATA2, MAP1B, and ARG1 were found to require miR-92a, placing this miRNA downstream of *ANRIL* for some atherogenic effects (69). Finally, *ANRIL* is related to inflammation: *ANRIL* is increased by pro-inflammatory factors NF-κ B and TNF-α in endothelial cells, and *ANRIL* was found to bind directly to the YY1 transcription factor to mediate TNF-a induction of cytokines IL-6 and IL-8 (26).

ANRIL and Obesity, Bone Mass, and Estrogen Signaling

Although GWAS studies do not suggest a link between *CDKN2A/B* locus SNPs and obesity risk in adult populations, intriguingly, *ANRIL* may be a genomic site of environmental epigenetic influence on obesity. The *ANRIL* promoter contains CpG methylation sites that are differentially regulated across samples. In human tissues taken at birth, lower CpG methylation in infancy predicted higher fat mass at 6 years of age, as well as increased bone size, mineralization and density (29, 30). *ANRIL* promoter methylation was also negatively correlated with BMI in contemporaneous samples of peripheral blood from adolescents and in adipose tissue from adults (29). Methylation of these CpG sites increased tissue abundance of *ANRIL* RNA, in a mechanism that might include increased activity of an estrogen response element. Functional studies in a liposarcoma cell line showed that transcription factor binding to an adjacent ERE was enhanced by methylation, and estradiol increased *ANRIL* expression (29).

ANRIL and Type 2 Diabetes

Multiple SNPs in different linkage blocks at the *CDKN2A/B* locus are associated with T2D risk; evidence in human populations suggests these SNPs impact pancreatic islet mass or function (10). Despite the fact that the T2D risk SNPs are located in or near the *ANRIL* gene, the field has largely assumed the effect was mediated by the protein coding genes at the locus, due to extensive published work implicating p16INK4A in the regulation of beta cell mass (10). However, although studies have found no association between *CDKN2A/B* T2D SNPs and transcript level of *p14ARF*, *p15INK4B,* or *p16INK4A* in human islets (10, 107), an age-dependent positive association was identified between distal T2D risk-SNPs (group F in **Table 1**) and *ANRIL* expression (68). On the other hand, a T2D risk-SNP in *ANRIL* exon 2 (group C in **Table 1**) was associated with reduced *ANRIL* expression, again with no change in *p14ARF*, *p15INK4B,* or *p16INK4A* expression (14, 48); however, these studies were carried out in blood rather than islets. In human islets, this exon 2 SNP was shown to remove a CpG methylation site; risk allele was associated with reduced islet insulin content but no change in locus gene expression (108). Risk allele at this SNP was associated with impaired beta cell proliferation response to high glucose (68). In a study relevant to diabetic retinopathy, high glucose exposure increased *ANRIL* expression in human retinal epithelial cells (25). In *ANRIL* was found to increase expression of VEGF, a critical element of the neovascularization that is central to damage from retinopathy, via a mechanism involving PRC2 and miR200b (25, 109).

SUMMARY

Studies suggest the *ANRIL* lncRNA influences risk of a number of diseases, including many types of cancer as well as metabolic disease. Current understanding of *ANRIL* biology indicates the primary function of this lncRNA is to regulate gene expression, both locally at *CDKN2A/B* as well as across the genome, via mechanisms including chromatin modulation, transcription factor binding, and miRNA regulation. Knowledge concerning ANRIL function in cancers is more solid and advanced than for metabolic tissues. Mechanisms by which SNPs influence ANRIL abundance remain uncertain and require more study; how DNA methylation regulates ANRIL in cancers also will benefit from more study. Much remains to be learned about the structural complexity of *ANRIL*; how the various identified linear and circular isoforms impact tissue biology to modulate disease risk is mostly unknown. There is an urgent need for deeper understanding of how *ANRIL* isoforms modulate cellular function in human organs and tissues, and to explore the differing roles of ANRIL in cancer and metabolic disease. Given the advent of RNA therapeutics, and the broad disease relevance of *ANRIL*, it is possible that these studies may lead to future disease prevention and treatment.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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Critical Enzymatic Functions of FTO in Obesity and Cancer

Xiaolan Deng[1,2], *Rui Su*[1], *Savanna Stanford*[1] *and Jianjun Chen*[1]*

[1] *Department of Systems Biology and The Gehr Family Center for Leukemia Research, Beckman Research Institute of City of Hope, Monrovia, CA, United States,* [2] *School of Pharmacy, China Medical University, Shenyang, China*

Fat mass and obesity-associated protein (FTO) single-nucleotide polymorphisms (SNPs) have been linked to increased body mass and obesity in humans by genome-wide association studies (GWAS) since 2007. Although some recent studies suggest that the obesity-related SNPs in *FTO* influence obesity susceptibility likely through altering the expression of the adjacent genes such as *IRX3* and *RPGRIP1L*, rather than *FTO* itself, a solid link between the SNP risk genotype and the increased FTO expression in both human blood cells and fibroblasts has been reported. Moreover, multiple lines of evidence have demonstrated that FTO does play a critical role in the regulation of fat mass, adipogenesis, and body weight. Epidemiology studies also showed a strong association of FTO SNPs and overweight/obesity with increased risk of various types of cancers. As the first identified messenger RNA N^6-methyladenosine (m^6A) demethylase, FTO has been shown recently to play m^6A-dependent roles in adipogenesis and tumorigenesis (especially in the development of leukemia and glioblastoma). Given the critical roles of FTO in cancers, the development of selective and effective inhibitors targeting FTO holds potential to treat cancers. This mini review discusses the roles and underlying molecular mechanisms of FTO in both obesity and cancers, and also summarizes recent advances in the development of FTO inhibitors.

Keywords: fat mass and obesity-associated protein (FTO), obesity, cancer, mRNA N^6-methyladenosine (m^6A), m^6A demethylase, AML, GBM, FTO inhibitors

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Università degli Studi di Padova, Italy

Correspondence:
Jianjun Chen
jianchen@coh.org

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INTRODUCTION

As the first genome-wide association studies (GWAS)-identified obesity susceptibility gene, the fat mass and obesity-associated gene (FTO) has been well known for the strong association of the multiple single-nucleotide polymorphisms (SNPs) located in its intron 1 with risk of obesity (1–10). Although there are some controversial reports regarding the association between *FTO* SNPs and *FTO* expression (11–13), mouse model studies have shown the pivotal role of FTO in the regulation of fat mass, adipogenesis, and body weight (14–20). The link between the SNP risk genotype and increased *FTO* expression in human fibroblasts and blood cells has also been demonstrated (21–23). Studies have demonstrated that a strong association exists between *FTO* SNPs and/or overweight/obesity with the increased risk of various types of cancers (24–29), implying a role of FTO in the pathogenesis of cancers. Indeed, the oncogenic role of FTO has been reported in leukemia and glioblastoma (GBM), where FTO is highly expressed (30–32). More importantly, FTO was reported as the first N^6-methyladenosine (m^6A) demethylase of eukaryotic messenger RNA (mRNA) (33), and the functions of FTO in adipogenesis and tumorigenesis have been linked to its m^6A demethylase activity (30–32, 34). As the most abundant internal modification in eukaryotic mRNAs, m^6A usually occurs at the consensus motif of

RRm^{6}ACH ([G/A/U][G>A]m^{6}AC[U>A>C]); enriched in 3′ untranslated region (UTR), gene coding regions, and especially near stop codons (35, 36). The m^{6}A modification is deposited by the METTL3-METTL14-WTAP methyltransferase complex (i.e., writer) (37–39) and can be removed by m^{6}A demethylases (i.e., erasers) such as FTO and ALKBH5 (33, 40). The m^{6}A modification functions as a post-transcriptional modulator of gene expression by decreasing or increasing mRNA stability, or promoting mRNA translation efficiency through its recognition of different m^{6}A reader proteins (41–48). The roles of m^{6}A modification and the associated machinery in the pathogenesis of various types of cancers have been reported recently (30–32, 48–59). This review focuses on the functions of FTO in both adipogenesis and tumorigenesis and on the underlying m^{6}A-dependent mechanisms, along with a brief discussion of recent advance in the development of FTO inhibitors and their therapeutic potential to treat cancers.

ASSOCIATION OF FTO WITH OVERWEIGHT/OBESITY AND ITS ROLE IN ADIPOGENESIS

Obesity and overweight populations have become a global crisis, with the numbers increasing every year in adults and children. In 2015, there were 603 million adults and 108 million children who were diagnosed obese in 195 countries, and the population suffering with obesity has increased two-fold in over 70 countries during 25 years (60). Obesity is commonly caused by inherited or behavioral factors (food intake, physical activities, etc.), and it may induce other chronic diseases: diabetes, heart disease, chronic kidney disease, bone disorders, and many types of cancer (10, 26, 60). SNPs of *FTO* in intron 1 was first found to be associated with human obesity in European populations in 2007 (1–3), and subsequently validated by different groups in other populations including Asians (4–6), Africans (7), Hispanics (8), and Native Americans (9, 10), demonstrating a strong association between *FTO* SNPs in intron 1 (rs9939609, rs17817449, rs3751812, rs1421085, rs9930506, and rs7202116) and overweight or obesity (61) (see **Figure 1**). People carrying FTO risk alleles typically have a high body mass index (BMI), which may be due to a higher food intake (62, 63) and diminished food satiety (64), but not related to energy expenditure (62). Meta-analysis studies (65–67) have validated and confirmed that the influence of FTO variants on obesity risk is attenuated through physical activities as well as dietary and drug-based interventions (68, 69), although the underlying mechanism remains elusive. Some recent studies have suggested that the association between FTO SNPs in intron 1 and obesity might be owing to their potential influence on expression of *IRX3*, *IRX5*, and *RPGRIP1L*, rather than on their expression of *FTO* (11–13). However, there is also compelling evidence showing that such *FTO* SNPs are associated with increased expression of FTO (21–23, 70, 71). Moreover, animal model studies have shown that FTO plays a critical role in regulating fat mass, adipogenesis, and total body weight (14–20). For instance, FTO-deficient mice develop postnatal growth retardation and show a reduction in both adipose tissue and lean body mass (14). Conversely, overexpression of FTO in mice develops obesity by increased food intake (15), demonstrating the pivotal role of FTO expression itself in obesity (58). Therefore, there is no doubt that there is still a robust association of the FTO expression level/function with obesity and increased body mass, though the underlying mechanism has yet to be fully elucidated.

The recent discovery of FTO acting as an m^{6}A eraser paved a novel way to reveal the molecular mechanism that links FTO with the increased susceptibility to overweight and obesity. A study in 2013 showed that the *FTO* obesity-risk allele (rs9939609 T/A) is associated with increased FTO expression, reduced m^{6}A ghrelin mRNA methylation, and increased ghrelin expression (22). Ghrelin, the "hunger hormone," is a key mediator of ingestive behavior, and its increased expression results in increased food intake and a preference for energy-dense foods, tending to lead to overweight and obesity (22, 72). A later study also reported that the *FTO* genotype (the AA (risk) genotype at the rs9939609 locus of *FTO*) impacts food intake and corticolimbic activation (73).

Excessive accumulation of adipose tissue under obese condition is a main mechanism for storage of excess energy (61). It has been reported that a positive correlation exists between the FTO level in subcutaneous adipose tissue and BMI, with a higher *FTO* mRNA level in adipose tissue from obese individuals than that in control populations (61, 74, 75). Zhao et al. demonstrated that FTO-mediated m^{6}A demethylation regulates mRNA splicing and plays a critical role in the regulation of adipogenesis (34). They showed that *FTO* expression is inversely correlated with the m^{6}A level during adipogenesis, and *FTO* depletion blocks differentiation and wild-type FTO (but not FTO mutant) restores adipogenesis; mechanistically, FTO mediates differentiation through the regulation of m^{6}A levels around splice sites, thereby controlling the exonic splicing of the adipogenic regulator factor RUNX1T1 (34, 76). Similarly, another study also revealed that the demethylase activity of FTO is functionally required for pre-adipocyte (3T3-L1) differentiation (77). Furthermore, Merkestein et al. showed FTO regulates adipocyte differentiation *in vivo*, and further revealed that FTO enhances adipocyte numbers during mitotic clonal expansion at an early stage of adipogenesis (19). The compelling evidence of these studies supports FTO-mediated m^{6}A demethylation playing a pivotal role on adipogenesis regulatory.

ASSOCIATION OF FTO WITH CANCERS AND ITS ONCOGENIC ROLE IN BOTH TUMORIGENESIS AND DRUG RESPONSE

Epidemiology studies show that FTO SNPs (including rs9939609, rs17817449, rs8050136, rs1477196, rs6499640, rs16953002,

Abbreviations: FTO, the fat mass and obesity-associated protein; SNP, single-nucleotide polymorphism; GWAS, genome-wide association study; mRNA, messenger RNA; m^{6}A, N^6-methyladenosine; GBM, glioblastoma; UTR, untranslated region; BMI, body mass index; CSCC, cervical squamous cell carcinoma; AML, acute myeloid leukemia; R-2HG, R-2-hydroxyglutarate; GSCs, glioblastoma stem(-like) cells; ATRA, all-trans-retinoic acid; AZA, azacitidine; αKG, α-ketoglutarate; MA, meclofenamic acid.

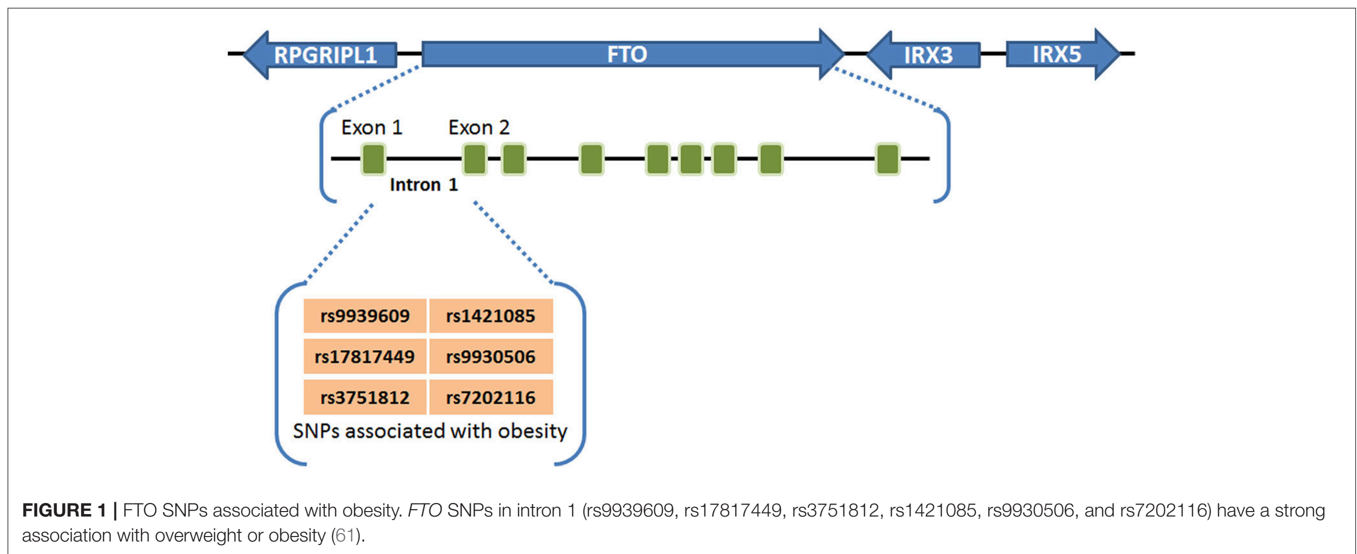

FIGURE 1 | FTO SNPs associated with obesity. *FTO* SNPs in intron 1 (rs9939609, rs17817449, rs3751812, rs1421085, rs9930506, and rs7202116) have a strong association with overweight or obesity (61).

rs11075995, and rs1121980) and overweight/obesity are strongly associated with an increased risk of various types of cancers, including breast cancer, prostate cancer, kidney cancer, endometrial cancer, pancreatic cancers, lymphoma, and leukemia (24–29). For instance, several SNPs of intron 1 of FTO (including rs7206790, rs8047395, rs9939609, and rs1477196) are all significantly associated with breast cancer risk, and rs1477196 shows the strongest association (29). Notably, SNPs outside of intron 1 of FTO could also be associated with cancer risk. For example, rs16953002 of intron 8 of FTO has been identified to be significantly associated with melanoma risk (28). It is possible that the obesity-associated SNPs lead to increased expression of FTO, which in turn contributes (at least to some extent) to an increased susceptibility to overweight and obese, as well as an increased risk of cancer development (30). Indeed, several recent studies have suggested that FTO plays an oncogenic role in various types of cancers such as leukemia, brain tumor, breast cancer, gastric cancer, endometrial carcinoma, and cervical squamous cell carcinoma (CSCC) where it is overexpressed (30–32, 78–82). Li et al. provided the first *in vivo* animal model study demonstrating a critical oncogenic role of FTO in cancer (30). They reported that FTO is highly expressed in certain subtypes of acute myeloid leukemias (AMLs) such as those carrying t(11q23)/*MLL*-rearrangements, t(15;17)/*PML-RARA*, *FLT3*-ITD, and/or *NPM1* mutation (30). They further showed that forced expression of FTO significantly promoted human AML cell survival and proliferation and inhibited human AML cell differentiation and apoptosis, and forced expression of FTO significantly promoted leukemogenesis in mice (30). The opposite was true when endogenous expression of FTO was depleted (30). Subsequently, Su et al. reported that by the inhibition of FTO's oncogenic role, R-2-hydroxyglutarate (R-2HG), a previously well-recognized oncometabolite (83–90), actually exhibits a broad and intrinsic antitumor activity in AML and GBM (31). Cui et al. reported that targeting glioblastoma stem(-like) cells (GSCs) with a FTO inhibitor in mice could significantly inhibit the development of GSC-initiated tumor *in vivo* (32). It was also reported that the depletion of *FTO* expression significantly inhibited cell proliferation, migration, and invasion of human gastric cancer cell lines, and the opposite phenomenon was observed when FTO was forced expressed (80).

FTO has also been reported to affect the response of cancer cells to drug treatment. Li et al. showed that a knockdown of FTO could significantly enhance the response of human AML cells to all-trans retinoic acid (ATRA) treatment and promote ATRA-induced AML cell differentiation (30). Su et al. reported that analogous to FTO depletion, R-2HG treatment also sensitized human AML cells to standard chemotherapeutic agents such as ATRA, azacitidine (AZA), Decitabine, and Daunorubicin *in vitro* (31). They further showed that R-2HG treatment also sensitized human AML cells to Decitabine and Daunorubicin *in vivo* in immunodeficient xenotransplantation recipient mice (31). Similarly, Zhou et al. reported that FTO enhanced the resistance of CSCC cells to chemo-radiotherapy (82). Consistent with the function of FTO in drug resistance, it was reported that overexpression of FTO is a marker for poor prognosis in cancers such as gastric cancer and endometrial carcinoma (80, 81).

Mechanistically, the roles of FTO in tumorigenesis and drug response have been linked to its m^6A demethylase activity. Li et al. reported that FTO negatively regulates expression of a set of tumor suppressor target genes, such as *ASB2* and *RARA* [two genes implicated in leukemia cell proliferation and drug response (91–93)], through post-transcriptionally modulating m^6A abundance of the target mRNA transcripts and thereby affecting their stability (30). Su et al. further reported that FTO also positively regulates expression of a set of oncogenic targets such as *MYC* and *CEBPA* through an m^6A-dependent mechanism (31). The suppression effect of the FTO inhibitor on GSC growth/proliferation and survival is also believed to be owing to the inhibition of the m^6A demethylase activity of FTO (32). In CSCC, FTO has been

reported to enhance chemo-radiotherapy both *in vitro* and *in vivo* through positively regulating expression of β-catenin (CTNNB1) via an m^6A-dependent mechanism (82). Collectively, evidence is emerging that FTO plays critical oncogenic roles in various types of cancers as an m^6A demethylase, and post-transcriptionally regulates expression of a number of functionally important target genes through m^6A-dependent mechanisms.

IDENTIFICATION OF SMALL MOLECULE INHIBITORS TARGETING FTO

Since the discovery of FTO as an m^6A demethylase in 2011 (33), efforts have been made to identify selective small-molecule inhibitors targeting FTO's m^6A demethylase activity (94–98). FTO belongs to the AlkB family, and the crystal structure of FTO resolved in 2010 (99) shows a strong Fe (II) and α-ketoglutarate (αKG) dependent activity as a dioxygenase, at N-terminals. Chen et al. reported in 2012 that rhein, a natural product, competitively binds to an FTO active site, and exerts an inhibitory activity on FTO-dependent m^6A demethylation in cells, through directly disrupting the bindings between FTO and the m^6A substrate (94). In 2014, Zheng et al. developed a selective FTO inhibitor that also selectively inhibits the m^6A demethylase activity of FTO and increases the m^6A levels in cells (95); a later study showed that this FTO inhibitor (i.e., MO-I-500) could significantly inhibit the survival and/or colony formation of human SUM149 cells, a triple-negative inflammatory breast cancer cell line (97). Meclofenamic acid (MA), a nonsteroidal anti-inflammatory drug, was discovered to specifically inhibit FTO's m^6A demethylase activity, while paring ALKBH5 (96). MA has been further proved to effectively inhibit the survival and growth of GBM cells through suppression of the m^6A demethylase activity of FTO (32). In addition, Compound 12 has been developed based on a α-KG tethering strategy, which could selectively inhibit FTO over other AlkB subfamilies (including ALKBH5) and α-KG oxygenases (98). Su et al. showed that R-2HG is also an inhibitor of FTO that binds direct to FTO protein and significantly inhibits the m^6A demethylase activity of FTO in a dose-dependent manner, leading to a significant increase of global m^6A abundance in R-2HG-treated sensitive leukemia cells (31).

DISCUSSION AND CONCLUSIONS

A growing body of evidence suggests that FTO plays critical roles in both overweight/obesity and cancers. As the first m^6A demethylase identified, FTO has been shown to regulate expression of a number of important target genes through post-transcriptionally reducing their m^6A levels and thereby affecting the stability and/or splicing of target mRNAs, in turn leading to promoting adipogenesis, tumorigenesis, and drug resistance of cancer cells. Therefore, although FTO may regulate expression of distinct sets of target mRNAs in different cell types, it affects overweight/obesity and cancers likely through similar, m^6A demethylase activity-dependent mechanisms (see **Figure 2**). The strong association between FTO SNPs or overweight/obesity with an increased risk of cancers suggests that the obesity-associated function of FTO in metabolism may also contribute to its effects in cancers (**Figure 2**). Indeed, the FTO gene variant related to cancer risk is unlikely independent of adiposity (100). In addition, it was reported that by targeting the PI3K/AKT signaling, FTO influences breast cancer cell energy metabolism including lactic acid, ATP, pyruvate kinase activity, and hexokinase activity (79).

Given the essential role of FTO in cancer development and drug resistance, targeting FTO holds therapeutic potential in treating cancers in which FTO is overexpressed. Thus far, FTO inhibitors have been tested *in vitro* and *in vivo*, and show potent antitumor effects in treating both GBM and breast cancer (32, 97). Similarly, Su et al. showed that by targeting

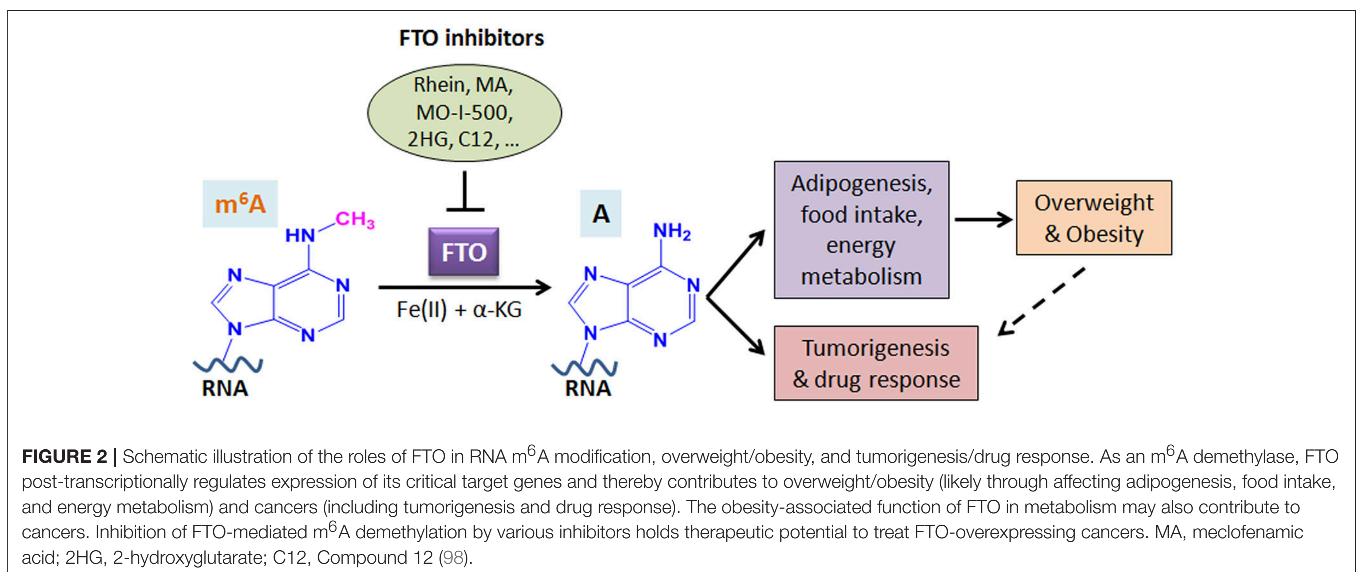

FIGURE 2 | Schematic illustration of the roles of FTO in RNA m^6A modification, overweight/obesity, and tumorigenesis/drug response. As an m^6A demethylase, FTO post-transcriptionally regulates expression of its critical target genes and thereby contributes to overweight/obesity (likely through affecting adipogenesis, food intake, and energy metabolism) and cancers (including tumorigenesis and drug response). The obesity-associated function of FTO in metabolism may also contribute to cancers. Inhibition of FTO-mediated m^6A demethylation by various inhibitors holds therapeutic potential to treat FTO-overexpressing cancers. MA, meclofenamic acid; 2HG, 2-hydroxyglutarate; C12, Compound 12 (98).

FTO directly, R-2HG exhibits a strong antitumor effect in both leukemia and GBM, especially when in combination with standard chemotherapeutic agents (31). These studies provide proof-of-concept evidence demonstrating that FTO is a realistic druggable target in treating cancers. In the near future, when more effective and selective inhibitors of FTO are developed, they could be applied, especially in combination with other therapeutic agents, into the clinic to treat various types of cancers. On the other hand, although FTO also plays a role in obesity, it was argued that FTO might not be a good pharmaceutical target to treat obesity, because the factors leading to obesity might be more complex (101, 102). Thus, a deeper understanding of the factors contributing to obesity could lead to the development of therapeutics targeting obesity.

AUTHOR CONTRIBUTIONS

XD and JC drafted and revised the manuscript, while RS and SS contributed to the revision of the manuscript.

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Conflict of Interest Statement: A patent has been filed by JC and RS based on their work on R-2HG/FTO.

The remaining 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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Heparan Sulfate and Heparan Sulfate Proteoglycans in Cancer Initiation and Progression

Arvindhan Nagarajan[1], *Parmanand Malvi*[1] *and Narendra Wajapeyee*[1,2]*

[1] Department of Pathology, Yale University School of Medicine, New Haven, CT, United States, [2] Yale Cancer Center, Yale University School of Medicine, New Haven, CT, United States

Heparan sulfate (HS) are complex unbranched carbohydrate chains that are heavily modified by sulfate and exist either conjugated to proteins or as free, unconjugated chains. Proteins with covalently bound Heparan sulfate chains are termed Heparan Sulfate Proteoglycans (HSPGs). Both HS and HSPGs bind to various growth factors and act as co-receptors for different cell surface receptors. They also modulate the dynamics and kinetics of various ligand-receptor interactions, which in turn can influence the duration and potency of the signaling. HS and HSPGs have also been shown to exert a structural role as a component of the extracellular matrix, thereby altering processes such as cell adhesion, immune cell infiltration and angiogenesis. Previous studies have shown that HS are deregulated in a variety of solid tumors and hematological malignancies and regulate key aspects of cancer initiation and progression. HS deregulation in cancer can occur as a result of changes in the level of HSPGs or due to changes in the levels of HS biosynthesis and remodeling enzymes. Here, we describe the major cell-autonomous (proliferation, apoptosis/senescence and differentiation) and cell-non-autonomous (angiogenesis, immune evasion, and matrix remodeling) roles of HS and HSPGs in cancer. Finally, we discuss therapeutic opportunities for targeting deregulated HS biosynthesis and HSPGs as a strategy for cancer treatment.

Keywords: heparan sulfate, heparan sulfate proteoglycans, cancer, immune evasion, signaling

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Che-Pei Kung,
Washington University in St. Louis,
United States

Reviewed by:
Dragana Nikitovic,
University of Crete, Greece
Sanjeev Das,
National Institute of Immunology (NII),
India

Correspondence:
Narendra Wajapeyee
narendra.wajapeyee@yale.edu

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INTRODUCTION

Normal cells acquire series of genetic and epigenetic aberrations to become cancerous. The acquired cancer growth and progression enabling attributes are collectively referred to as hallmarks of cancer (1). Several hallmarks of cancer, such as sustained growth signaling, suppression of apoptosis, deregulated metabolism, immune evasion and angiogenesis can also be enhanced through pathological alterations of normal physiological processes (1).

Heparan sulfates (HS) are unbranched chains of disaccharide repeats that are heavily sulfated at various positions on their sugar residues (2, 3). HS can occur either conjugated to amino acids, creating heparan sulfate proteoglycans (HSPGs), or as unconjugated chains (4). Both HS and HSPGs play important roles in cancer initiation and progression. Previous studies have implicated

the role of HS and HSPGs in several types of solid tumors as well as hematological malignancies (5–11).

HSPGs are complex biopolymers whose synthesis is orchestrated by many enzymes, which catalyze the various steps of HS synthesis with very little redundancy (**Figure 1**). The majority of HS deregulation in cancer occurs due to alterations in the expression of HS-synthesizing and HS-modifying enzymes, however, alterations in HSPGs can also contribute to HS deregulation (12) (also see **Table 1**) (10, 11, 13–84).

In this review, we provide an overview of the cell-autonomous and cell-non-autonomous roles of HS and HSPGs in cancer initiation and progression. In addition, we will also discuss opportunities to develop cancer therapies by targeting the HS and HSPG axis.

CELL-AUTONOMOUS ROLE OF HS AND HSPGs IN CANCER INITIATION AND PROGRESSION

HS and HSPGs regulate diverse cell-autonomous functions, including oncogenic signaling, apoptosis, and cellular differentiation. In this section, we describe the cell-autonomous functions of HS and HSPGs in cancer initiation and progression.

Growth Factor Signaling and Regulation of Proliferation

Previous studies have shown important roles of HS and HSPGs in oncogenic signaling (85–88). In this regard, FGF binding interactions are best characterized by the role of HS in altered Receptor Tyrosine Kinase (RTK) signaling. For example, HS-modified HSPGs bind FGF ligands and receptors to form a ternary complex and enhance signaling by promoting FGF receptor (FGFR) dimerization (89–91). This in turn results in receptor activation and enhanced FGFR signaling, which consequentially promotes tumor growth (89–91). In addition to FGF, HS binds to several different mitogenic growth factors such as PDGF, Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF), and Hepatocyte Growth Factor (HGF) and modulates their signaling in a context dependent manner (86).

Breast cancer cells are also shown to overexpress HSPGs, such as Glypican 1 (GPC1) and Syndecan 1 (SDC1), which enhance the proliferative response after treatment with various growth factors due to prolonged signaling (86). Similar to breast cancer, GPC1 also has been shown to have growth-promoting effects in pancreatic cancer and gliomas (49, 92, 93). Collectively, these studies highlight wide-spread deregulation of HSPGs in different cancers that exert tumor promoting roles.

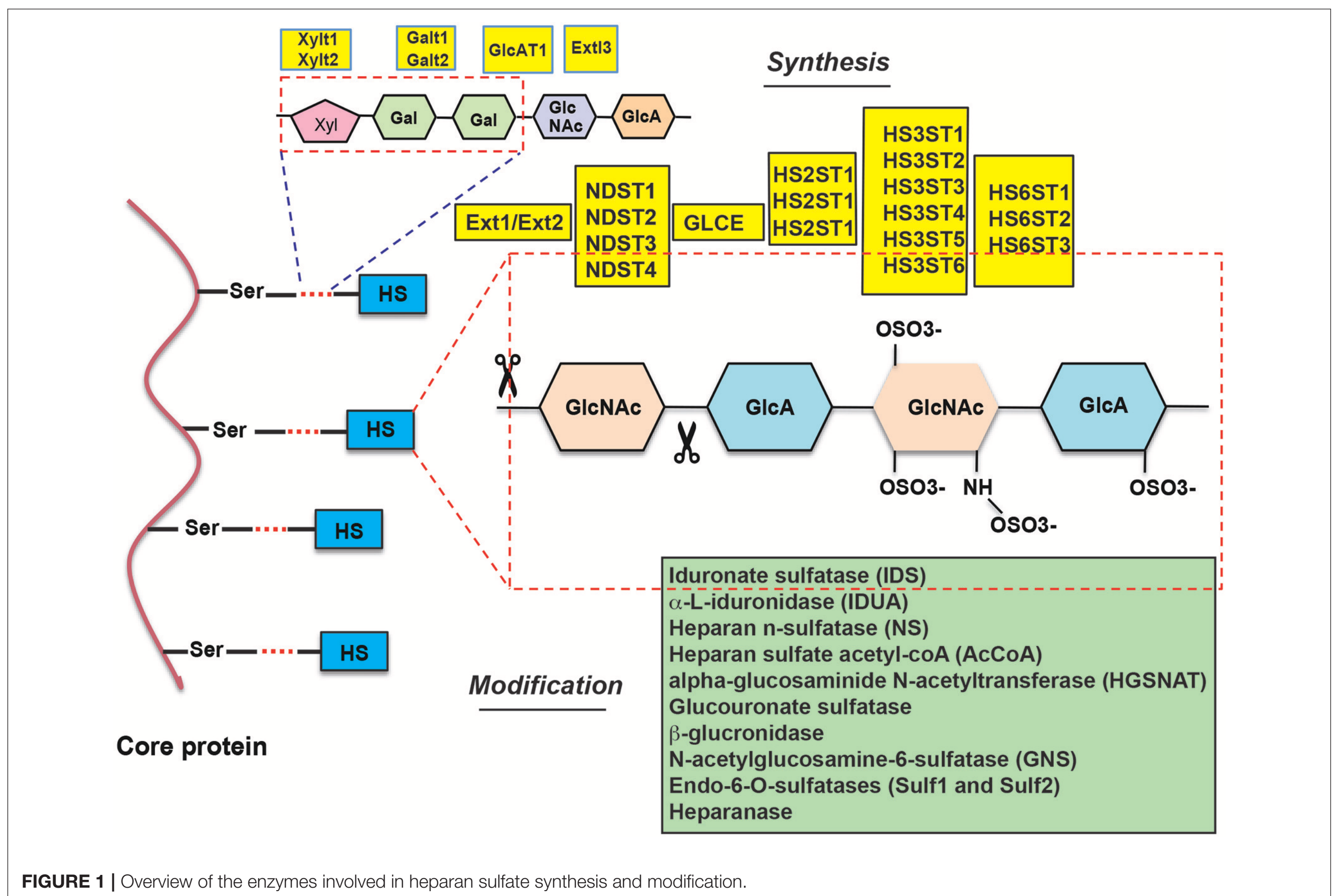

FIGURE 1 | Overview of the enzymes involved in heparan sulfate synthesis and modification.

TABLE 1 | Deregulation of HS and HSPGs and enzymes involved in HSPG metabolism in cancer.

	Alteration in cancer	Functional consequence(s)	Cancer type(s)
Enzyme involved in HSPG metabolism			
HS2ST1	Upregulated	Promote cell proliferation, invasion and growth factor signaling	Prostate cancer (13)
HS3ST2	Epigenetic silencing	Suppression of tumor growth and invasion	Lung cancer (14)
HS3ST2	Upregulated	Invasion and migration	Breast cancer (15)
HS3ST3B1 and HS3ST4	Upregulated	Promote cell proliferation, invasiveness, and tumor angiogenesis	acute myeloid leukemia (16) Colorectal cancer (17) Pancreatic cancer (18)
HS6ST1 and HS6ST2	Upregulated	Increased tumor Angiogenesis	Ovarian cancer (19)
HS6ST2	Upregulated	Poor survival of patients	Colorectal cancer (20)
HS6ST2	Upregulated	Bone metastasis	Breast cancer (21)
HPSE	Upregulated	Tumor metastasis and angiogenesis	Neuroblastoma (22), breast cancer (23), prostate cancer (24), colon cancer (25), lung cancer (26), liver cancer (27), ovarian cancer (28), and pancreatic cancer (29), human myeloma (30)
NDST1 and NDST2	Upregulated	Tumor progression	Hepatocellular carcinoma (31)
SULF1	Downregulated	Suppress tumor cell proliferation and invasion	Breast cancer, Pancreatic, Ovarian and head and neck cancers (32) Hepatocellular carcinoma (33)
SULF2	Unaltered	Tumor progression	Hepatocellular carcinoma and glioblastoma (34)
SULF2	Upregulated	Tumor growth	Hepatocellular carcinoma (33, 35, 36)
HSPG			
Agrin	Elevated	Angiogenesis	Hepatocellular carcinoma (37, 38), glioblastoma (39), cholangiocarcinoma (37)
CD44	Elevated	Adhesion, invasion, cancer stem cell	Breast cancer (40), colorectal cancer (41), oral squamous cell carcinoma (42), melanoma (43) Neuroblastoma (44)
Collagen XVIII	Reduced	Angiogenesis	Cutaneous squamous cell carcinoma (45, 46)
GPC1	Elevated	Proliferation	Breast cancer (47), pancreatic ductal adenocarcinoma (48), glioma (49)
GPC3	Elevated	Proliferation	Hepatocellular carcinoma (50), follicular thyroid cancer (51), testicular germ cell tumor, neuroblastoma (52), Wilms' tumor (53), yolk sac tumor (54), lung squamous cell carcinoma (55), hepatoblastoma (56)
GPC5	Elevated	Proliferation, invasion	Rhabdomyosarcoma (10), non-small cell lung cancer (57)
	Reduced	Initiation	Non-small cell lung cancer (58)
Perlecan	Elevated	Proliferation, angiogenesis	Prostate cancer (59), hepatoblastoma (60), pancreatic ductal adenocarcinoma (61), melanoma (62)
SDC1	Elevated	Proliferation	Breast cancer (63), pancreatic ductal adenocarcinoma (64), ovarian cancer (65), multiple myelom (66)
SDC2	Elevated	Adhesion, proliferation	Breast cancer (67), prostate cancer (68), colorectal cancer (69), bladder cancer (70), glioma (71), sarcoma (72)
SDC3	Elevated	Perineural invasion and poor prognosis	Pancreatic ductal adenocarcinoma (73)
SDC4	Reduced	Differentiation	Neuroblastoma (11)
TbRIII	Elevated	Migration, proliferation	Colon cancer (74), non-Hodgkin's lymphoma (75),
	Reduced	Invasion, proliferation, differentiation, immune response	Breast cancer (76), prostate cancer (77), ovarian cancer (78), multiple myeloma (79), neuroblastoma (11), non-small cell lung cancer (80), pancreatic ductal adenocarcinoma (81), endometrial cancer, renal cell carcinoma (82), melanoma (83)

Additionally, HSPGs also influence cell-matrix interactions by binding matrix proteins such as fibronectin, laminin, thrombospondin, and collagen (89, 94). For example, SDC2 has been shown to be overexpressed in colon cancer cell lines and inhibition of SDC2 in these cells results in cell cycle arrest (69). Similarly, RKIP and HMGB2-dependent breast cancer survival and metastasis was shown to be regulated in SDC2 dependent manner (67). However, it is important to note that in addition to the pro-tumorigenic effects, some HSPGs, such as SDC2, exert tumor suppressive effects depending on the cancer type (95, 96).

Interestingly, HSPGs on the cell surface can also shed, generating soluble proteins that influence cellular proliferation by accumulating in intercellular spaces and sequestering growth factors (89). HSPGs are also often expressed in the tumor stroma and affect several cancer cell growth-enabling features (89). For example, stromal SDC1 that is released into the tumor microenvironment promotes breast carcinoma growth by enhancing FGF2 signaling (97). Interestingly, SDC1 shedding into the stroma is enhanced by heparanase expression, in part through removal and reduction of heparan sulfate chains (30). Thus, various components of the HS signaling pathway coordinate to promote carcinogenesis.

HSPGs secreted into the stroma can also inhibit cancer cell proliferation. For example, increased FGF2 signaling due to soluble HSPGs suppresses neuroblastoma proliferation (11, 98). Specifically, it has been shown that growing neuroblastoma cells with soluble HSPGs promote its differentiation by enhancing both basal and FGF1 mediated phosphorylation of ERK1/2 and expression of transcription factor ID1 (11). Another study has shown that the HSPG, type III TGF-β receptor (TGFBR3) acts as a co-receptor in FGF2 mediated neuroblastome differentiation (98). Similarly, SDC1 that is expressed in multiple myeloma has been shown to activate WNT signaling by two mechanisms (99). First, Wnts bind to the SDC1 HS side chains and activates WNT pathway in a paracrine manner via Frizzled. Second, SDC1 binds to R-spondins produced in osteoblast and stabilizes Frizzled in a LGR4-dependent manner (99). In other instances, soluble HSPGs sequester growth factors, reducing certain pro-proliferative signals. For example, GPC3 promotes hepatocellular carcinoma growth by activating WNT signaling (100). However, contrary to this, soluble GPC3 has been shown to block hepatocellular carcinoma growth by blocking WNT signaling and MAP kinase and AKT pathways (101). Taken together, these studies underpin that HS and HSPGs can exert diverse cancer promoting or inhibitory functions depending upon the context.

Apoptosis and Cellular Senescence Regulation

HS and HSPGs can also play important role in the regulation of apoptosis and cellular senescence. For example, the upregulation of the RTK signaling pathway by HSPGs induces an anti-apoptotic effect through upregulating phosphatidylinositol 3-kinase (PI3K)- and Mitogen-Activated Protein Kinase (MAPK)-mediated survival pathways (102). Additionally, HS and chondroitin sulfate directly inhibit H_2O_2-induced apoptosis by blocking cytochrome c release and caspase-3 and -9 activation (103). Death receptor-mediated apoptosis pathway, which is mediated through the cell surface receptors for Fas ligand (FasL) and Tumor Necrosis Factor-related Apoptosis-Inducing Ligand (TRAIL) can also be regulated by HSPGs. For example, SDC1 suppresses TRAIL-mediated apoptosis in multiple myeloma cells (104). The same study also reported that SDC1 knockdown in lymphoma cells protected them against FasL-mediated apoptosis. In addition to the regulation of apoptosis, a recent study also revealed that heparan sulfation is essential for preventing senescence (105). This study revealed that the depletion of 3'-phosphoadenosine 5′-phosphosulfate synthetase 2 (PAPSS2), an enzyme that synthesizes the sulfur donor PAPS, and the small molecule inhibitor-mediated repression of HS sulfation led to premature cell senescence (105). Collectively, these studies further demonstrate the importance of HS and HSPGs in the regulation of cancer growth relevant cellular processes, such as apoptosis and senescence.

Cellular Differentiation Regulation

HS, HSPGs, and HS modifiers have also been shown to determine the cellular differentiation state. In this regard, the role of HS modifiers in regulating epithelial-to-mesenchymal transition (EMT) is noteworthy. EMT plays an important role in metastatic progression and drug resistance (106). Cells overexpressing the HS modifier sulfatase 2 (SULF2) present with reduced levels of the trisulfated disaccharide UA(2S)-GlcNS(6S). This reduction is followed by an increase in EMT markers and WNT signaling (107). Tumor cell-mediated tumor stroma modulation can also suppress differentiation and increase proliferation. The expression of several HSPGs is low in neuroblasts and high in the Schwannian stroma, and neuroblastomas with a high TβRIII, GPC1, and SDC3 expression have improved prognosis (11). The same study also found that soluble HSPGs and heparin promoted differentiation and decreased proliferation through FGFR1 and ERK phosphorylation. Similarly, another study has shown that neuroblastoma differentiation is promoted by release of a GPI-anchored HSPG, Glypican-6 (GPC6) through via Glycerophosphodiesterase (GDE2). This study also found that high GDE2 or low GPC6 level in neuroblastoma predicted significantly increased patient survival (108). These studies are of high significance as they make two major points; first, that the differentiation state of the cancer cells predict survival, and second, that HS and HSPGs are among the key regulators of cancer differentiation states.

CELL-NON-AUTONOMOUS ROLES OF HS SIGNALING IN CANCER

Several features of cancer such as sustained angiogenesis, tissue invasion and migration and immune evasion require a complex interplay between more than one cell type and involve multiple organ systems. In this section, we describe the cell-non-autonomous functions of HS and HSPGs in cancer initiation and progression.

Role in Angiogenesis

Angiogenesis is considered a key requirement for cancer growth and progression (109). This is highlighted by the fact that several angiogenesis inhibitors are in clinical trials for cancer treatment (110). HS and HSPGs modify angiogenesis due to their effect on angiogenic factors, such as FGF, PDGF, and VEGF. For exmaple, SDC1 binds to VEGF, and SDC1 shedding increases the VEGF concentration in the matrix and promotes angiogenesis in myeloma (111). The same study also showed that heparanase expression increases SDC1 shedding (112). SDC1 is overexpressed in endothelial cells derived from patients with multiple myeloma. In addition to suppressing cell proliferation, RNAi silencing of SDC1 in patient-derived endothelial cells reduces capillary-like structure organization, which is correlated with reduced VEGF receptor (VEGFR)-2 surface expression (111). Other members of the syndecan family, such as SDC2 and SDC3, also affect tumor angiogenesis (113, 114).

Another HSPG with an opposing effect on angiogenesis is Perlecan. Perlecan is a secreted HSPG which is also found on cancer cell surface and in cancer microenvironment (115). Perlecan is shown to promotes angiogenesis in its intact form (115). However, Perlecan can also be partially cleaved by proteases, which results in a C-terminal fragment, called endorepellin, which has been shown to exert anti-angiogenic effects (116). Thus, HSPGs modulate tumor angiogenesis in multiple ways: they increase the tumor microenvironment VEGF concentration, affect VEGFR surface localization, and fine-tune interaction of VEGF with its receptor and co-receptor.

Role in Immune Evasion

Immune response is the first line of systemic defense against tumorigenesis (117). Recent success of immunotherapeutic approaches to treat cancer further highlights the importance of immune evasion mechanisms for cancer initiation and progression (118, 119). HSPGs can serve as cancer biomarkers, which can also be used to target antibodies for immunotherapies (120, 121). At the same time, evidence suggests that HSPGs in the extracellular matrix (ECM) or those expressed on bystander cells are involved in reducing immune signaling to dendritic cells (DCs) (122). One of the well-studied HSPGs roles in melanoma immunity involves myeloid-derived suppressor cells (MDSCs) that suppresses immunity against melanoma (122). Previous studies have shown that melanoma immune evasion involves myeloid-derived suppressor cells (MDSCs) that express an immune-suppressive molecule called dendritic cell-associated, HSPG-dependent integrin ligand (DC-HIL) (122). DL-HIL engages Syndecan-4 on effector T cell causing anergy (122). Furthermore, targeting DC-HIL with neutralizing antibody or its genetic knockout delayed the growth of transplantable B16 melanoma in syngeneic mice, which further strengthen the role of DC-HIL as a potential target for enhancing the immune response and cause tumor eradication (123).

HSPGs also affect innate immune response against cancer cells by modulating Natural Killer (NK) cell-mediated activity against cancer cells. NK cells exert their cytotoxic activity on cancer cells through recognition of specific ligands, one group of which is called the natural cytotoxicity receptors (NCR) (124). The NCRs bind to HSPGs and their interaction promotes NK cell-mediated cancer cell eradication (125). Additionally, it has been shown that cancer cells upregulate heparanase through activation of bromodomain PHD finger transcription factor (BPTF), leading to reduced NCR-HSPG interaction, which results in dampened NK cell response (126). Collectively, these studies demonstrate that by activating immune tolerance, enhancing signaling pathways, and interfering with immune cell-tumor interactions, HSPGs regulate immune evasion functions in cancer cells.

Role in the Regulation of Extracellular Matrix Modification

HSPGs, free HS chains and heparin are structural components of extracellular matrix (ECM) (12). The ECM is a major part of the tumor microenvironment and influences tumor progression by several mechanisms, including growth factor concentrations, angiogenesis, and immune infiltration (127). The changes in HSPGs and HS metabolizing enzymes vary widely with cancer type and have varying context dependent roles.

Right-sided colorectal cancers show that the expression of the HSPGs glypican-1,-3, and -6 and betaglycan are altered in non-metastatic tumors, whereas in metastatic tumors, only glypican-1 and SDC1 are modified. Interestingly, alterations were found in only non-metastatic tumors, affecting N-sulfation, and the isoforms of heparan sulfate 6-O-sulfotransferase 1 (HS6ST1), heparan sulfate-glucosamine 3-sulfotransferase 3B1 (HS3ST3B1) and heparan sulfate-glucosamine 3-sulfotransferase 5 (HS3ST5) (128). The HSPG SDC2 induces MMP-7-mediated E-cadherin shedding in colorectal cancer. E-Cadherin shedding led to reduced cell-to-cell contacts and the acquisition of a fibroblast-like morphology, which are both associated with cancer metastasis (129). Another important study showed that SDC1-positive human mammary fibroblasts (HMF) induced extracellular matrix remodeling by promoting an aligned fiber architecture, which promoted directional migration and invasion of breast cancer cells (130).

Apart from syndecans, perlecan and agrin, two other basement membrane constituents are also involved in cancer progression (131–133). Antisense RNA against perlecan inhibits tumor growth and angiogenesis in colon carcinoma (134). Moreover, the ECM protein agrin stimulated osteosarcoma cell growth and migration. Agrin also induces a switch from topoisomerase I to topoisomerase II (135). Therefore, these studies collectively reveal the role of HSPG ECM constituents and cell surface HSPGs in regulating cell-to-cell and cell-matrix adhesion, which in turn control tumor cell migration and shedding.

TARGETING HS AND HSPGs FOR CANCER TREATMENT

Understanding the biology behind HS and HSPG deregulation in cancers has enabled the development of

various therapeutic strategies aimed at various HS- and HSPG-mediated cancer growth and progression enabling features. Small molecule inhibitors, which interfere with the activities of various enzymes involved in HSPG synthesis and modification, have been developed (6). Additionally, small molecule inhibitors and monoclonal antibodies, which target interactions between HSPGs and their targets, are being developed (136, 137). Below, we describe some of these agents and their value as anti-cancer agents.

Antibody and Small Molecule Targeting HS-Modifying Enzymes, HS, and HSPGs

Among the enzymes involved in HS synthesis and modifications, heparanases, and sulfatases are considered good drug targets. Heparanase is overexpressed in a wide-variety of solid tumors and hematological malignancies (29). A previous study assessed the therapeutic value of heparanase targeting using heparanse-neutralizing antibodies for the treatment of diffuse non-Hodgkin's B-cell lymphoma and follicular lymphoma (138). This study found that heparanase inhibition blocked xenograft tumors and growth of lymphoma cells in the bones of mice (138). Additional studies have shown that antibody-mediated anti-heparanase-therapies inhibit cell invasion and tumor metastasis (138–140). Recently, a small molecule inhibitor of hepranase was developed and was shown to reduce metastatic attributes in a model of hepatocellular carcinoma (141). Thus, these studies collectively establish heparanase as a potential drug target for cancer therapy.

Small molecule inhibitors, which prevent growth hormone binding to HSPG, reduce the proliferative HSPG-mediated signal. A similarity-based screening of small molecule libraries identified bi-naphthalenic compounds, which can inhibit FGF binding to both, HSPGs and FGFR1 binding. *In vitro* and *ex vivo,* these compounds inhibit FGF2 activity in angiogenesis models, with improved therapeutic potency (142). Monoclonal antibodies developed against the HS chain on GPC3 inhibit Wnt3a/β-catenin activation, recapitulating GPC3 knockdown by reducing HCC migration and motility (137).

Small molecule inhibitors against sulfatases have shown promise in inhibiting tumor growth. A disulfonyl derivative of phenyl–tert–butyl nitrone (PBN) called OKN-007 inhibited Sulf2 activity in hepatocellular carcinoma (HCC) cell lines and blocked HCC tumor xenograft growth in mice (136).

HS signaling modulation also affects immune cell trafficking and associated immune responses. Deletion of the glycosyltransferase gene exostosin glycosyltransferase 1 (Ext1), which is essential for HS chain formation, in myxovirus resistance-1 (Mx-1)-expressing bone marrow stromal cells increased hematopoietic stem cells (HSCs) efflux from the bone marrow to the spleen in response to granulocyte colony-stimulating factor. Thus, a therapeutic that targets Ext1 may help mobilize immune cells to target cancer cells (143). For detailed review on the role of different enzymes in HS synthesis and modification readers are referred to a review by Bishop et al. (12).

Heparan Sulfate Mimetics

HS mimetics were also used as anti-cancer agents. HS mimetics induce an immune response against lymphoma through activation of natural killer (NK) cells (144). The HS mimetic PG545, in addition to its anti-heparanase and anti-angiogenic effect shows pleiotropic effect by enhancing toll-like receptor 9 (TLR9) activation through increasing the TLR9 ligand CpG in DCs. It was shown that treatment with PG545 resulted in the accumulation of CpG in the lysosomal compartment of DCs. This in turn enhanced the IL-12 production, which was essential for the ability of PG545 to activate NK cells (144). Furthermore, PG545 was also shown to directly bind to WNT3A and WNT7A and inhibits WNT/β-catenin signaling, inhibiting proliferation in pancreatic tumor cell lines (145). These studies further highlight the possibility of using heparin sulfate mimetics as agents for cancer therapy.

HSPGs as Immunotherapeutic Targets

Some recent studies have also indicated that the upregulation of HSPGs on cancer cells can be used as unique biomarkers that can be targeted to selectively deliver cytotoxic drugs (146, 147). A recent study that analyzed differential expression of cell surface proteins on neuroblastoma identified the HSPG, Glypican-2 (GPC2) as selectively expressed on neuroblastoma where it enhances neuroblastoma proliferation (148). The researchers were able to develop an antibody drug conjugate that selectively eradicated GPC2 positive neuroblastoma (148). This is another exciting area of emerging research where HSPGs can be exploited to serve as targets for selective drug delivery to cancer cells.

CONCLUSION

Recent cancer therapies have largely focused on targeting driver mutations and their downstream effectors. However, the emerging body of evidence now shows that driver-mutations are, in fact, enhanced and modified by a host of other modifications as cancer evolves. HS and HSPG deregulation are major contributing factors to cancer evolution. This review has covered some of the well-established and emerging roles of HS and HSPGs in cancer. However, new, non-canonical functions of HSPGs are still being discovered. For instance, in addition to modulating growth factors and RTK interactions, HSPGs also transport growth factors directly to the nucleus, where these factors modify gene regulation (149). HSPGs have also been shown to influence cancer exosome shedding and uptake, thereby modulating cell-to-cell communication between cancer and healthy fibroblasts, immune cells, and endothelial cells (150, 151). HSPGs can also influence actin cytoskeleton remodeling and cancer cell motility (95). The HSPG, SDC2 binds Ezrin, a cytoskeletal protein (152) and serves as adapter molecules for IGF1 mediated activation of ERK (95). Additionally, HSPGs are implicated in lipoprotein uptake and cellular stress signaling (153, 154). As more researchers validate these findings, newer

areas of HS- and HSPG-mediated regulation will be discovered. Additionally, as cancer treatment moves from single target to combination therapies, HS- and HSPG-targeting therapies will likely emerge as a major new direction for cancer therapeutics.

AUTHOR CONTRIBUTIONS

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

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Deoxyribonucleotide Triphosphate Metabolism in Cancer and Metabolic Disease

*Raquel Buj and Katherine M. Aird**

Department of Cellular and Molecular Physiology, Penn State College of Medicine, Hershey, PA, United States

The maintenance of a healthy deoxyribonucleotide triphosphate (dNTP) pool is critical for the proper replication and repair of both nuclear and mitochondrial DNA. Temporal, spatial, and ratio imbalances of the four dNTPs have been shown to have a mutagenic and cytotoxic effect. It is, therefore, essential for cell homeostasis to maintain the balance between the processes of dNTP biosynthesis and degradation. Multiple oncogenic signaling pathways, such as c-Myc, p53, and mTORC1 feed into dNTP metabolism, and there is a clear role for dNTP imbalances in cancer initiation and progression. Additionally, multiple chemotherapeutics target these pathways to inhibit nucleotide synthesis. Less is understood about the role for dNTP levels in metabolic disorders and syndromes and whether alterations in dNTP levels change cancer incidence in these patients. For instance, while deficiencies in some metabolic pathways known to play a role in nucleotide synthesis are pro-tumorigenic (e.g., p53 mutations), others confer an advantage against the onset of cancer (G6PD). More recent evidence indicates that there are changes in nucleotide metabolism in diabetes, obesity, and insulin resistance; however, whether these changes play a mechanistic role is unclear. In this review, we will address the complex network of metabolic pathways, whereby cells can fuel dNTP biosynthesis and catabolism in cancer, and we will discuss the potential role for this pathway in metabolic disease.

Keywords: purines, pyrimidines, c-Myc, p53, mTORC1, diabetes, obesity

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Curtin University, Australia
Eva Surmacz,
Temple University, United States

Correspondence:
Katherine M. Aird
kaird@psu.edu

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INTRODUCTION

The maintenance of deoxyribonucleotide triphosphate (dNTP) pools is critical for multiple cellular pathways. For instance, imbalances in dNTPs are associated with genomic instability (1). Likewise, they have also been shown to disturb mitochondrial DNA (mtDNA) and consequently mitochondrial fitness, which may lead to mitochondrial diseases (MDs), such as diabetes, obesity, and cancer (2). Additionally, disorders of purine and pyrimidine metabolism (DPPM) profoundly affect cell metabolism, underlying the importance of nucleotides for cell behavior (3). Thus, both nucleotide synthesis and degradation must be exquisitely fine-tuned. In this review, we will focus on synthesis of dNTPs and the consequences of dNTP pool imbalances in cancer and MDs.

HEALTHY dNTP POOLS

A correct balance of dNTPs is necessary for the prevention of multiple pathologies. A healthy cell must maintain two asymmetric and spatial-temporal dNTP pools; one for nuclear DNA synthesis and repair and another for mtDNA replication and repair. Disruptions in dNTP balance are associated with enhanced mutagenesis, leading to genomic instability, which promotes cancer (4), and may have a role in metabolic disease (5).

Cytosolic dNTP pool concentrations positively correlate with the cell cycle. In fact, the amount of dNTPs at the beginning of S-phase is not enough for a complete DNA duplication (6). The S-phase increase in dNTPs is necessary for faithful nuclear DNA replication. mtDNA is replicated continuously in post-mitotic cells, and faithful maintenance of mtDNA also depends on correctly balanced dNTPs (7). Thus, both proliferating and non-proliferating cells need to fine-tune nucleotide and dNTP synthesis to allow for both nuclear and mtDNA replication and repair to maintain the health of the cell.

Anabolism and Catabolism of Nucleotides

Cells possess two biosynthetic pathways to produce dNTPs: *de novo* and salvage (8). Purines and pyrimidines arise from two different *de novo* pathways that generate nucleotides starting from raw material (glucose, glutamine, aspartate, and HCO_3) (9). The *de novo* nucleotide synthesis pathway is highly energy-intensive (9). Therefore, cells have developed a more energy-efficient route to synthesize nucleotides, termed the salvage pathway (10). The salvage pathway acts as a recycling plant taking free nitrogen bases and nucleosides arising from nucleic acid breakdown and diet (9). Nucleosides are hydrophilic compounds, thus proper function of nucleoside transporters (SLC29 and SLC28 families) is an essential requirement for salvage pathway function (11). Ribonucleotides obtained by either pathway can be reduced to their deoxyribonucleotide counterpart in a reaction catalyzed by ribonucleotide reductase (RNR) (12).

Turnover of RNA and other nucleotides occurs regularly to maintain homeostasis. Human cells cannot break down the purine ring. Purine catabolism involves a sequence of three reactions in which nucleotides are stripped step-by-step from their phosphates and sugar to finally become oxidized to the end product uric acid (UA), which is excreted into the urine (13). Conversely, uracil and thymidine rings can be completely degraded to β-alanine and β-aminoisobutyrate, respectively. Subsequently, both metabolites can be excreted or transformed into intermediates of the tricarboxylic acid (TCA) cycle (14). Biosynthesis and catabolism of nucleotides and dNTPs are highlighted in **Figure 1**.

IMPAIRED NUCLEOTIDE METABOLISM IN CANCER AND METABOLIC DISEASE

Deregulation of nucleotide metabolism is associated with a broad spectrum of pathological conditions, including cancer and MDs (15–17). Virtually all metabolic pathways have been implicated in dNTP biosynthesis. Thus, *de novo* and salvage pathways, as well as all involved anapleurotic reactions (**Figure 1**), need to be highly cross-regulated.

It is well known that cancer cells must increase dNTP biosynthesis (18) to ensure rapid replication of the genome (17). This occurs through a variety of pathways (discussed below). In contrast, MDs are caused by congenital or acquired genetic defects in metabolic enzymes. DPPM are due to abnormalities in the biosynthesis, interconversion, and degradation of nucleotides (19). DPPM have a wide variety of clinical presentations, highlighting the importance of proper nucleotide metabolism for cell and organism function (15). Alterations in nucleotide metabolism are also present in other metabolic-related pathological conditions, such as diabetes, obesity, and insulin resistance (20–22) (**Table 1**). In this section, we will summarize some important features affecting nucleotide metabolism in cancer and MDs.

Deregulation of Major Growth Signaling Pathways Leads to Nucleotide Pool Imbalances in Cancer and Metabolic Disease

The main growth signaling pathways (PI3K-AKT and ERK1/2-MAPK) are induced and maintained during metabolic reprogramming of cancer (18). Additionally, deregulation of these pathways may contribute to different MDs, including diabetes, obesity, or steatosis resistance (33, 89, 90). These pathways sense and orchestrate nutrient utilization; therefore, is not surprising that alterations in these pathways affect energy and biomass production and cause a broad variety of diseases.

mTOR is a central signaling pathway that integrates environmental inputs (e.g., nutrients and hormones) into downstream pathways to control many cellular processes (91). This includes regulation of metabolism, growth, and survival (32). Indeed, the mTORC1/2 pathway not only promotes glucose uptake and protein and lipid biosynthesis, but also promotes nucleotide biosynthesis (29, 30) and uptake of nucleosides through transporters (88). At least one member of this pathway is altered in 38% of human cancer (92). Altered metabolism induced by aberrant mTORC1 activation has also been shown to play a role in diabetes and obesity (32, 93).

c-Myc, one of the most commonly altered proteins in human cancer, is also regulated by PI3K-AKT and ERK1/2-MAPK pathways (94). c-Myc is a highly pleiotropic transcription factor considered a master regulator of cell metabolism (34, 35) through regulation of glycolysis, glutamine metabolism, and mitochondrial biogenesis (95, 96). Indeed, c-Myc has been shown to induce hepatic glucose uptake and utilization, while blocking gluconeogenesis and ketogenesis, suggesting a counteracting effect of c-Myc in obesity and insulin resistance (36, 97). In addition to regulating glucose and glutamine, substrates for purine and pyrimidine biosynthesis (**Figure 1**) (98), c-Myc also transcriptionally regulates nucleotide metabolic enzyme gene expression (35). Thus, deregulation in c-Myc acutely alters nucleotide homeostasis in cancer (99), and it is interesting to speculate that the role of c-Myc in MDs is also related to nucleotide metabolism.

Previous publications from our laboratory and others have shown that DNA damage and DNA damage response (DDR) proteins regulate dNTP biosynthesis in the context of cancer (80, 100, 101). Interestingly, upregulation of p53, a key player in the DDR, in adipose tissue is associated with increased inflammation and insulin resistance (102). Notably, wild-type p53 negatively regulates G6PD activity (37), the rate-limiting enzyme of the pentose phosphate pathway and one of the most important sources of nucleotides (103). Upregulation of G6PD correlates with functional defects in liver, heart, and pancreas of obese and diabetic animals (104). Although the relationship between G6PD upregulation and increased oxidative stress has been studied in MD (105), the implication for nucleotide metabolism has not

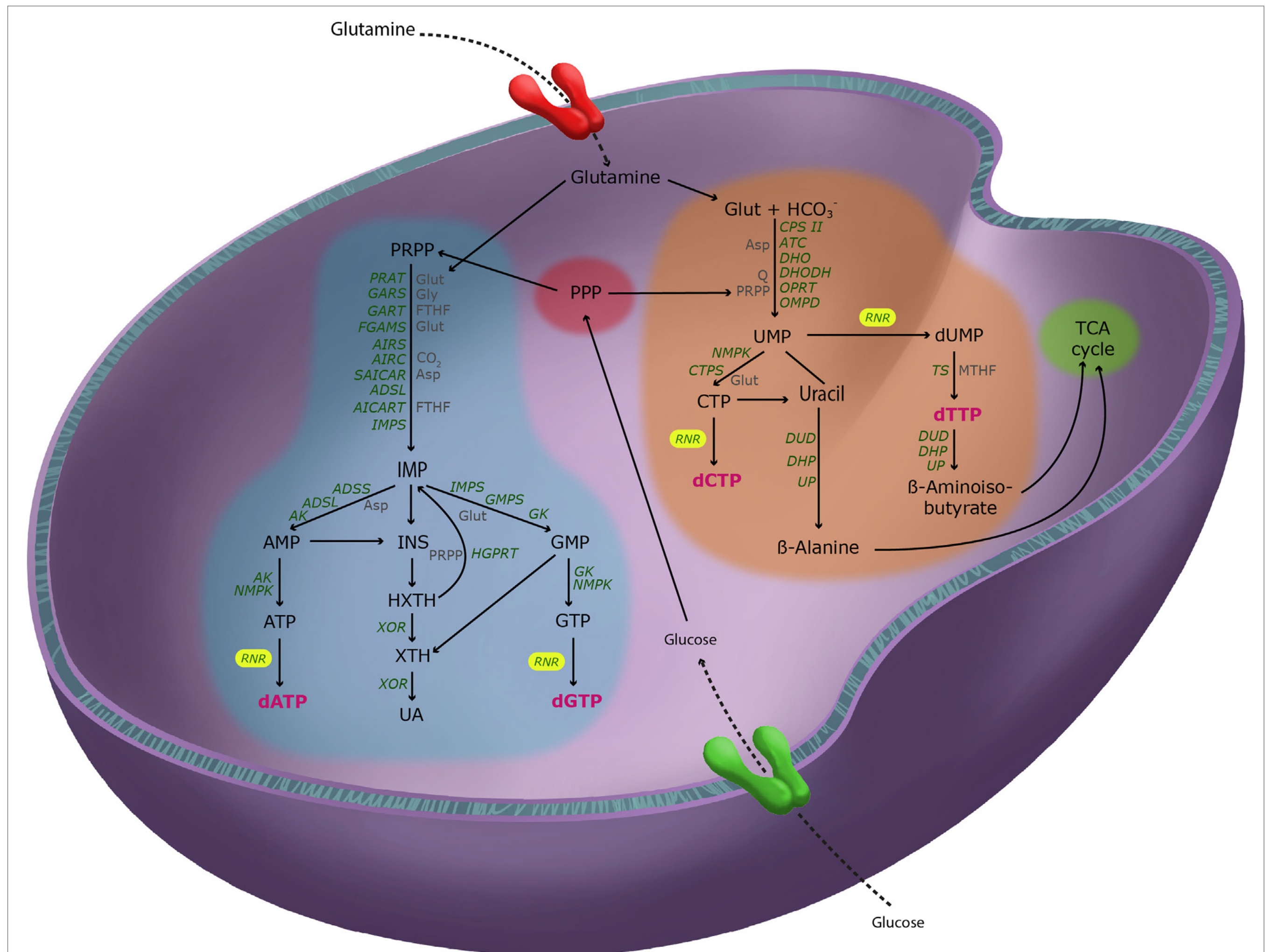

FIGURE 1 | Pathways of deoxyribonucleotide metabolism in mammalian cells. Simplified representation of purine (blue) and pyrimidine (orange) metabolism and their crosstalk with the major metabolic pathways, the pentose phosphate pathway [(PPP), red] and the tricarboxylic acid cycle (green). Key metabolic enzymes (green), their principal reactive substrates (gray), and the four deoxyribonucleotide triphosphate (dNTP) end-products (magenta) are shown. Glucose and glutamine feed into both purine and pyrimidine metabolism to donate carbons and nitrogens to all dNTPs. Abbreviations: RAT, phosphoribosylpyrophosphate amidotransferase; GARS, glycinamide ribonucleotide synthetase; GART, glycinamide ribonucleotide transformylase; FGAMS, phosphoribosylformyl-glycineamide synthetase; AIRS, phosphoribosylaminoimidazole synthetase; AIRC, phosphoribosylaminoimidazole carboxylase; SAICAR, phosphoribosylaminoimidazole-succinocarboxamide; ADSL, adenylosuccinate lyase; AICART, phosphoribosylaminoimidazolecarboxamide formyltransferase; IMPS, inosine monophosphate synthase; ADSS, adenylosuccinate synthetase; AK, adenylate kinase; NMPK, nucleotide monophosphate kinase; IMPS, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; GK, guanylate kinase; XOR, xanthine oxidoreductase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; RNR, ribonucleotide reductase; CPS II, carbamoyl phosphate synthetase II; ATC, aspartate carbamoyltransferase; DHO, dihydroorotase; DHOD, dihydroorotase dehydrogenase; OPRT, orotate phosphoribosyltransferase; OMPD, orotidine monophosphate decarboxylase; CPTS, cytidine triphosphate synthetase; TS, thymidylate synthase; DUD, dihydrouracil dehydrogenase; DHP, dihydropyrimidinase; UP, ureidopropionase; Glut, glutamine; Gly, glycine; FTHF, *N*10-formyltetrahydrofolate; Asp, aspartate; PRPP, phosphoribosylpyrophosphate; Q, ubiquinone; MTHF, *N*5,*N*10-methylenetetrahydrofolate.

yet been addressed. More research is needed to understand the contribution of dNTP imbalances due to G6PD deregulation in diabetes and obesity.

An imbalance in nucleotides has been shown in two different studies related to diabetes (106, 107). Additionally, pyrimidine metabolism has been linked to fatty liver (26). Interestingly, increasing evidence suggests a link between obesity, a risk factor for non-alcoholic fatty liver disease (108), and cancer. Obese patients show many cancer-promoting features, such as chronic low-level inflammation (109), insulin-resistance/diabetes (110), and deregulation of mTORC1 (111). Although the contribution of deregulated nucleotide pools promoting cancer has been extensively demonstrated (18, 112–115), their role in MD and metabolic-related diseases has not yet been elucidated. Based on these recent studies, we speculate that deregulation of nucleotide pools may in part contribute to the altered metabolic landscape promoting obesity and diabetes. Studying the implications of altered nucleotide pools in these diseases would open a therapeutic window based on modulation of nucleotide metabolism.

TABLE 1 | Genes, protein families, and pathways discussed in this review: role in deoxyribonucleotide triphosphate (dNTP) metabolism and expression in cancer and metabolic disease.

Gene/family/pathway	Known role in dNTP metabolism	Expression in cancer	Expression in metabolic disease
Purine/pyrimidine synthesis pathway	Necessary for *de novo* dNTP biosynthesis (8)[a]	Increased (23) or mutated (24, 25)	Heptatic steatosis (uridine metabolism) (↓) (26) Diabetes[b] (↓) (27)
MTOR	Promotes glucose uptake (28); promotes *de novo* nucleotide biosynthesis (29, 30)	Increased (31)	Diabetes (↑) (32) Obesity (↑) (33)
MYC	Induces glucose uptake and utilization (34); transcriptionally regulates nucleotide metabolic enzymes (23, 35)	Increased (oncogene) (23)	Insulin resistance (↑)[c] Obesity (↑)[c] (36)
TP53	Negative regulator of pentose phosphate pathway through G6PD (37); gain-of-function mutations increase gene transcription of genes for dNTP synthesis (38)	Decreased or mutated (tumor suppressor) (39)	Insulin resistance (↑) Glucose intolerance (mut) (5) Mitochondrial changes (mut)[d] (40)
PI3K-AKT pathway	Oncogenic activation promotes glucose and glutamine uptake and catabolism (41)	Increased (oncogenes) (41)	Diabetes (↑) (42) Nonalcoholic fatty liver disease (↑) (43) Obesity (↑) (44)
ERK-MAPK pathway	Regulation of CPS II in *de novo* pyrimidine synthesis (45)	Increased (oncogenes) (46)	Diabetes (↑) (47) Obesity (↑) (48)
G6PD	Rate-limiting for ribose-5-phosphate synthesis from the PPP (49)[a]	Increased or mutated (50)	Obesity (↑) (51) Diabetes (↑) (52)
RRM1	Catalytic subunit of the ribonucleotide reductase (RNR); catalyzes the reduction of deoxyribonucleotides from ribonucleotides (12)[a]	Increased or decreased (53)	Unknown
RRM2	Regulatory subunit of RNR (12); S-phase regulated (54); rate-limiting enzyme in the reduction of deoxyribonucleotides from ribonucleotides (55)[a]	Increased (oncogene) (53)	Unknown
RRM2B	Regulatory subunit of the RNR (56); formation of deoxyribonucleotides from ribonucleotides for DNA damage repair and mitochondrial DNA (mtDNA) replication (57–59)[a]	Increased or decreased (53)	Mitochondrial disorders (↓) (60)
SLC25 family	Mitochondrial nucleoside transporters (61) Important for mtDNA pools through the salvage pathway (62)	Increased (63)	Mitochondrial disease (mut)[e] Mitochondrial dysfunction (↓)[f] (61)
SLC29 and SLC28 families	Nucleoside transporters that are important for the salvage pathway (11, 64, 65)	Increased (11)	Diabetes (mut)[g] (66)
TK2	Phosphorylates deoxycytidine to generate dCTP (67)	Unknown	Mitochondrial disease (↓) (68)
DGUOK	Catalyzes the conversion of deoxyguanosine to dGMP (67)[a]	Mutated[h] (69, 70)	Mitochondrial disease (mut) (69)
TWNK	Mitochondrial helicase (71)	Unknown	Mitochondrial dysfunction (mut) (72, 73)
POLG	Catalytic subunit of the mitochondrial DNA polymerase (74)	Mutated (75–77)	Mitochondrial disease (mut) (77–79)
Ataxia-telangiectasia mutated	Increases glucose/glutamine uptake and inhibits the PPP (80)	Mutated (81)	Mitochondrial dysfunction (mut)[i] Insulin resistance (mut)[i] (82)
XOR	Catalyzes the conversion of xanthine to uric acid (83)[a]	Increased[j] or decreased (84)	Metabolic syndrome (mut)[k] Insulin resistance (mut)[k] Diabetes (mut)[k] Fatty liver disease (mut)[k] (85)

[a] *These genes/pathways are shown in **Figure 1**.*
[b] *These studies show that purines and pyrimidines are downregulated in diabetes. It is not known whether changes in purine or pyrimidine synthesis genes are the mechanism behind this observation.*
[c] *Increased MYC expression counteracts insulin resistance and obesity.*
[d] *Occurs in patients with Li–Fraumeni syndrome.*
[e] *SLC25A4 (86).*
[f] *SLC25A33 and SLC25A36 have only been tested in mouse models (87, 88).*
[g] *SLC29A3 is the only gene in this family that has been found to affect metabolic disease.*
[h] *While the data are limited, some patients with DGUOK mutations have hepatocellular carcinoma.*
[i] *Occurs in patients with ataxia-telangiectasia.*
[j] *Increased XOR expression/activity is likely important for cancer initiation; however, XOR expression is decreased in most established tumors.*
[k] *Occurs in patients with XOR deficiency.*

RNR in Cancer and Metabolic Disease

Ribonucleotide reductase reduces ribonucleotides to the corresponding deoxyribonucleotides (116, 117). In mammals, RNR is a tetrameric enzyme composed of two homodimeric subunits, RRM1 and RRM2. Whereas, RRM1 is continuously expressed throughout the cell-cycle, expression of RRM2 is activated upon entry into S-phase (54, 118). Additionally, RRM2 is rapidly degraded *via* the proteasome in G2 (12, 119). Thus, RRM2 is

considered rate-limiting for RNR activity. RRM2B (RNR subunit M2B) is an alternative M2 subunit that is induced by p53 activation in response to DNA damage (56). RRM2B is not cell-cycle regulated *per se*, but it plays key roles in enhancing dNTP synthesis in cells under stress (120–122) and mediating mtDNA synthesis and repair (57–59).

The role of RNR in cancer is clear as it was one of the first identified DNA damage-induced enzymes (123). While RRM2 overexpression is tumorigenic, leading to lung neoplasms *in vivo*, RRM1 reduces tumor formation, migration, and metastasis [reviewed in Ref. (53)]. Previous studies from our lab and others have shown the potential of RRM2 as a prognostic and diagnostic biomarker in multiple cancers (112, 124–127). However, the utility of RRM1 and RRM2B as a tumor biomarker is still unclear [reviewed in Ref. (53)].

Although there is no study directly linking RNR with MD, RRM2B is required for mtDNA synthesis and healthy mitochondrial function (57). Deregulated mitochondria are associated with a higher risk of diabetes and obesity (discussed below). Therefore, it is possible that RNR function is linked to these MDs (**Table 1**). More mechanistic studies will be needed to determine the role for RNR in obesity and diabetes.

Mitochondrial Dysfunction in dNTP Pool Disruption During Cancer and Metabolic Disease

The mitochondria are one of the most important organelles for eukaryotic function (128). In addition to the production of ATP through oxidative phosphorylation, mitochondria are also the scaffold of several metabolic reactions for cellular building block synthesis (e.g., fatty acid beta-oxidation, one-carbon/folate cycle, TCA cycle, amino acid metabolism, etc.) (129). Hence, altered mitochondrial behavior has a broad impact on cellular metabolism.

Maintenance of mitochondrial dNTP pools is critical for proper mtDNA function. Alterations in nuclear genes involved in transport of cytosolic dNTPs (e.g., SLC25A4), the salvage nucleotide biosynthesis in the inner mitochondrial membrane (e.g., TK2 and DGUOK), and genes involved in mtDNA replication (e.g., TWNK and POLG) are implicated in both cancer and metabolic syndromes (63, 68, 77–79, 130–133). Moreover, dysfunction in the electron transport chain induces oxidative stress, which has been associated with impaired one-carbon metabolism (134, 135), an essential anapleurotic pathway for both purine and pyrimidine nucleotides. Mitochondrial genomic instability due to increased levels of reactive oxygen species (ROS) and/or mutations in mtDNA or nuclear genes involved in mitochondria function are underlying factors of MDs, and contribute to cancer and diabetes (136). Alterations in genes discussed above that are important for dNTP homeostasis and mitochondrial function are highlighted in **Table 1**.

Although the link between mitochondrial dysfunction and MD has been studied for the past two decades, the results are contradictory (137). These contradictory results mainly arise from the complex relationship between mitochondria and metabolism, but also from the lack of global and standardized methodological strategies to phenotype insulin-resistance in humans (138). Dysregulation of nucleotide metabolism is an important aspect of mitochondrial dysfunction; therefore, their role in MDs should not be ignored.

Relationship Between DPPM and Cancer

It is clear that cancer is a metabolic disease; however, a predisposition to cancer is not a foregone conclusion in patients with DPPM, who by definition have alterations in nucleotide supplies. Interestingly, while deficiencies in some metabolic pathways known to play a role in nucleotide synthesis are pro-tumorigenic, others confer an advantage against the onset of cancer. This highlights the large variability in the clinical presentation of these disorders.

Alterations in p53 or ataxia-telangiectasia mutated (ATM) lead to metabolic changes and predispose patients to cancer. Patients with germline *TP53* (encoding for p53) mutations have Li–Fraumeni syndrome and are predisposed to cancer (139, 140). Interestingly, a recent report showed that nucleotide metabolism is regulated by the gain-of-function activity of mutant p53 (38). Consistently, wild-type p53 negatively regulates G6PD and PPP activity to decrease dNTP synthesis (37). Similarly, our group has previously shown that ATM (mutated in some ataxia-telangiectasia patients) inactivation increases glucose uptake and enhances glucose flux through the PPP and ultimately increases dNTP biosynthesis (**Figure 1**) (80, 141). Indeed, patients with ATM mutations show alterations in glucose homeostasis (142, 143). It is well-known that these patients have an increased susceptibility to cancer (144). It is interesting to speculate that alterations in dNTP metabolism may play a role in the cancer predisposition in these patients; however, further studies are needed to support this notion.

Other DPPM confer a tumor suppressive benefit. For instance, patients with G6PD deficiency have a reduced risk of some cancers (145–147) (**Table 1**). This suggests that hyperactivity of dNTP synthesis is more likely to increase cancer risk than deficiencies in synthesis.

Finally, some DPPM have both a pro- and anti-tumorigenic effect. Deficiency in xanthine oxidoreductase (XOR), the enzyme that catalyzes the last step in purine catabolism (**Figure 1**), increases UA (148). There is a dual role for UA in cancer, the so-called the oxidant–antioxidant UA paradox (149). On one hand, extracellular UA is a potent ROS scavenger, thus protecting cells against oxidative stress (150). On the other hand, high intracellular levels of UA in a XOR-deficient cellular background promote dNTP biosynthesis and tumor growth by shuttling XOR precursors (xanthine and hypoxanthine) into the purine salvage pathway (149). Additionally, intracellular UA is pro-inflammatory by inducing NADPH-oxidases that lead to oxidative stress and cancer (151, 152). This again emphasizes the complex nature of these disorders in relation to cancer (**Table 1**).

Together, the lack of consensus in predisposition to cancer in DPPM patients points to the significant redundancy in the dNTP biosynthetic pathways. This should not be surprising due to the fact that dNTP synthesis is critical for organismal survival and, therefore, we have evolved to have multiple metabolic arms feeding into the same pathway. Understanding whether these patients

are predisposed or not to cancer will be incredibly important for the clinical management of these patients.

THERAPEUTIC MODULATION OF DEOXYRIBONUCLEOTIDE METABOLISM IN CANCER AND METABOLIC DISEASE

As described in this review, the balance of dNTPs must be tightly regulated in the cell. Many cancer types show alterations in dNTP levels, supporting their rapid proliferation. Likewise, defective mutations in anabolic and catabolic nucleotide enzymes, causing imbalances in the dNTP pools or in their precursors, are associated with different grades of disease severity in DPPM. Thus, it is not surprising that therapies for both cancer and DPPM focus on restoration of the normal balance of intracellular nucleotides.

Some of the first chemotherapeutic agents were cytotoxic nucleoside analogs and nucleobases (e.g., thiopurines and fluoropyrimidines) (153). These antimetabolites have a similar molecular structure to endogenous nucleotides and interfere with nucleotide metabolic pathways and DNA/RNA synthesis (154). Inhibitors of RNR were one of the first cancer therapies [reviewed in Ref. (53)] and are still used today. For instance, gemcitabine, a chemotherapeutic nucleoside analog, is used in pancreatic adenocarcinoma, but also in breast, bladder, and non-small cell lung cancer (155). Unfortunately, resistance to gemcitabine in common, often through an increase in nucleotide synthesis pathways or transport of nucleosides (156). Other successful chemotherapeutic regimens include methotrexate, which reduces substrates for purine and pyrimidine biosynthesis (157). Finally, specific inhibition of enzymes in the *de novo* pathway and/or in anapleurotic reactions (glucose and glutamine metabolism) has also been used as adjuvant therapies in cancer (154).

The spectrum of nucleotide therapies for DPPM is much broader in scope due to the high variability of deficiencies (3). Thus, deficiencies resulting in the overproduction of UA are treated with allopurinol, an inhibitor of xanthine oxidase (16). In other cases, patients can be treated with oral supplements of specific nucleotides they are lacking (16). What is clear is that cancer patients with DPPM cannot be treated with antimetabolites such as 5-fluoro-uracil due to severe side effects (19). This suggests that cancer patients, DPPM must remain above a certain threshold of nucleotide pools to remain healthy. Finally, no nucleotide therapies are currently used for MDs, such as diabetes or obesity. More studies will need to be performed to determine whether nucleotide metabolism plays a contributing role to these pathologies before these types of therapies can be tested.

CONCLUSION

For decades researchers and clinicians alike have recognized the importance of fine-tuned dNTP levels for cellular homeostasis, as shown by the number of anti-cancer therapies based on the abolishment of nucleotide synthesis. In addition, the broad range of pathologies associated with congenital defects in nucleotide metabolic enzymes further demonstrates the importance of healthy intracellular dNTP levels. However, the association between cancer and MD and whether nucleotide pools are interconnected in these pathologies remains unclear. Future work will need to focus on mechanistic and population-based studies to determine whether nucleotide pool imbalances in MD lead to changes in cancer predisposition and whether targeting these pathways for cancer therapy affects metabolic homeostasis and function in normal cells.

AUTHOR CONTRIBUTIONS

RB and KA conceived of and wrote the manuscript.

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Mitochondrial Dynamics in Type 2 Diabetes and Cancer

*Michelle Williams and M. Cecilia Caino**

Department of Pharmacology, University of Colorado School of Medicine, Aurora, CO, United States

Mitochondria are bioenergetic, biosynthetic, and signaling organelles that control various aspects of cellular and organism homeostasis. Quality control mechanisms are in place to ensure maximal mitochondrial function and metabolic homeostasis at the cellular level. Dysregulation of these pathways is a common theme in human disease. In this mini-review, we discuss how alterations of the mitochondrial network influences mitochondrial function, focusing on the molecular regulators of mitochondrial dynamics (organelle's shape and localization). We highlight similarities and critical differences in the mitochondrial network of cancer and type 2 diabetes, which may be relevant for treatment of these diseases.

Keywords: mitochondria, fission, fusion, diabetes, cancer

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***Correspondence:**
M. Cecilia Caino
cecilia.caino@ucdenver.edu

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INTRODUCTION

All living organisms rely on cellular and physiological mechanisms of homeostasis in order to maintain an internal environment optimal for life and function. Mitochondria are the foundation of cellular homeostasis, *via* their multiple roles in energy production, biosynthesis, calcium regulation and signaling, redox balance, and generation of reactive oxygen species. Not surprisingly, cells have evolved multiple mechanisms of quality control to ensure that mitochondria function at their best. These include protein import (1), folding and degradation (2), antioxidant defense mechanisms (3), mitochondrial turnover *via* autophagy (4), mitochondrial biogenesis (5), mitochondrial shape changes and cristae remodeling (6), and communication with the nucleus to coordinate transcriptional responses (7).

Emerging evidence indicate that mitochondrial dysfunction is associated with disparate diseases, including aging (8), neurodegenerative diseases (9), mitochondrial diseases (10), obesity (11), diabetes, and cancer. Although some controversies remain regarding whether functional or dysfunctional mitochondria are responsible for metabolic disorders, there is a resurgence of interest in understanding the mechanisms responsible for such mitochondrial alterations in disease. This review focuses on the molecular regulators of mitochondrial dynamics (organelle's shape and localization) in cancer and metabolic pathologies.

REGULATION OF MITOCHONDRIAL DYNAMICS

Mitochondria constantly undergo shape and number changes thanks to the two opposing processes of fission and fusion (12). In turn, changes in gross mitochondrial morphology and the interconnectivity of the mitochondrial network impact on energy production (13), calcium signaling, mitochondrial DNA distribution, apoptosis, mitophagy, and segregation of mitochondria between daughter cells (6). The fine-tuning of the fusion–fission balance is crucial for cellular fitness in response to extracellular stimuli and environmental stress (14). Thus, alterations of the fission–fusion balance lead to oxidative stress, mitochondrial dysfunction, and metabolic alterations.

At the molecular level, dynamin-like GTPases orchestrate mitochondria shape changes. The fission protein dynamin-related protein 1 (DRP1) assembles into ring-like structures to constrict mitochondrial membranes in a GTP-dependent manner (6). DRP1 is recruited to mitochondria by

fission protein 1 (FIS1), mitochondrial fission factor (MFF), and the mitochondrial dynamic proteins of 49 (MiD49) and 51 kDa (MiD51). On the other hand, the fusogenic proteins mitofusin 1 and 2 (MFN1/2) are located in the outer mitochondrial membrane, and tether two mitochondria through homo- and heterotypic dimerization (13). A single GTPase, optic atrophy protein 1 (OPA1), achieves fusion of the IMM.

An expanding number of degenerative disorders are associated with mutations in the genes encoding MFN2 and OPA1, including Charcot–Marie–Tooth disease type 2A and autosomal dominant optic atrophy (15). Defective mitochondrial dynamics seem to play a more general role in the molecular and cellular pathogenesis of common neurodegenerative diseases (Alzheimer's and Parkinson's) (14), as well as in cardiovascular disease (16), type 2 diabetes (T2D), and cancer.

MITOCHONDRIAL DYNAMICS IN T2D

The clinical complications of T2D include dyslipidemia, hyperglycemia (17), insulin resistance, and defects in insulin secretion from pancreatic beta cells (18). A major cause of such clinical complications is the increased production of mitochondrial ROS by hyperglycemia (17, 19). A common feature of mitochondrial morphology in T2D is an increased fragmentation (**Figure 1**), achieved *via* activation/upregulation of DRP1 and/or downregulation of MFN2 levels. In turn, increased fission and fragmentation of mitochondria was linked to HG-induced overproduction of ROS (20) and insulin secretion in mouse and human islets (21). Importantly, both HG-induced ROS and insulin secretion were blocked by inhibiting DRP1-induced fission. Furthermore, impaired mitochondrial fusion has been associated with insulin resistance in skeletal muscle (22) and with glucose intolerance and enhanced hepatic gluconeogenesis in a liver-specific MFN2 knockout (KO) mice (23). Interestingly, MFN2 KO led to increased ROS production, activation of JNK and endoplasmic reticulum (ER) stress response. Studies in rat models show that MFN2 overexpression improved insulin sensitivity and reduced lipid intermediates in muscle (24) and liver (25). At the molecular level, liver expression of MFN2 was associated with increased expression of the insulin receptor and the glucose transporter GLUT2, and activation of the PI3K/AKT2 pathway.

In addition, dyslipidemia models of T2D show increased mitochondrial fission (**Figure 1**). Excess palmitate (PA)-induced mitochondrial fragmentation and increased mitochondrion-associated DRP1 and FIS1 in differentiated muscle cells (26). In addition, PA induced mitochondrial depolarization, lower ATP synthesis and increased oxidative stress, and reduced insulin-stimulated glucose uptake (**Figure 1**). Both genetic and pharmacological inhibition of DRP1 attenuated PA-induced mitochondrial fragmentation and insulin resistance. In another study, DRP1 was induced in rat islets after stimulation by free fatty acids (FFAs), and this DRP-1 upregulation was accompanied by increased pancreatic β cell apoptosis (27).

Mitochondrial fission is associated with various processes that contribute to atherosclerosis in T2D (**Figure 1**), including endothelial dysfunction (28), collagen matrix alteration (29), and motility and proliferation of vascular smooth muscle cells (30). From a therapeutic standpoint, silencing FIS1 or DRP1 in venous endothelial cells isolated from patients with T2D blunted HG-induced mitochondrial fission and ROS production (28). Furthermore, metformin attenuated the development of atherosclerosis in diabetic mice by reducing DRP1-mediated mitochondrial fission in an AMP-activated protein kinase (AMPK)-dependent manner (31). Mitochondrial fission induced by DRP1 also plays a critical role in the pathogenesis of microvascular [nephropathy (32), retinopathy (33), and neuropathy] and macrovascular [stroke and myocardial ischemia (34)] complications of diabetes.

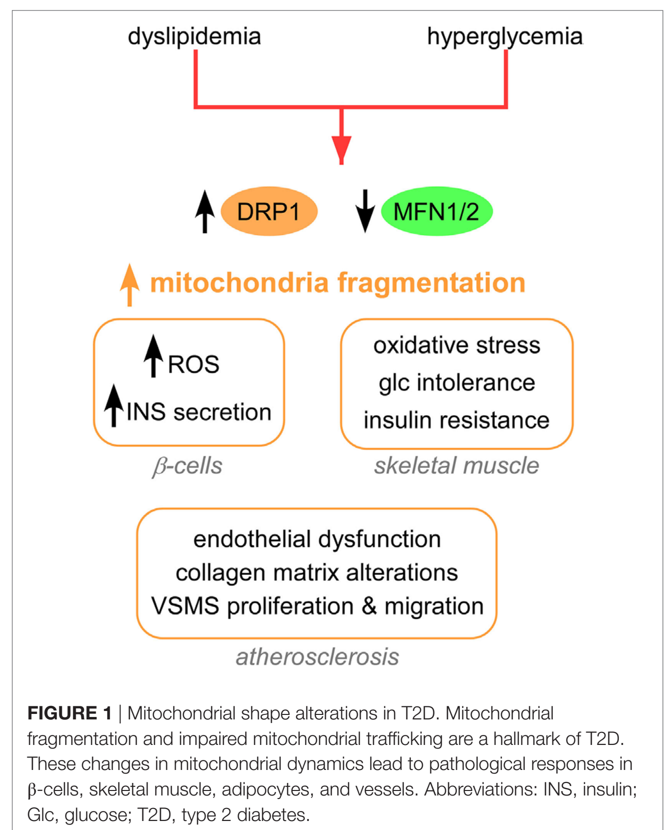

FIGURE 1 | Mitochondrial shape alterations in T2D. Mitochondrial fragmentation and impaired mitochondrial trafficking are a hallmark of T2D. These changes in mitochondrial dynamics lead to pathological responses in β-cells, skeletal muscle, adipocytes, and vessels. Abbreviations: INS, insulin; Glc, glucose; T2D, type 2 diabetes.

In summary, we know that many of the clinical complications of T2D are associated with mitochondrial fragmentation. We also know that tipping the balance toward increased mitochondrial fragmentation in mice leads to models of T2D. Furthermore, blocking DRP1 (or increasing MFNs) ameliorated hyperglycemia, dyslipidemia, and atherosclerosis in T2D models. Less clear are the mechanisms of alterations in expression and/or activity of DRP1/MFNs. Up to date, most of the studies have shown correlation between the hallmarks of T2D and increased fragmentation of mitochondria (**Table 1**). However, more studies should focus on understanding the spatiotemporal regulation of DRP1 and MFN1/2 levels during the natural progression of T2D. In this context, there are a number of open questions. For example, are there alterations on the regulation of DRP1/MFNs at the transcriptional, translational, or posttranslational level? Are DRP1/MFNs regulated by insulin, glucose, FFA signaling

TABLE 1 | Mitochondrial dynamics in T2D and cancer.

Disease	Regulatory event	Molecular pathway	Cell function	Reference
T2D	DRP1 enrichment in calcified human carotid arteries	DRP1 controls matrix mineralization, cytoskeletal rearrangement, mitochondrial dysfunction, and reduced type 1 collagen secretion and alkaline phosphatase activity	Extracellular matrix changes in cardiovascular complications	(29)
	FFA	DRP1 leads to cytC release, caspase-3 activation, and generation of ROS	Apoptosis	(27)
	Hyperglycemia	ROCK1 phosphorylates DRP1	Nepropathy	(32)
	PA	Fragmentation was associated with increased oxidative stress, mitochondrial depolarization, loss of ATP production, and reduced insulin-stimulated glucose uptake	Insulin stimulated glucose uptake in skeletal muscle	(26)
	FIS1 and DRP1 increased in T2D patients	DRP1 induced ROS, and nitric oxide synthase activation	Endothelial dysfunction	(28)
	Hyperglycemia	HG leads to DRP1-mediated fragmentation and ROS	Cellular respiration	(20)
	Inflammatory signaling (TNF-α)	TNF-α induced MiR-106b which led to MFN2 downregulation	Insulin resistance	(23)
	Insulin	Unknown	Unknown	(30)
	Dyslipidemia	MFN2 prevents accumulation of lipid intermediates, including diacylglycerol and ceramides	Insulin resistance in skeletal muscle	(24)
	Dyslipidemia	MFN2 promotes the insulin signaling pathway (INSR/IRS2/GLUT2PI3K/AKT)	Insulin resistance in liver	(25)
	Hyperglycemia	MFN2 deficiency impaired insulin signaling in muscle and liver, induced ER stress, ROS production, and JNK activation	Insulin and glucose homeostasis	(23)
Cancer	Oncogenic MAPK signaling	RasG12V or $BRAF^{V600E}$ activate ERK1/2, which then phosphorylates and activates DRP1	Mitochondria function and cell survival	(56)
	mTOR	mTORC1/4E-BP-dependent translation of MTFP1 leads to activation and recruitment of DRP1 to mitochondria	Cell survival	(58)
	Nestin	Nestin binds DRP1 and enhances DRP1 recruitment	Proliferation and invasion	(59)
	EHD1	EHD1 and Rabankyrin-5 interact with the retromer complex and induce VPS35-mediated removal of inactive DRP1 from mitochondrial membranes	Unknown	(60)
	AMPK	AMPK phosphorylates MFF, which increases DRP1 recruitment to mitochondria	Unknown	(61)
	SPOP loss-of-function mutants	SPOP mutations allow localization of INF2 to mitochondria, where it recruits DRP1	Cell migration and invasion	(62)
	SIRT4	SIRT4 inhibited Drp1 phosphorylation and weakened Drp1 recruitment to the mitochondrial membrane *via* an interaction with FIS1	Cell migration and invasion	(63)
	Estradiol	Estradiol stimulates mitochondria fission by decreasing MFN1/2 levels	Cell migration and proliferation	(66)
	Androgen	Androgens increase DRP1 expression *via* the AR	Cell proliferation	(65)

The upstream regulators of mitochondrial shape are presented along with the molecular mechanisms at play.
SPOP, speckle-type POZ protein; FFA, free fatty acid; FIS1, fission protein 1; DRP1, dynamin-related protein 1; AMPK, AMP-activated protein kinase; MFF, mitochondrial fission factor; T2D, type 2 diabetes; ER, endoplasmic reticulum; MFN1/2, fusogenic proteins mitofusin 1 and 2; AR, androgen receptor.

pathways? What are the tissue- and cell-specific differences in the regulation of mitochondrial shape in T2D? Identifying such molecular pathways controlling DRP1/MFN alterations in T2D might enable therapeutic efforts in prediabetic patients to prevent full-blown settlement of the disease.

Another question that warrants further investigation is whether genetic susceptibility variants of DRP1 or MFNs are associated with T2D. A recent study in type 1 diabetes patients identified genetic factors associated with kidney disease (35). We propose that a similar approach in T2D patients could address to what extent genomic alterations of the mitochondrial shape genes are associated with disease. A potential association between genomic alterations of mitochondrial shaping genes and T2D might allow for better screening of susceptibility and/or risk prediction of certain T2D complications.

MITOCHONDRIAL DYNAMICS IN CANCER

Recent evidence indicates that mitochondrial shape, size, and localization regulate several of the hallmarks of cancer. For instance, mitochondrial shape dynamics have been linked to metabolic adaptation, cell cycle progression (36), necroptosis (19), apoptosis (37–39), autophagy (40), tumor growth, tumor cell motility (41, 42), invasiveness, and metastasis (43). The role of mitochondrial shape changes as regulators of cancer biology is reviewed in Ref. (44). Here, we will discuss recent insights into how mitochondrial dynamics are regulated in cancer.

When considering the common alterations in mitochondria shape, we find a dichotomy between tumors with enhanced mitochondrial fragmentation versus tumors with enhanced mitochondrial fusion. For instance, hepatocellular carcinoma (45),

osteosarcoma (46), medulloblastoma (47), thyroid (42), colorectal (48), endometrial (49), and breast cancer (43) show increased mitochondrial fragmentation, due to upregulation of DRP1 levels and a concomitant reduction in MFN1/2 levels. On the other hand, tumors of the prostate (50), neuroblastoma (51), leukemia (52), glioblastoma (53), and lung (54) are associated with down-regulation of DRP1 and increased MFN1/2 levels. What could be driving these contrasting preferences of fission versus fusion of the mitochondrial networks in cancer? Plausible explanations could lie on the genomic landscape, hormonal/growth factor context, tumor microenvironmental conditions, and therapy responses of the tumors in question.

Oncogenic and tumor suppressor signaling converge on mitochondria to reprogram cellular metabolism (55); thus, the particular genomic events driving a tumor might favor mitochondrial shape changes to meet the metabolic demands of the tumor cells. According to this hypothesis, oncogene-induced metabolic reprogramming should induce changes in mitochondrial shape. Indeed, recent studies show that oncogenic RasG12V, $BRAF^{V600E}$ and MAPK/ERK (56, 57), mTOR (58) Nestin (59), and the endocytic protein EDH1 (60) increase DRP1-mediated mitochondrial fission. Similarly, the energy-sensing AMPK increased recruitment of DRP1 to mitochondria *via* phosphorylation of the MFF and (61). Speckle-type POZ protein loss-of-function mutations commonly found in primary prostate cancer were associated with increased DRP1 activation, mitochondrial fission, and prostate cancer cell invasion (62). Recently, loss of expression of the sirtuin SIRT4 was shown to lead to increased mitochondrial fragmentation (63). The signaling events that lead to DRP1 activation downstream of genomic and epigenetic alterations are summarized in **Table 1**.

In addition to the increasing number of oncogenes and tumor suppressors, growth factors and hormones regulate mitochondrial shape. Examples include Sonic Hedgehog (47), non-canonical Wnt ligands, pro-inflammatory cytokines, transforming growth factor-β, estradiol (64), and androgens (65). Estradiol promotes mitochondrial fragmentation through a reduction of MFN2 with parallel increase of FIS1 levels in ER+ breast cancer (66). From a translational standpoint, overexpression of MFN2 prevented estradiol-induced cell proliferation and motility (66). On the other hand, DRP1 is a transcriptional target of the androgen receptor, and androgen-stimulated DRP1 expression sensitizes prostate cancer cells to therapy-induced apoptosis (65). The possibility that other hormone-related malignancies exploit similar mechanisms of mitochondrial shape awaits further confirmation.

Tumor microenvironmental conditions exert yet another layer of regulation of mitochondrial shape. For instance, mitochondrial elongation is induced by nutrient deprivation in cancer cells (67). A hypoxic environment enhances mitochondrial fission in breast cancer (68) and glioblastoma (69). In this context, DRP1 was essential for hypoxia-stimulated cell motility. Indeed, silencing or expression of a dominant-negative mutant of DRP1 inhibited hypoxia-induced migration in both tumor cell models.

Finally, cancer cells also remodel their mitochondrial network in response to therapy. For instance, DRP1-mediated mitochondrial fragmentation is associated with cisplatin (68, 70), cytarabine and methotrexate (71), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (70) treatment among others. However, other therapeutic agents such as histone deacetylase inhibitors (72) produce the opposite effect, namely increased elongation of mitochondria. These opposite effects of therapy upon mitochondria morphology can be reconciled when considering the divergent signaling pathways elicited by the drugs. In the case of HDAC inhibitors, a decreased expression of FIS1 impaired DRP1 recruitment to mitochondria. These effects were independent of apoptosis induction. On the other hand, increased mitochondrial fragmentation on cisplatin and TRAIL-treated cells is coupled to apoptosis. Also worth considering, HDAC inhibitors could have additional roles in regulating mitochondrial morphology, due to non-histone-acetylating activity (acetylation of non-histone proteins, regulation of signaling kinases). A final consideration is the influence of the genomic background and tumor microenvironment on eliciting fission versus fusion upon therapy.

In summary, emerging evidence suggests that the contribution of the mitochondrial shaping genes to tumor cell biology is tumor type dependent and may reflect the genetic makeup, hormonal/growth factor context, tumor microenvironment conditions, and therapy responses of the tumor. Future efforts should aim to integrate these novel regulatory pathways and reach a comprehensive picture of the regulation of mitochondrial shape and function in cancer. Second, more emphasis should be directed toward identifying metabolic-dependent versus -independent functions of DRP1 and MFNs in cancer. For instance, which of the phenotypes associated with DRP1 activation in cancer are explained on basis of metabolism (increased glycolysis versus respiration)? Is it DRP1's function on apoptosis (or mitochondrial localization) also important? A third area of interest for future research would be the development of anti-cancer therapies targeting mitochondrial dynamics. Encouraging fresh evidence indicates that modulating mitochondria morphology enhances anti-cancer therapies (73), particularly death receptor ligands (74–76) and antimitotic drugs (77).

TARGETING MITOCHONDRIAL DYNAMICS

The involvement of DRP1-mediated fission in disparate diseases settings has fueled the development of pharmacological approaches to inhibit mitochondrial fission. Mitochondrial division inhibitor-1 (mdivi-1) selectively impairs the GTPase activity of DRP1, without affecting the activity of dynamin-1, MFN1/2, or OPA1 (78). The mechanism of action of mdivi-1 involves allosteric binding and stabilization of a conformational form of unassembled DRP1 that cannot polymerize. mdivi-1 treatment induces rapid mitochondrial fusion, dampens ROS production and increases ATP production. Interestingly, the original report described a second function of DRP1 in mitochondrial outer membrane polarization (MOMP). DRP1 facilitated BAX/BAK-dependent MOMP in response to C8-BID or staurosporine, independently of mitochondrial fragmentation. Thus, mdivi-1 impaired staurosporine-induced apoptosis (78). Interestingly, mdivi-1 can induce apoptosis in DRP1-KO cells (79), suggesting that mdivi-1 has off-target effects. In contrast to these initial studies in which mdivi-1 prevented apoptosis, later studies showed

that mdivi-1 sensitized cells to TRAIL-dependent apoptosis (74). This potentiation of apoptosis by mdivi-1 occurred through activation of mitochondrial and ER apoptosis pathways. Thus, these controversial results suggest that mdivi-1 can act either as pro- or anti-apoptotic pharmacologic agent, depending on the cell types and apoptotic stimuli in question (80).

In T2D models, mdivi-1 prevented mitochondrial fragmentation, oxidative stress and inflammation, and improved endothelial cell function (31). Another study showed that mdivi-1 prevented HG-stimulated insulin secretion in mouse and human islets (21). Furthermore, mdivi-1 rescued palmitate-induced mitochondrial dysfunction and ROS generation, as well as insulin resistance in skeletal muscle (26). Inhibition of Drp1 with mdivi-1 improved mitochondrial function and cardiac function in a model of myocardial ischemia/reperfusion of diabetic hearts (34).

In cancer cells, DRP1 inhibition has been shown to modulate therapy sensitivity, tumor metabolism, growth, and invasiveness. For instance, mdivi-1 suppressed mitochondrial autophagy, metabolic reprogramming, cancer cell viability, and motility of breast cancer cells (81). In regards to therapy modulation, mdivi-1 potentiated TRAIL-induced apoptosis in melanoma (74, 76) and ovarian cancer models (75). Furthermore, mdivi-1 induced cell death (75) and synergized apoptotic effects of platinum agents in drug resistant ovarian tumor cells (79). However, mdivi-1 prevents apoptosis induced by cisplatin in breast cancer (68) and leukemia (52). As discussed above, these controversial results suggest that mdivi-1 can act either as pro- or anti-apoptotic agent, depending on the cell types and apoptotic stimuli in question [reviewed in Ref. (80)]. Further investigations should address the precise mechanisms dictating the differential effects of mdivi-1 on cell survival.

Regarding the potential utility of mdivi-1 in the clinic, a number of questions remain open. For instance, what are the consequences of sustained *in vivo* inhibition of mitochondrial fission? What are the pharmacokinetics and cytotoxicity profiles for mdivi-1? Another point to consider is that mdivi-1 has poor solubility in water (80). This fact might limit the utility of mdivi-1 and might open the door for the design of new DRP1 inhibitors with improved solubility, specificity, and potency. In this regard, another pharmacological agent targets the recruitment of DRP1 to mitochondria *via* its interaction with FIS1. The small peptide inhibitor P110 blocks DRP1/FIS1 binding (82) and has shown promising results in neurodegenerative disease models. When tested in hepatocellular carcinoma, P110 blocked cell proliferation *in vitro* and *in vivo* (83). Future research will be needed to evaluate the utility of P100 both in T2D and cancer models.

CONCLUSION

Given the metabolic alterations that are a hallmark of both T2D and cancer, it is not surprising that mitochondrial alterations are a shared feature in these disparate diseases. Over the past few years, we have learnt that mitochondria are not static, solitary organelles, but they rather undergo constant changes in morphology and subcellular distribution to meet the metabolic demands of the cell. Defects in mitochondrial dynamics play a role in the molecular and cellular pathogenesis of both T2D and cancer. Now, how similar or different are these two pathologies in regards to mitochondrial dynamics? In T2D, the literature unanimously reports an increase of mitochondrial fission mediated by DRP1. In cancer, most tumors follow this same pattern of increased DRP1-mediated mitochondrial fission. However, although less frequently, tumors might display augmented mitochondrial fusion *via* an increase of MFN1/2 levels and/or activity. How are these differences and similarities in the mitochondrial network explained at the molecular level? Up to date, most of the studies have shown correlation between T2D and altered mitochondrial shape. More studies should focus on understanding the spatiotemporal regulation of DRP1 and MFN1/2 levels and activity during the natural progression of T2D. Likewise, there is limited information on how the genetic, epigenetic, and microenvironmental factors influence mitochondrial dynamics, or which signaling pathways integrate extracellular stimuli with mitochondrial shape in T2D. Thus, due to this limited information, is not possible to conclude if T2D and cancer utilize similar or divergent mechanisms of control of mitochondrial shape. In this regard, it would be interesting to address how metabolic pathways commonly altered both in T2D and cancer impinge on mitochondrial morphology. Examples of such pathways include PI3K/AKT and AMPK. Another question that warrants further investigation is whether other aspects of mitochondrial biology are dysregulated in these diseases. For instance, are there alterations in mitochondrial quality control, mitochondria crosstalk to other organelles, or mitochondrial localization present in both T2D and cancer?

Regarding the use of DRP1 inhibitors as anti-T2D and -cancer agents, further studies should determine long-term effects of targeting mitochondrial dynamics *in vivo*, and establish the pharmacokinetics and cytotoxicity profiles for mdivi-1. In addition, the involvement of potential compensatory or resistance mechanisms to mdivi-1 has not been explored yet and should be addressed in the future. An area in need of further investment is the development of selective MFN1/2 inhibitors. Despite the existence of a few DRP1 inhibitors, there is no equivalent therapeutic agent to target fusion. The fact that several tumors show increased fusion might warrant further effort in this area.

AUTHOR CONTRIBUTIONS

MW performed literature search and review; MC conceived the project, designed the figures, and wrote the paper.

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REVIEW
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The Role of RNA Editing in Cancer Development and Metabolic Disorders

Che-Pei Kung[1,2]*, *Leonard B. Maggi Jr.*[1,2] and *Jason D. Weber*[1,2,3]*

[1] ICCE Institute, Washington University School of Medicine, Saint Louis, MO, United States, [2] Division of Molecular Oncology, Department of Medicine, Washington University School of Medicine, Saint Louis, MO, United States, [3] Siteman Cancer Center, Department of Cell Biology and Physiology, Washington University School of Medicine, Saint Louis, MO, United States

Numerous human diseases arise from alterations of genetic information, most notably DNA mutations. Thought to be merely the intermediate between DNA and protein, changes in RNA sequence were an afterthought until the discovery of RNA editing 30 years ago. RNA editing alters RNA sequence without altering the sequence or integrity of genomic DNA. The most common RNA editing events are A-to-I changes mediated by adenosine deaminase acting on RNA (ADAR), and C-to-U editing mediated by apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1). Both A-to-I and C-to-U editing were first identified in the context of embryonic development and physiological homeostasis. The role of RNA editing in human disease has only recently started to be understood. In this review, the impact of RNA editing on the development of cancer and metabolic disorders will be examined. Distinctive functions of each RNA editase that regulate either A-to-I or C-to-U editing will be highlighted in addition to pointing out important regulatory mechanisms governing these processes. The potential of developing novel therapeutic approaches through intervention of RNA editing will be explored. As the role of RNA editing in human disease is elucidated, the clinical utility of RNA editing targeted therapies will be needed. This review aims to serve as a bridge of information between past findings and future directions of RNA editing in the context of cancer and metabolic disease.

Keywords: RNA editing, ADAR, APOBEC1, cancer, metabolic disease

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Veronica Vella,
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Marco Ghezzi,
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Luis Del Valle,
LSU Health Sciences Center New Orleans, United States

Correspondence:
Che-Pei Kung
patkung@wustl.edu
Jason D. Weber
jweber@wustl.edu

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INTRODUCTION

Genetic complexity, or plasticity, is the foundation to develop complicated biological functions in living organisms. To maximize the versatility of limited amounts of genetic material, a variety of changes take place at the genomic level, including RNA metabolism and modification (1). RNA is involved in some of the most evolutionarily conserved cellular processes, including transcription and translation. The mechanisms of RNA regulation, including modification, processing and degradation, have been extensively studied. Among these mechanisms, site-specific substitution of RNA, or "RNA editing," has garnered increasing attention in recent years, despite its discovery more than 30 years ago.

The year of 1987 marked the first milestone for the journey of RNA-editing. A cytidine (C) to uridine (U) conversion in the mRNA of human apolipoprotein B (apoB) was identified to

be responsible for the production of a shorter version of apoB (apoB48) by creating a new stop codon (2). This alteration is mediated by an enzyme complex that contains the catalytic deaminase, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1) (3).

Meanwhile, a curious phenomenon of destabilization of double-stranded RNA was observed during the early embryogenesis of *Xenopus laevis* (4, 5). Hoping to use antisense RNA inhibition to study genetic factors in the embryonic development, investigators were surprised to learn that the same technique that works well in early-stage oocytes was not successful in later-stage oocytes and embryos due to failed formation of RNA duplex. This observation prompted speculations of a RNA-unwinding mechanism that either controls RNA stability or helps RNAs shape their secondary structures. It was further characterized by the loss of RNA's base-pairing properties and attributed to the conversion of adenosines (A) to inosines (I), an activity later found to be mediated by members of the adenosine deaminase acting on RNA (ADARs) family (6–8).

In the last 30 years, the physiological functions of APOBEC and ADAR protein family members have been gradually revealed (9, 10). These RNA editing enzymes (referred to as editases herein) can shuttle between the nucleus and cytoplasm, and homodimerization is required for their catalytic activity. APOBEC-mediated RNA editing has been implicated in maintaining homeostasis in digestive organs, such as the liver and small intestine, while ADAR-mediated RNA editing is thought to play a crucial role in regulating the innate immune response to infection. More recently, next generation sequencing has expedited the identification of specific RNA-editing targets and their associated functional consequences in human diseases.

The functional impact of RNA editing on cell biology is demonstrated through (i) changing amino acid sequences of proteins (recoding); (ii) altering splicing patterns of pre-mRNA; (iii) causing changes in seed sequences of microRNAs (miRNAs) or in sequences of miRNA targeting sites; and (iv) influencing the stability of targeted RNAs (9, 10) (**Figure 1**).

This review aims to (1) provide a summary of recently identified RNA-editing events that regulate both cancer development and metabolic dysfunctions, (2) highlight the existing gaps in our knowledge of RNA-editing mechanisms, and (3) describe the potential implications for the development of novel therapeutic approaches to regulate RNA editing.

RNA Editing in Cancer Development

Biogenesis of RNAs and RNA-regulated functions have been well-established in playing important roles in tumorigenesis (11). With the ability to change DNA-encoded genetic information after transcription, deregulated RNA-editing could be an important contributor in cancer development. Studies of RNA editing in a variety of cancer types (mostly in the context of A-to-I editing) have generated conflicting reports regarding the exact role RNA-editing plays.

The consistent finding from these reports is that RNA editing is a common phenomenon in cancer helping to drive transcriptomic and proteomic diversity, and overall levels of RNA editing mirror the expression levels of editases in cancers compared to normal tissues (ex. overexpression = general hyper-editing; reduced expression = general hypo-editing) (12–15). In contrast, the relationship between the overall editing level and tumorigenic potential of cancers appears to be unsettled. Increased level of RNA editing has been found to correlate with enhanced tumorigenesis in some cancers but reduced tumorigenesis in others, sometimes with both correlations in the same cancer type (12–14, 16–18).

These conflicting reports suggest that the relationship between RNA editing and cancer development is complicated and potentially influenced by other factors such as the origin, stage and microenvironment associated with the studied cancer. Instead of attempting to connect an individual cancer with the global level of RNA editing, connecting specific RNA editing events to cancer-related functions could prove to be more informative.

ADAR1

Currently, three *ADAR* gene family members have been identified and studied for their RNA-editing functions: ADAR1 (encoded by *ADAR*), ADAR2 (encoded by *ADARB1*) and ADAR3 (encoded by *ADARB2*) (9).

The mRNAs of multiple proteins have been identified as direct targets of ADAR1 and undergo nonsynonymous amino-acid substitutions associated with cancer development (**Figure 2**). In hepatocellular carcinoma (HCC), esophageal squamous cell carcinoma (ESCC), colorectal cancer (CRC) and breast cancer (BC), overexpression of ADAR1 leads to the creation of an oncogenic version of antizyme inhibitor 1 (AZIN1; S367G). Edited AZIN1 is stabilized and serves as an analog of ornithine decarboxylase (ODC) to block antizyme-mediated degradation of ODC and cyclin D1. Accumulations of ODC and cyclin D1 lead to increased cell proliferation and metastatic potential, as well as tumor initiating capacity (17, 19–21). In cervical cancer (CC), ADAR1 promotes tumorigenesis by editing multiple sites within the YXXQ motif of bladder cancer-associated protein (BLCAP), a tumor suppressor. Edited BLCAP loses its ability to interact with and inactivate signal transducer and activator of transcription 3 (STAT3), resulting in increased cell proliferation (22).

ADAR1 can exert anti-tumorigenic activities through RNA editing-mediated protein recoding, too. In BC, $GABA_A$ receptor alpha 3 (GABRA3) activates the Akt pathway and promotes cell migration, invasion and metastasis. ADAR1-mediated editing of GABRA3 (I342M) reduces its cell surface expression and suppresses Akt activation and metastatic potential of cancer cells (23).

A recent study revealed a novel function of ADAR1 in cancer-associated immune environment. In subsets of tumor samples, including ovarian cancer (OC), melanoma and BC, increased levels of ADAR1-edited peptides are presented by human leukocyte antigen (HLA) molecules (24). Presentations of these edited peptides, such as cyclin I (CCNI; R75G), elicit antigen-specific killing of tumor cells through cytotoxic ($CD8^+$) T cells. It presents an intriguing possibility to explore immunotherapeutic approaches utilizing information of RNA editing. Alternatively, it

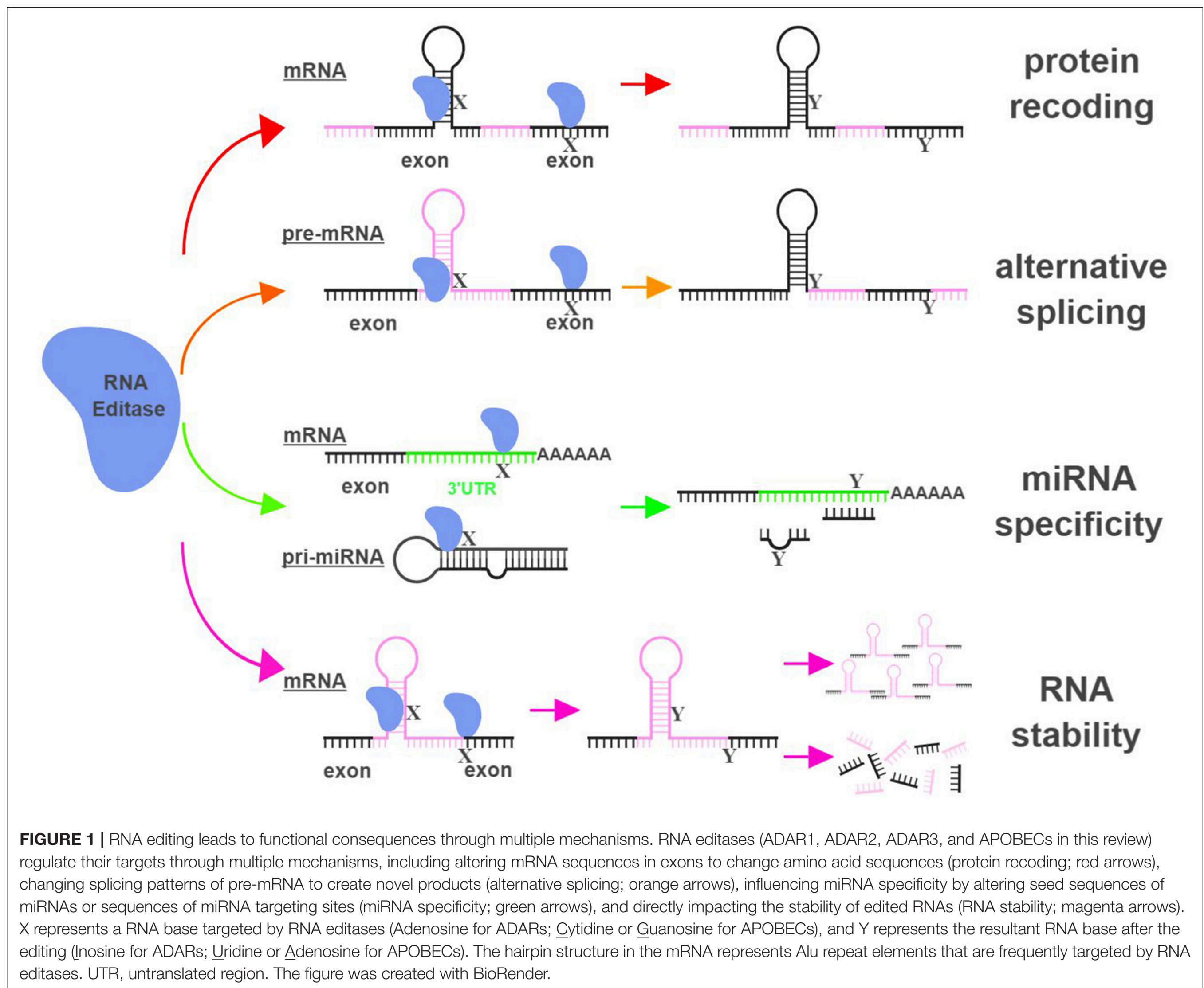

FIGURE 1 | RNA editing leads to functional consequences through multiple mechanisms. RNA editases (ADAR1, ADAR2, ADAR3, and APOBECs in this review) regulate their targets through multiple mechanisms, including altering mRNA sequences in exons to change amino acid sequences (protein recoding; red arrows), changing splicing patterns of pre-mRNA to create novel products (alternative splicing; orange arrows), influencing miRNA specificity by altering seed sequences of miRNAs or sequences of miRNA targeting sites (miRNA specificity; green arrows), and directly impacting the stability of edited RNAs (RNA stability; magenta arrows). X represents a RNA base targeted by RNA editases (Adenosine for ADARs; Cytidine or Guanosine for APOBECs), and Y represents the resultant RNA base after the editing (Inosine for ADARs; Uridine or Adenosine for APOBECs). The hairpin structure in the mRNA represents Alu repeat elements that are frequently targeted by RNA editases. UTR, untranslated region. The figure was created with BioRender.

also poses a potential mechanism that cancers can hijack in order to avoid effective immune surveillance.

The boundary between pro-tumorigenic and anti-tumorigenic functions of ADAR1 can be blurry even with the same amino-acid-sequence-altering event. The glioma-associated oncogene 1 (GLI1) activates the Hedgehog (HH) signaling pathway to promote cell proliferation (25). In multiple myeloma (MM), amplified ADAR1 edits GLI1 (R701G) and stabilizes GLI1 expression by preventing the binding of its negative regulator, suppressor of fused (SUFU). Edited GLI1 displays higher transcriptional activity to drive HH signaling and promote malignant regeneration and drug resistance of MM (26). Interestingly, this exact same editing event has the opposite effect in medulloblastoma (MB) and basal cell carcinoma (BCC) to inhibit tumorigenesis. ADAR1-edited GLI1 (R701G), despite its resistance to SUFU binding, also becomes much less accessible to one of its activators, Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A). The net result is reduced oncogenic potential of edited GLI1 in MB and BCC (27).

In metastatic melanoma, ADAR1 confers tumor-suppressive activities by editing miRNA sequences to alter their target specificity. ADAR1-mediated editing of miR455-5p results in lack of inhibition of the tumor suppressor cytoplasmic polyadenylation element-binding protein 1 (CPEB1), while edited miR378a-3p targets the oncogene, α-Parvin, for downregulation (28, 29). During the course of melanoma progression, transcriptional repressors of ADAR1, such as cyclic AMP-responsive element binding protein (CREB), are upregulated to reduce ADAR1 expression to promote malignancy.

Not surprisingly, ADAR1 is also capable of hijacking the miRNA biogenesis process to promote tumorigenesis. In blast crisis chronic myeloid leukemia (BC CML), JAK2 activation and BCR-ABL1 amplification was shown to increase ADAR1 expression promoting leukemia stem cell (LSC) self-renewal (30).

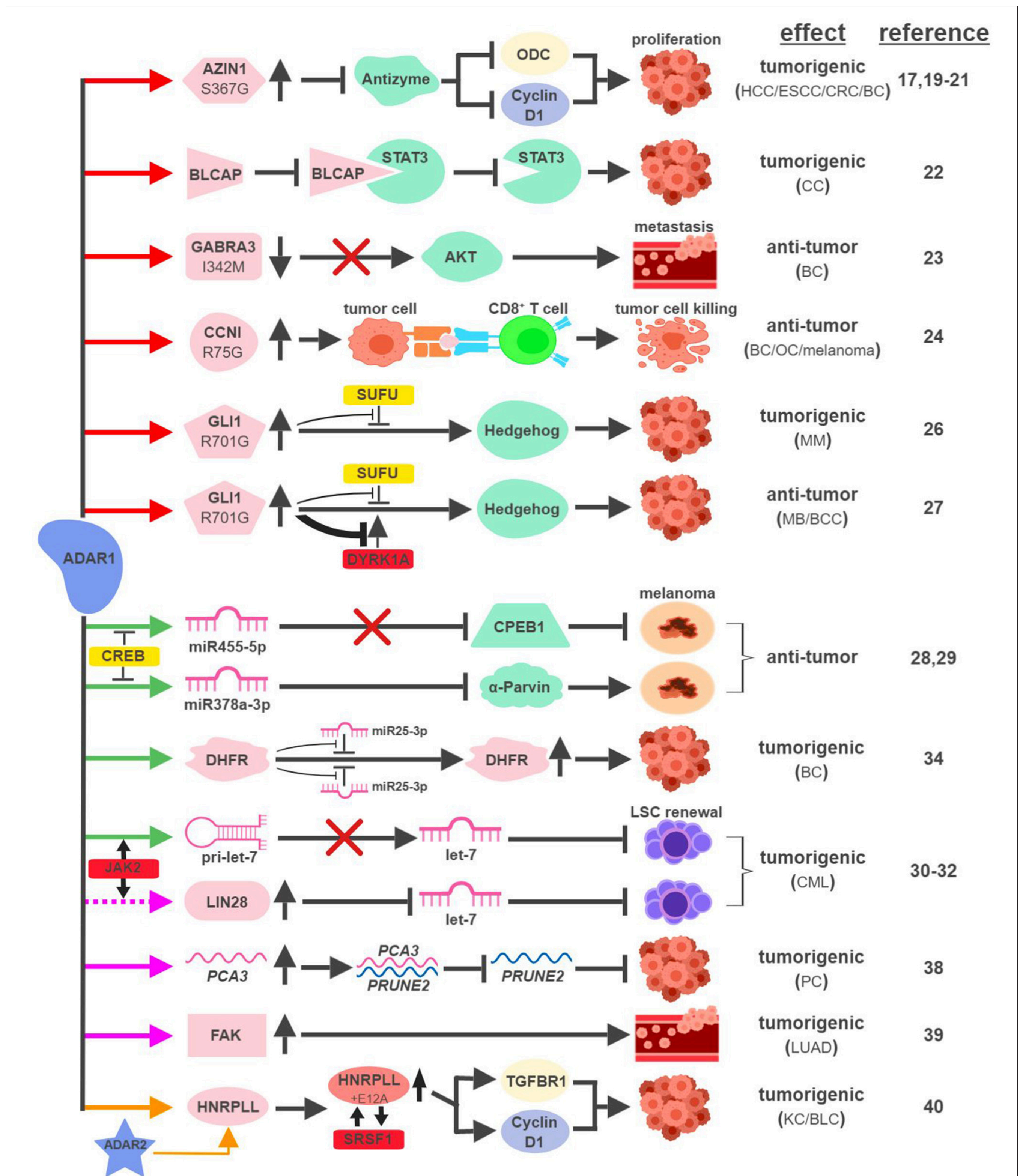

FIGURE 2 | ADAR1-mediated RNA editing in cancer development. The color of the first arrow in each pathway indicates the mechanism (refer to **Figure 1**) by which ADAR1 regulates its direct targets, depicted in pink icons or shapes. Dashed lines indicate suggested/unproven functions/relationships. Additional activators and inhibitors of specific pathway steps are depicted in red and yellow rounded rectangles, respectively. Specific diseases and phenotypes/functions affected by ADAR1-mediated RNA editing are only labeled in the first appearance (ex. proliferation…etc). HCC, hepatocellular carcinoma; ESCC, esophageal squamous cell

(Continued)

FIGURE 2 | carcinoma; CRC, colorectal cancer; BC, breast cancer; CC, cervical cancer; OC, ovarian cancer; MM, multiple myeloma; MB, medulloblastoma; BCC, basal cell carcinoma; CML, chronic myeloid leukemia; PC, prostate cancer; LUAD, lung adenocarcinoma; KC, kidney cancer; BLC, bladder cancer. The figure was created with BioRender.

A follow-up study revealed that ADAR1 accomplishes this feat by reducing the expression of the tumor-suppressive miRNA let-7 (31). Mechanistically, overexpression of ADAR1 promotes the induction of the pluripotency gene *LIN28* and the editing of the primary miRNA pri-let-7 at multiple sites, both events reduce the production of let-7 family members (32, 33).

In addition to altering miRNAs directly, ADAR1 also edits miRNA targets to affect their susceptibility to miRNA-mediated repression. ADAR1 was shown to edit over two dozen sites on the three prime untranslated region (3'UTR) of dihydrofolate reductase (DHFR). In BC, edited DHFR becomes resistant to the targeting of miR25-3p and miR125a-3p. As a result, the protein levels of edited DHFR increase to promote cell growth and resistance to chemotherapeutic agents like methotrexate (34).

ADARs have been shown to regulate RNA stability through a variety of mechanisms, including alterations of subcellular localization or changing the secondary structure of edited RNAs (35–37). Although the detailed molecular processes remain elusive, several recent studies highlighted ADAR1's role in this capacity to impact cancer development. In prostate cancer (PC), ADAR1-mediated editing of *prostate cancer antigen 3* (*PCA3*), an intronic long noncoding RNA, increases its stability and expression. *PCA3* acts as a dominant-negative oncogene and forms a double-stranded RNA with precursor mRNA (pre-mRNA) of *prune homolog 2* (*PRUNE2*), a tumor suppressor gene (38). The formation of the *PCA3-PRUNE2* complex promotes tumorigenesis in cell and mouse models through the downregulation of PRUNE2, whose expression also inversely correlates with *PCA3* in human PC samples.

ADAR1-mediated RNA editing also occurs within the introns of protein-coding RNAs. One such example involves an important facilitator of tumor metastasis, focal adhesion kinase (FAK). In lung adenocarcinoma (LUAD), the most common form of non-small cell lung cancer (NSCLC), ADAR1-mediated editing of intron 26 of FAK results in increased stabilization of FAK mRNA and protein. Induction of FAK contributes to cell invasiveness and is associated with tumor recurrence in LUAD patients (39).

A recent study linking ADAR1 to intron-editing showed that ADAR1 edits the intron of heterogeneous nuclear ribonucleoprotein L-like (HNRPLL) to create an additional exon (E12A) for *HNRPLL* (40). E12A-containing *HNRPLL* acts as an enhancer of oncogenic splicing factor serine/arginine rich splicing factor 1 (SRSF1), resulting in a positive feed-back loop to increase the abundance of E12A-containing *HNRPLL* transcript. E12A-containing *HNRPLL* regulates expressions of cyclin D1 and transforming growth factor beta receptor 1 (TGFBR1) to promote cell proliferation in kidney and bladder cancers. Interestingly, this editing event is also mediated by ADAR2, pointing to potential interactions between ADAR1- and ADAR2-mediated RNA editing.

ADAR2

First cloned in 1996, ADAR2 is the second identified A-to-I RNA editase that is also capable of editing itself (41, 42). ADAR2 was first identified as the main RNA editase of glutamate receptor subunit B (GluR-B) (**Figure 3**). Underediting of GluR-B (Q607R) results in early-onset epilepsy in a mouse model (43). Mice with mutant ADAR2 are also seizure-prone and experience early postnatal death, establishing the functional significance of ADAR2-mediated editing of GluR-B (44, 45). The translational impact of this connection was found in malignant human brain tumors in both adults and children, where ADAR2-mediated editing of GluR-B is reduced compared to control samples. Although brain tumors are not present in ADAR2-mutant mice, likely due to early postnatal death, these observations potentially explain the aggressive nature of these cancers and neurologic symptoms suffered by human patients (16, 46).

These early studies inspired mechanistic investigations to directly link ADAR2 with malignant brain tumors such as high-grade astrocytoma or glioblastoma multiforme (GBM). The same team that made the initial connection between ADAR2-mediated RNA editing and brain tumors identified several pathways downstream of ADAR2 regulating GBM pathogenesis. By editing multiple sites within intron 7 of the CDC14B phosphatase pre-mRNA, ADAR2 promotes upregulation of CDC14B, subsequently causing the degradation of E3-ligase S-phase kinase-associated protein 2 (SKP2) (47, 48). In GBM, downregulation of ADAR2-mediated RNA editing of CDC14B results in overexpression of SKP2. Increased level of SKP2 then leads to ubiquitin-mediated degradation of cell cycle inhibitors, $p27^{Kip1}$ and CDKN1A/$p21^{Cip1/Waf1}$, to promote cell cycle progression and tumorigenesis.

Interestingly, ADAR2-mediated downregulation of p27 is also connected to ADAR2's ability to edit selected miRNAs in the brain. ADAR2-mediated editing reduces expression of oncogenic miRNAs, such as miR21 and miR221/222. In GBM, failed editing/reduction of miR21 and miR221/222 lead to downregulation of their respective targets, tumor suppressors programmed cell death protein 4 (PDCD4) and $p27^{Kip1}$ (49).

ADAR2 was found to edit numerous miRNAs, regulating tumorigenesis by balancing the functions of oncogenic and tumor-suppressive miRNAs (49, 50). This "balancing act" of ADAR2 can also be achieved by switching miRNAs between their oncogenic and tumor-suppressive activities via altering target specificities. The first such example was demonstrated by ADAR2's ability to edit miR376a to inhibit GBM progression (51). ADAR2-edited miR376a targets and downregulates autocrine motility factor receptor (AMFR) to inhibit tumor migration and invasion. Unedited miR376a, however, switches its affinity from AMFR to Ras-related protein RAP2A, a tumor suppressor protein acting on actin

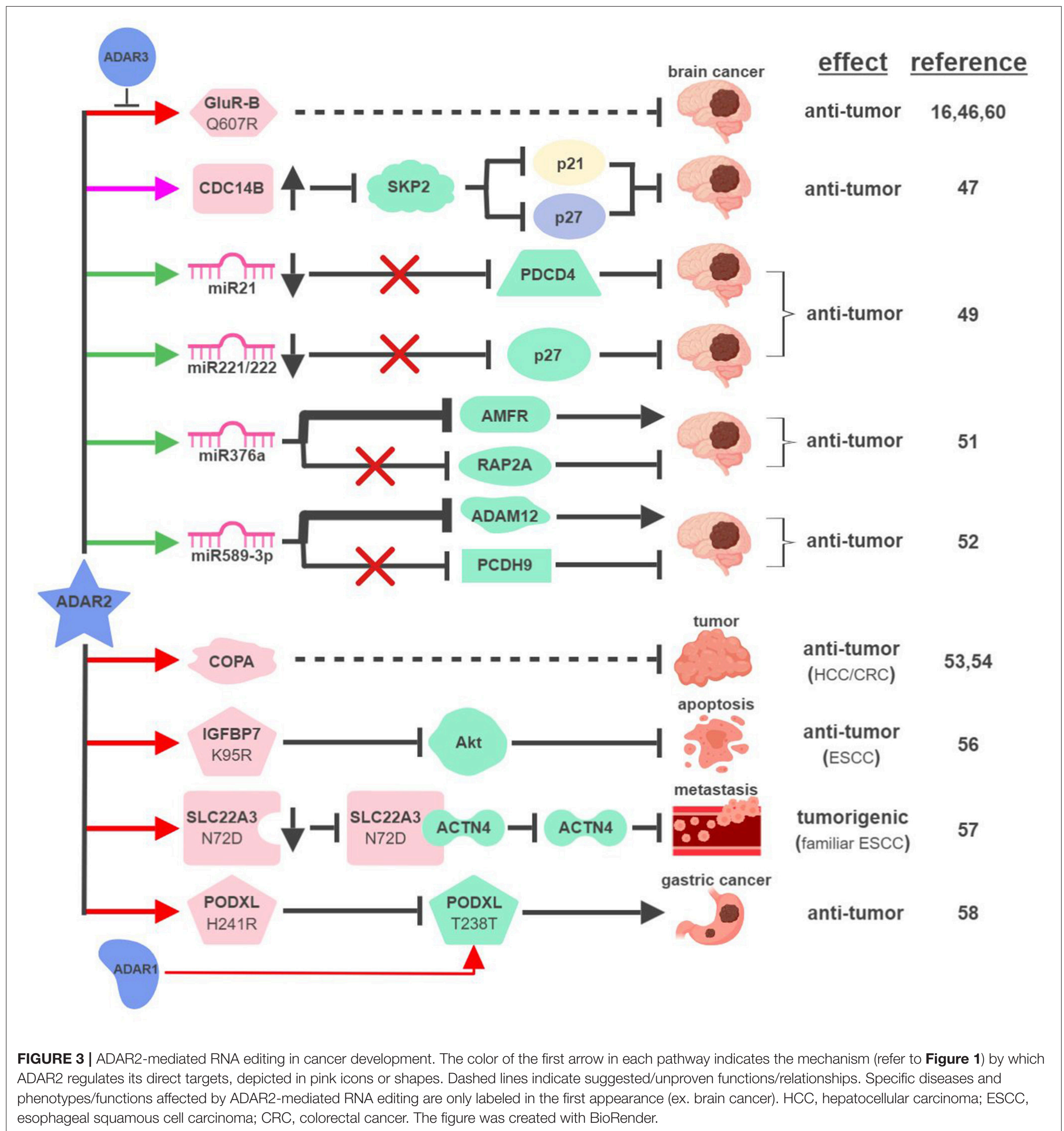

FIGURE 3 | ADAR2-mediated RNA editing in cancer development. The color of the first arrow in each pathway indicates the mechanism (refer to **Figure 1**) by which ADAR2 regulates its direct targets, depicted in pink icons or shapes. Dashed lines indicate suggested/unproven functions/relationships. Specific diseases and phenotypes/functions affected by ADAR2-mediated RNA editing are only labeled in the first appearance (ex. brain cancer). HCC, hepatocellular carcinoma; ESCC, esophageal squamous cell carcinoma; CRC, colorectal cancer. The figure was created with BioRender.

remodeling. In GBM, deactivated ADAR2 flips the switch through miR376a to tilt the balance toward malignant tumor progression.

A recent study revealed another ADAR2-controlled switch through miRNA editing in GBM. In normal brain tissues, ADAR2 edits nearly 100% of miR589-3p to target and reduce expression of disintegrin and metalloproteinase domain-containing protein 12 (ADAM12), a metalloprotease that promotes cancer metastasis. In high-grade GBM, editing of miR589-3p decreases dramatically and unedited miR589-3p targets protocadherin 9 (PCDH9), a tumor suppressor protein, instead (52). These studies likely represent only a small

percentage of such "switches" regulating pathogenesis of GBM and other cancers.

In addition to GBM, ADAR2 also displays tumor-suppressive functions in other types of cancer. ADAR2 is downregulated in ∼50% of HCC and overexpression of ADAR2 in ADARs-deficient HCC cells reduces their oncogenic potential (53). ADAR2's HCC-suppressing function is thought to be mediated through editing coatomer protein complex subunit α (COPA) mRNA (I164V), whose editing level inversely correlates with HCC pathogenesis. The lack of ADAR2-mediated editing of COPA was also observed in aggressive subtypes of CRC with an epithelial-to-mesenchymal (EMT) phenotype leading to liver metastasis (54).

Curiously, this seemingly straightforward assertion of "ADAR2 edits COPA to suppress tumorigenesis" is complicated by recent studies showing that (i) ADAR2 is overexpressed in a small subset of HCC (55); and (ii) edited COPA has been shown to promote a malignant phenotype of BC cells *in vitro* (15). This suggests that RNA-editing mechanism is subject to complex regulations to contribute to cancer-specific phenotypes.

ADAR2 can also serve as a dual-role regulator in esophageal cancers. In ESCC where ADAR2 is downregulated, reduced editing and expression of insulin-like growth factor-binding protein 7 (IGFBP7; K95R) lead to activation of Akt and inhibition of programmed cell death (56). In contrast, ADAR2 is a potential predisposing factor in familiar ESCC. Elevated levels of ADAR2 lead to editing and reduction of solute carrier family 22 member 3 (SLC22A3; N72D), which in turn promotes metastasis in familial ESCC by inhibiting the interaction between SLC22A3 and its inhibitory target α-actinin-4 (ACTN4), an actin-binding protein facilitating filopodia formation (57).

A recent study described an interesting "tug-of-war" relationship between ADAR2 and ADAR1 in gastric cancer (GC). Unedited podocalyxin-like protein (PODXL) promotes GC tumorigenesis by regulating cell adhesion mechanisms. PODXL can be targeted by both ADAR1 and ADAR2 to cause synonymous (ACA>ACG; T238T) and nonsynonymous (CAC>CGC; H241R) amino acid substitutions, respectively. In normal tissues, high ratio of ADAR2/ADAR1-mediated editing keeps PODXL in its edited form (H241R) preventing oncogenic onset. In GC, amplification of ADAR1 and reduction of ADAR2 cause imbalanced editing of PODXL to accumulate unedited PODXL to drive tumorigenesis (58).

ADAR3

Few studies have focused on investigating ADAR3's functional importance in RNA editing. ADAR3 shares significant sequence and structural similarities with ADAR1 and ADAR2, including nuclear localization, deaminase, and RNA-binding domains (59). Despite these similarities, ADAR3 has not been shown to display deaminase activity to influence physiological functions such as cancer development. A recent study demonstrated that ADAR3 may instead act as a dominant-negative form of other ADARs. Overexpressed in GBM compared to normal brain tissues, ADAR3 competes with ADAR2 for binding and editing of GluR-B through its RNA-binding domain (60). Given the observation that ADAR3 is expressed primarily in the brain, it is reasonable to speculate that ADAR3 regulates tumorigenesis of brain cancers by modulating ADAR2/ADAR1-mediated RNA editing (61).

APOBECs

In the human genome, there are eleven genes that belong to the APOBEC protein family. Functionally, APOBECs are cytidine deaminases and evolutionally conserved in vertebrates. The majority of research activities regarding APOBECs have focused on their ability to restrict viral infections by creating mutational imbalances in the viral genome (62). Recent developments, however, identified several APOBEC proteins, particularly APOBEC3 subfamily members (A3A, A3B, and A3H), being capable of catalyzing hypermutations in cancers to drive tumorigenesis and therapy resistance (63, 64). Interestingly, these connections have mostly been made with APOBECs' ability to modify single-stranded DNA instead of mRNA, despite what their names indicate (65).

So far, APOBEC1 is the most well-established APOBEC member that displays RNA editing activities (**Figure 4**). The first direct connection between APOBEC1-mediated RNA editing and tumorigenesis was made in 1995, when transgenic rabbits and mice expressing rabbit APOBEC1 in livers developed HCC (66). This outcome was found to be associated with hyperediting and reduced expression of a translational repressor NAT1 (novel APOBEC1 target no.1; also known as eukaryotic translation initiation factor 4 gamma 2, or EIF4G2), which regulates the expression of cell cycle inhibitor CDKN1A/p21$^{Cip1/Waf1}$ (67, 68). APOBEC1 was also shown to bind the 3′UTR of *c-myc* mRNA to increase its stability (69). These results suggest that APOBEC1 might affect expression levels of tumor-associated genes via its RNA-binding and –editing capabilities.

Similar to its ability to edit *apoB* mRNA to create a truncated form apoB48 (Q2153Stop), APOBEC1 was found to edit the mRNA of neurofibromin 1 (NF1) to generate a truncated NF1 (R1306Stop) in a subset of peripheral nerve–sheath tumor (PNSTs) samples (70). It results in inhibition of the tumor-suppressor function of NF1, and could be responsible for development of neuronal tumors associated with Neurofibromatosis (NF) Type 1 (71, 72). Interestingly, increased expression of APOBEC1 and editing of NF1 were also found in CRC, suggesting that other tumor types could also be affected by this pathway (73).

Unconventional G-to-A RNA edits were identified in the mRNA of Wilms Tumor 1 (WT1) (74). These modifications increase in non-progenitor umbilical cord blood mononuclear cell samples (CBMCs) compared to acute myeloid leukemia (AML), implicating their roles in tumorigenesis. RNA interference screening identified APOBEC3A as the responsible RNA editase, opening up the possibility to investigate the relationships between all APOBEC members and cancer-associated RNA editing events. Interestingly, a functionally-important RNA conversion between C and U was also observed in WT1 in rat kidney during development (75). It is unclear which RNA editase is responsible for this conversion, but the fact that APOBEC1 is the only known C-to-U editase

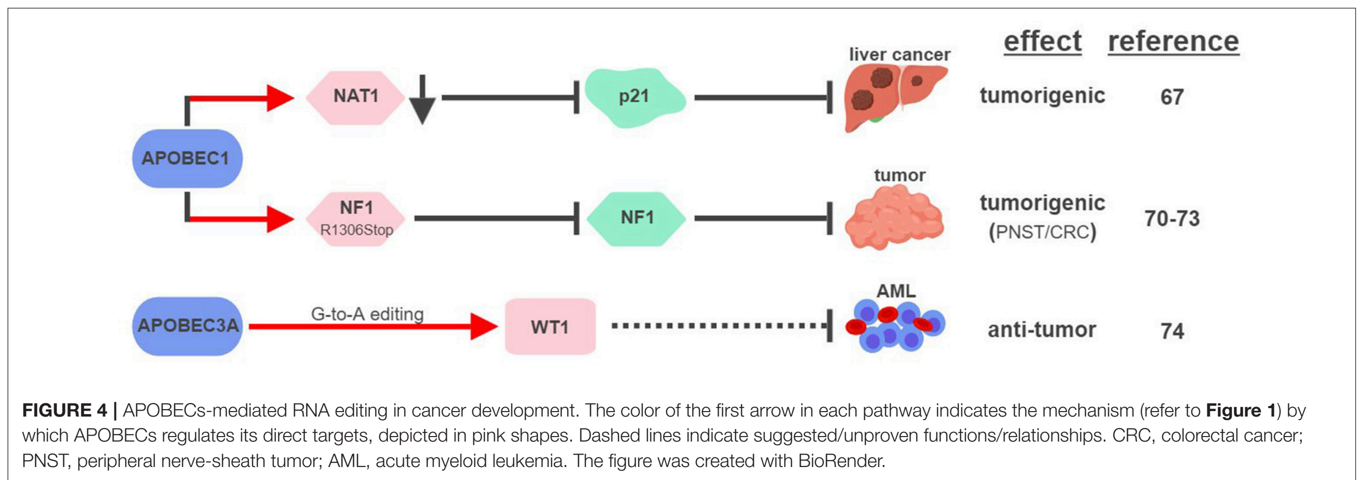

FIGURE 4 | APOBECs-mediated RNA editing in cancer development. The color of the first arrow in each pathway indicates the mechanism (refer to **Figure 1**) by which APOBECs regulates its direct targets, depicted in pink shapes. Dashed lines indicate suggested/unproven functions/relationships. CRC, colorectal cancer; PNST, peripheral nerve-sheath tumor; AML, acute myeloid leukemia. The figure was created with BioRender.

suggests that WT1 could be subject to RNA editing mediated by multiple APOBECs.

RNA Editing in Metabolic Functions and Disorders

The fact that RNA editases (ADARs and APOBEC1) are expressed in major metabolic organs, such as the liver and pancreas, offers an initial clue that RNA-editing might play an important role in metabolic regulation (3, 76, 77). Deep-sequencing data collected longitudinally from one individual predisposed to and diagnosed with type 2 diabetes suggested that RNA-editing could have predictive and diagnostic values across healthy and diseased states (78). Moreover, RNA editing events in diabetes-associated genes were identified in human pancreatic islets from 89 deceased donors in an effort to uncover genetic mechanisms affecting glucose metabolism (79).

There is evidence to suggest that the RNA editing machinery is one of the evolutionarily-adaptive mechanisms developed while living organisms were becoming more complex. A-to-I RNA editing occurs mainly in primate-specific Alu repetitive elements that form secondary structures of dsRNA (80, 81). APOBEC1-mediated editing of apoB, thus the production of apoB48, only occurs in mammals (82). Evolutionarily-aligned genetic alterations are thought to have important metabolic consequences (83). Such speculation is prevalent when debating the origins of human metabolic diseases, hence the proposal of the thrifty gene hypothesis (84). Due to the lack of proper experimental models, few genetic events have been functionally proven to influence metabolic disorders in human. One recent example is the codon 72 polymorphism (Pro72Arg or P72R) in the tumor suppressor protein TP53 (85). The ancestral variant of this polymorphism (P72) is only present in primates, while the diversion from P72 to R72 only arose during the modern human evolution (86). Using cell and transgenic mouse models, the P72 variant was found to be a stronger responder to metabolic stresses to cause cell death, and the R72 variant is a predisposing factor for diet-induced obesity and diabetes (87, 88). Through its ability to regulate numerous target genes, RNA editing has potential to cause broader effects on human metabolic health than a single genetic alteration such as a mutation or a genetic polymorphism.

ADAR1

It has been suggested that ADAR1 expression in the liver is important for early embryonic development. The absence of ADAR1 in the mouse liver results in impaired embryonic erythropoiesis, liver disintegration, and early death of the fetus (76, 89). Mechanistically, ADAR1 protects liver homeostasis by inhibiting inflammation (**Figure 5**). Silencing ADAR1 in liver cells induces levels of pro-inflammatory cytokines and type I interferons, partially through the NFκB pathway, to cause liver damage through inflammation, lipid accumulation, hepatitis and fibrosis (90, 91). ADAR1's ability to maintain liver homeostasis partially relies on its RNA-editing function, but no specific editing target has yet been identified as the responsible effector (90).

ADAR1-mediated RNA editing has recently been shown to contribute to cardiovascular disease (CVD). One mechanism for ADAR1 to promote CVD is through the phenotypic modulation of smooth muscle cells (SMC), a pivotal step during the development of CVD. The signature characteristic for the phenotypic modulation of SMC is the downregulation of SMC-specific genes, such as smooth muscle myosin heavy chain (SM-MHC) and smooth muscle α-actin (ACTA2), often mediated by platelet-derived growth factor (PDGF)-BB. In response to PDGF-BB, ADAR1 edits pre-mRNA of these SMC genes to cause abnormal splicing and subsequent downregulation of their mRNAs (92).

ADAR1's role to attenuate an aberrant innate immune response has been well established (93, 94). Its ability to inhibit unwanted inflammation also manifests in the form of Aicardi-Goutières syndrome (AGS), an autoimmune disease caused by ADAR1 mutations (95). Interestingly, ADAR1 acts as a promoting factor in the context of inflammation-driven CVD. Under hypoxia or pro-inflammatory conditions, ADAR1 expression is induced in endothelial cells to edit the 3′UTR of cathepsin S (CTSS) mRNA. This editing event promotes

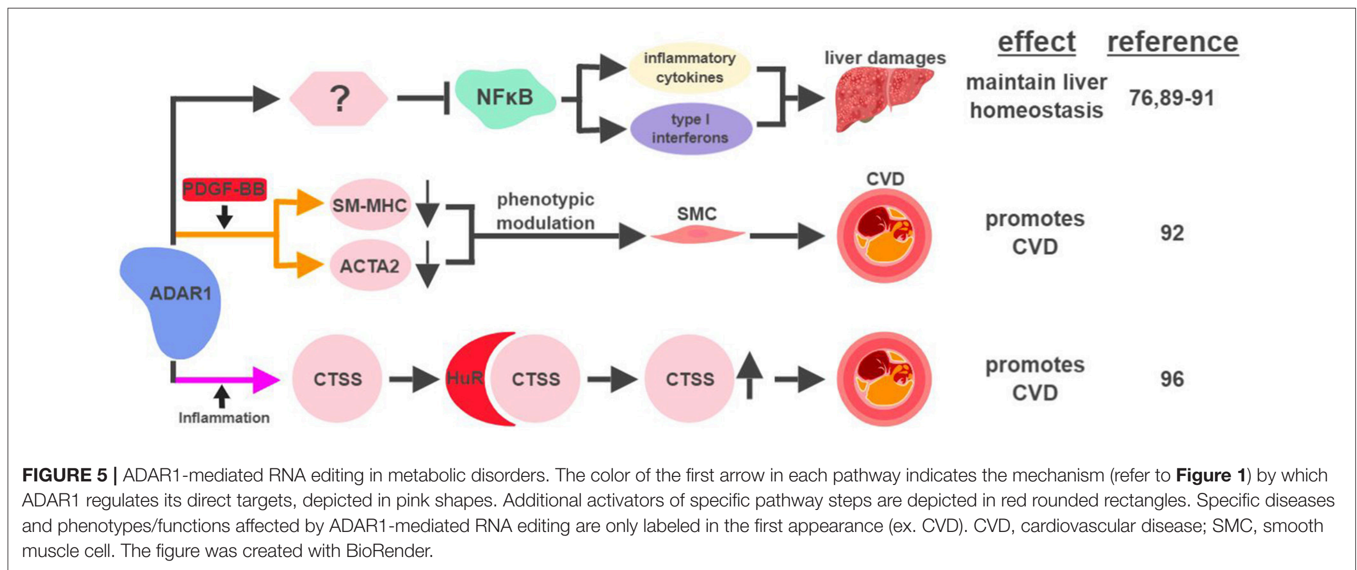

FIGURE 5 | ADAR1-mediated RNA editing in metabolic disorders. The color of the first arrow in each pathway indicates the mechanism (refer to **Figure 1**) by which ADAR1 regulates its direct targets, depicted in pink shapes. Additional activators of specific pathway steps are depicted in red rounded rectangles. Specific diseases and phenotypes/functions affected by ADAR1-mediated RNA editing are only labeled in the first appearance (ex. CVD). CVD, cardiovascular disease; SMC, smooth muscle cell. The figure was created with BioRender.

the recruitment of the RNA binding protein human antigen R (HuR) to stabilize the mRNA of CTSS, a cysteine protease known to be associated with atherosclerosis (96). Independent of its involvement in the regulation of the immune system, ADAR1 also plays critical roles regulating the development and homeostasis of multiple organs, including the spleen, small intestine, and kidney (97). These observations suggest that ADAR1 could impact metabolic functions in a systematic manner affecting multiple organs, possibly through its RNA-editing capability.

The links between circadian rhythms, sleep, and metabolism have been strengthened in recent years and presented as viable targets of therapeutic intervention for metabolic diseases (98, 99). A recent study, using *Drosophila melanogaster* as the model, demonstrated that deficiencies in ADAR1 results in synaptic dysfunction in glutamatergic neurons and sustained release of neurotransmitter to promote sleep (100). It underlines the variety of mechanisms ADAR1 could exploit to control an individual's susceptibility to metabolic disorders.

ADAR2

The first identified target of ADAR2-mediated RNA editing is GluR-B, a subunit of the glutamate receptor (41). Around the same time of this discovery, glutamate receptors were found to regulate functions of pancreatic β-cells (101, 102). Despite this hint, a decade would pass before ADAR2 was directly connected to the metabolic functions of the pancreas (**Figure 6**). Using levels of ADAR2 expression and GluR-B editing as indicators in a mouse model, ADAR2 was found to be deactivated in pancreatic β-cells during fasting and activated in response to a high-fat diet (77). This regulation is mediated through the JNK-c-Jun pathway, as JNK-phosphorylated c-Jun acts as the transcription factor to induce ADAR2 expression in response to nutrient stimulation (103). Activated ADAR2 in turn promotes the secretion of insulin from the pancreas by influencing the expression of key factors involved in exocytosis (104). It remains to be seen if ADAR2-mediated editing of GluR-B is solely responsible for ADAR2's function in the pancreas, or if it involves other ADAR2 targets.

Certain metabolic diseases, such as diabetes or obesity, can manifest in the condition of hyperuricemia (abnormally high uric acid level). Interestingly, increased levels of uric acid were detected in the cortex of ADAR2-knockout mice (105). Hyperuricemia mediated by the loss of ADAR2 in the cortex correlates with the induction of phosphoribosyl pyrophosphate synthetase 1 (PRPS1), an essential enzyme involved in the synthesis of uric acid. Expression of PRPS1 is downregulated by miR376, whose seed sequence is edited by ADAR2 to increase its hybridization with PRPS1 mRNA.

Due to ADAR2's role in facilitating insulin secretion upon nutrient stimulation and reducing uric acid levels, one would assume that ADAR2 might be an active gate-keeper to prevent metabolic diseases. ADAR2-transgenic mice, however, develop hyperglycemia and severe obesity (106). ADAR2-induced obesity in transgenic mice is the result of altered behavior patterns presented in the form of addictive overeating (hyperphagia) (106, 107). Whether ADAR2-mediated RNA editing is necessary for this phenotype is unclear, as transgenic mice expressing mutant ADAR2 (E396A), defective for RNA-editing ability, developed similar levels of obesity compared to wild-type ADAR2 (106). On the contrary, strong evidence does exist to support the connection between ADAR2-mediated RNA editing and hyperphagia. For example, expression and editing levels of the ADAR2 target, serotonin 2C receptor ($5\text{-HT}_{2c}R$), correlate with ADAR2 expression in the brains of ADAR2-transgenic mice and other mouse models of obesity (107, 108). In another study, transgenic mice solely expressing the fully-edited isoform of $5\text{-HT}_{2c}R$ developed phenotypic characteristics of Prader-Willi syndrome (PWS), including hyperphagia (109). PWS is a genetic imprinting disorder that manifests in hyperphagia, early-onset obesity and diabetes. One of the imprinting genes lost from the paternal copies in PWS is the small nucleolar RNA (snoRNA) HBII-52 (MBII-52 in

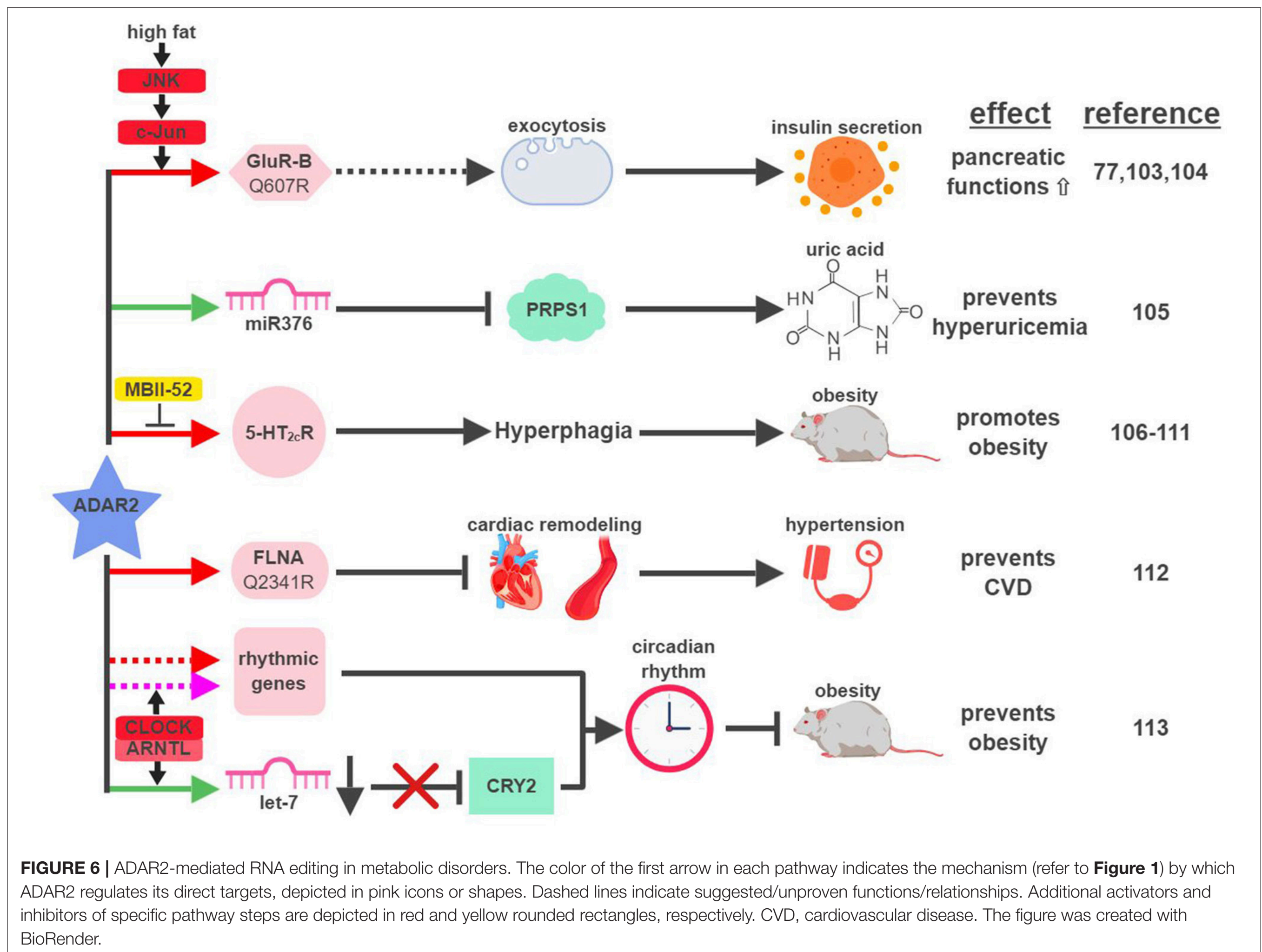

FIGURE 6 | ADAR2-mediated RNA editing in metabolic disorders. The color of the first arrow in each pathway indicates the mechanism (refer to **Figure 1**) by which ADAR2 regulates its direct targets, depicted in pink icons or shapes. Dashed lines indicate suggested/unproven functions/relationships. Additional activators and inhibitors of specific pathway steps are depicted in red and yellow rounded rectangles, respectively. CVD, cardiovascular disease. The figure was created with BioRender.

mouse). MBII-52 was found to specifically inhibit ADAR2-mediated RNA editing of 5-HT_{2C}R, and loss of MBII-52 results in elevated 5-HT_{2C}R RNA editing and PWS phenotypes (110, 111).

In CVD patients, RNA editing of Filamin A (FLNA), an actin crosslinking protein whose inactivation is linked to vascular abnormalities, is significantly reduced. ADAR2 was identified as the editase of FLNA, and ADAR2-edited FLNA (Q2341R) prevents cardiac remodeling and hypertension (112). This is the first known example linking ADAR2-mediated RNA editing to development of CVD through regulation of vascular function and blood pressure.

Like ADAR1, ADAR2 was also recently found to play a role in regulating circadian rhythm. CLOCK (Circadian Locomotor Output Cycles Kaput) – ARNTL (Aryl hydrocarbon Receptor Nuclear Translocator-Like protein (1) protein complex, a critical transcription factor during circadian cycles, was found to regulate the expression of ADAR2 and ADAR2-mediated RNA editing corresponding with the circadian rhythm in the liver (113). ADAR2 contributes to circadian clock maintenance through a couple mechanisms. First, ADAR2 regulates the recoding and stability of a subset of "rhythmic genes," whose expressions align with the circadian cycle. Secondly, ADAR2 alters expression levels of major "clock proteins" (whose expression is essential for the circadian rhythmicity), such as Cryptochrome 2 (CRY2), by regulating biogenesis of their targeting miRNAs (in the case of CRY2, let-7) (114). ADAR2-knockout mice display disrupted rhythms of fatty acid metabolism and gain excessive weight with a high-fat diet, highlighting the significance of ADAR2 at the intersection between circadian rhythm and metabolic regulation.

ADAR3

Other than its preferred presence in the brain and inability to catalyze RNA editing on any proven target, little is known about ADAR3's functional connections to human diseases, including metabolic disorders. The aforementioned study in glioblastoma suggests a similar role for ADAR3 in metabolic diseases, as an inhibitor of RNA editing mediated by other active editases (60).

Despite the lack of mechanistic data, the large amount of genetic information available from the general population has shed some light on potential connections between ADAR3 and metabolism. Multiple single nucleotide polymorphisms (SNPs) in *ADARB2* (encodes ADAR3) were found to be associated with human longevity, using genetic information

collected from centenarians in the US (115). Moreover, this association was later linked to a variety of metabolic parameters, including abdominal circumference, body mass index and serum triglyceride level (116). While the correlation between ADAR3 and aging was preliminarily demonstrated in mutant strains of *Caenorhabditis elegans*, more sophisticated models are needed to establish ADAR3's functional role in aging and metabolic regulation (115).

APOBECs

The first identified, and most studied, RNA editing target of APOBEC1 is apolipoprotein B (apoB; Q2153Stop) (2). Unedited and edited *apoB* encodes for a full-length form apoB100 and a truncated form apoB48, respectively. ApoB100 is synthesized in the liver and is a part of the assembly of low density lipoprotein (LDL) and very low density lipoprotein (VLDL), while apoB48 is mostly produced in the intestine and is required for chylomicron formation and fat absorption (117).

An elevated level of apoB100-containing LDL in the plasma is one of the major characteristics in patients with atherosclerosis, a disease state mimicked by mouse models that either lose APOBEC1's RNA editing activity or express apoB100 exclusively (118–120) (**Figure 7**). A recent genome-wide association study (GWAS) identified novel SNPs at *APOBEC1* that are associated with cholesterol composition, signifying APOBEC1's role in cholesterol-linked human diseases, such as atherosclerosis (121).

A transgenic rabbit model with reduced expression of APOBEC1 presented a lean phenotype compared to wild type when challenged with a high-fat diet (122). This phenotype is consistent with (i) apoB48's role in promoting chylomicron formation and lipid absorption, and (ii) observations in earlier studies that apoB48/apoB100 ratio and APOBEC1 expression were higher in obese and diabetic rats (123, 124).

However, the relationship between apoB and metabolic diseases is more complicated than just apoB48 (edited) leading to diabetes/obesity and apoB100 (unedited) leading to atherosclerosis. In an apoE-deficient mouse model, apoB48 promotes higher levels of cholesterol accumulation and atherosclerotic lesion formation. This phenotype is the manifestation of apoB48 being cleared exclusively through apoE, while apoB100 can be cleared through the LDL receptor alone (119, 125). On the other hand, links between apoB100 and obesity and diabetes have also been established. In rodent models fed high-fat diets, accumulation of apoB100 in the liver induces endoplasmic reticulum (ER) stress and insulin resistance (126, 127). This phenotype is caused by JNK-mediated phosphorylation of insulin receptor substrate (IRS-1), a connection known to link ER stress, obesity and diabetes (128).

These studies highlight the significant connections between apoB regulation, thus APOBEC1-mediated RNA editing, and metabolic disorders. Despite efforts made to identify other RNA editing targets of APOBEC1, no other target has yet been shown to play a role connecting APOBEC1-mediated RNA editing with metabolic disease (129). Data from one patient with a predisposition to type 2 diabetes showed that C-to-U editing is the second most frequent RNA editing event (next to A-to-I), indicating that RNA editing mediated by APOBEC1 or other C-to-U editases is an important factor in metabolic homeostasis (78).

Gap in Knowledge and Future Directions

The Complexity Between RNA Editases

An established link of "Enzyme-Target-Function" provides the clearest blueprint to plan effective interventions of the RNA editing machinery. Numerous examples mentioned in this review fit this description providing multiple intervention points, including modulation of the levels and activity of editases, as well as correction of the edited target(s). There are, however, plenty of ambiguities in the world of RNA editing.

In the context of A-to-I editing, many disease-relevant editing targets lack clear identification of the responsible editase(s). Such examples include hyperediting-mediated alternative splicing of protein tyrosine phosphatase PTPN6 in AML, and hyperediting-activated ras homolog family member Q (RHOQ) in CRC (130, 131). In cases where involvement of editases were confirmed, the relationships among editases could be complicated. For example, ADAR1 and ADAR2, the two major A-to-I editases, can display either collaborative or antagonistic functions with each other. One example is the aforementioned editing of PODXL in GC. The disease outcome is not controlled by the function of one editase, but rather by the ratio between ADAR1- and ADAR2-mediated PODXL editing (58). More complications could come from ADAR3, whose role in RNA editing is just starting to be appreciated (60, 115). The possibility remains that further identifications and characterizations of proteins closely related to ADARs, such as ADAD1 (Adenosine Deaminase Domain Containing 1) and ADAD2, can further increase the complexity of A-to-I RNA editing (9).

The functional difference between the two ADAR1 isoforms, p150 and p110, is also an important factor to consider when determining ADAR1's role in human disease. Earlier studies characterized p110 as constitutively expressed, while p150 is interferon-inducible and the main isoform responsible for innate immune response modulation and AGS (93, 95, 132, 133). In the context of cancers and metabolic diseases, not all ADAR1-related studies have clearly differentiated the involvement between p150 and p110, creating potential issues to pinpoint the underlying mechanisms. As our understanding of functional distinctions between p150 and p110 improves overtime, there will be a need to revisit their individual roles in different diseases (97, 134–136).

As mentioned previously, APOBECs are better known for their abilities to edit DNAs in viral and tumor genomes. Recent identifications of APOBEC3A-mediated G-to-A RNA editing and APOBEC3G as a novel RNA editase signaled that (i) other APOBECs could also engage in RNA editing activities; and (ii) APOBEC-mediated RNA editing is not limited to the conversion from C to U (74, 137). In fact, APOBEC3A was recently shown to be a C-to-U RNA editase in immune cells, making it the first proven RNA editase capable of performing multiple RNA

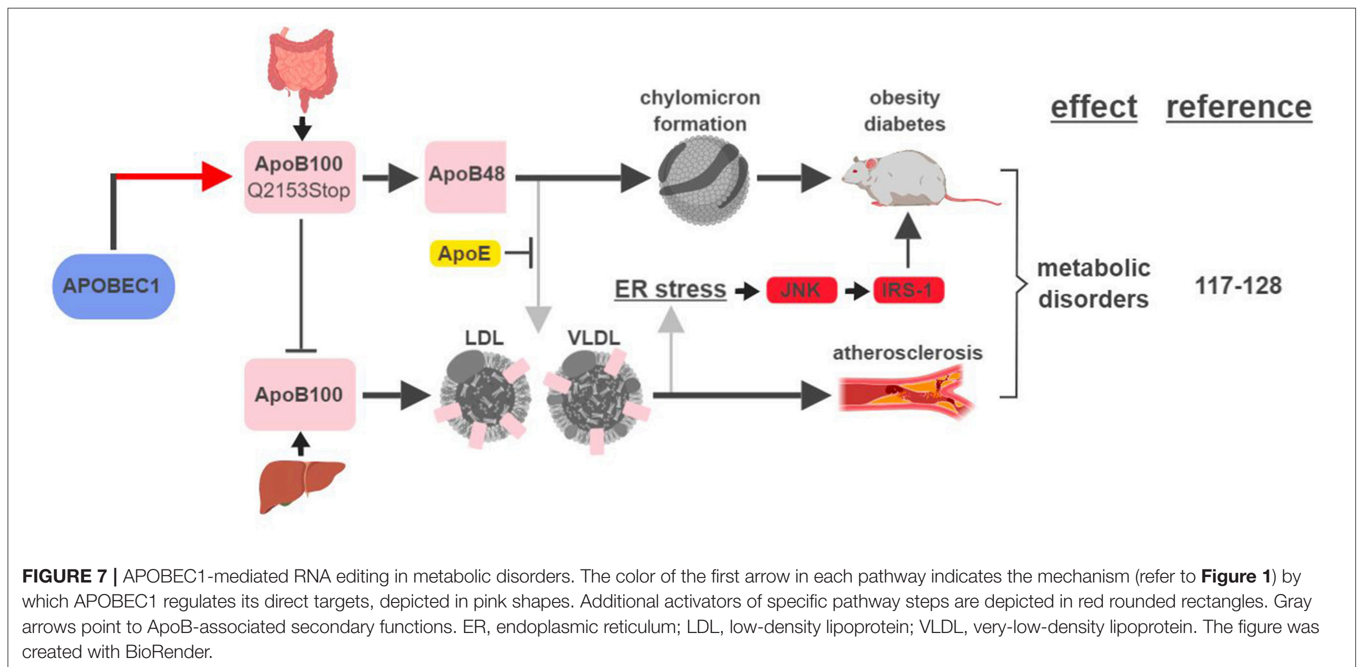

FIGURE 7 | APOBEC1-mediated RNA editing in metabolic disorders. The color of the first arrow in each pathway indicates the mechanism (refer to **Figure 1**) by which APOBEC1 regulates its direct targets, depicted in pink shapes. Additional activators of specific pathway steps are depicted in red rounded rectangles. Gray arrows point to ApoB-associated secondary functions. ER, endoplasmic reticulum; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein. The figure was created with BioRender.

editing conversions (138). Further, albeit indirect, evidence to support these hypotheses is the recent realization that ADARs, well-known for their RNA editing functions, are also capable of performing DNA editing (139, 140). These developments spotlight not only the significance of carefully establishing the "Enzyme-Target-Function" connections, but also the untapped potential in uncovering the vast network of RNA editing in the context of human disease.

RNA Editing-Independent Functions

The effects between RNA and DNA editing can be distinguished through careful planning and execution of sequencing strategies. RNA editases, however, possess functions that are independent of their RNA-editing abilities. RNA editing-independent functions of ADARs were first noted in their effects on miRNA expression. Both ADAR1 and ADAR2 are capable of influencing miRNA expression by either directly interacting with miRNAs or affecting miRNA biogenesis through regulation of important factors, such as Dicer, Drosha or DGCR8 (DiGeorge Syndrome Critical Region 8) (141–143). RNA editing-independent functions of RNA editases play prominent roles in the processes of proliferation, metastasis, and immune evasion during tumorigenesis (49, 144–146).

The RNA editing-independent functions of ADARs have been demonstrated by utilizing their catalytically inactive forms. This approach has helped identify these non-catalytic functions beyond the confines of miRNA biogenesis. The MAPK-phosphorylated ADAR1 p110 isoform can be shuttled by Exportin-5 to the cytoplasm, where it protects the expression of anti-apoptotic genes by competitively inhibiting binding of Staufen1 to their 3′UTRs (134). In metastatic melanoma, RNA editing-incompetent ADAR1 is able to negatively regulate the expression of the metastatic enhancer integrin beta-3 (ITGB3) by (i) inhibiting the transcriptional activator of ITGB3, PAX6, and (ii) promoting FOXD1-mediated induction of miR22 to block ITGB3 translation (147). As mentioned previously, catalytically inactive ADAR2 mimics WT ADAR2 in an overexpression mouse model causing hyperphagia and obesity, dissociating this phenotype from ADAR2's RNA-editing capability (106).

Little information is available regarding RNA editing-independent functions of C-to-U editases, such as APOBEC1. However, APOBEC-mediated functions that don't require its deaminase domains have been reported in humans and other species (148, 149). As more functional studies of RNA editases are reported, clear differentiations between RNA editing-dependent and –independent mechanisms will be necessary to adequately assess their contributions to the development of cancers and metabolic diseases.

Regulations of RNA Editing

RNA-editing events are subjected to highly precise regulatory mechanisms. Mechanisms that regulate general localization and expression of RNA editases have been well-studied (9, 10). Functional regulation of RNA editing in the context of cancer and metabolic disease, however, remains a gap in our knowledge. Depending on the tissue of origin and disease stage, different cancers have been associated with overall induction or reduction of RNA-editing levels (12, 13, 18). Even in diseases with either a clear overall editing profile (hyper- vs. hypo-editing) or an apparent alteration of RNA editase expression, many targets are edited in the opposite manner (12). Moreover, alterations of a single editase do not always yield the same result, as demonstrated by the aforementioned pro- and anti-tumorigenic functions displayed by both ADAR1 and ADAR2 in different cancers.

More dramatic examples can be found in situations where the same editing event leads to completely different functional or phenotypic outcomes. Such examples include ADAR1-mediated editing of GLI1 and ADAR2-mediated editing of COPA in tumorigenesis (15, 26, 27, 53). APOBEC1-produced apoB48 and its full-length counterpart apoB100 contribute to developments of atherosclerosis and obesity to different extents based on the surrounding regulatory environment (119, 125). Even replicating a complex editing profile on one target, such as editing of 5-$HT_{2c}R$, could result in opposite phenotypes (obesed vs. lean) in two different animal models (109, 150).

Several regulatory mechanisms of RNA editing have been recently identified. In aggressive forms of BC, such as triple-negative BC and metaplastic BC, the presence of ADAR1 is important for their tumorigenic capacity. Recent studies found that in these cancers, the expression and activity of ADAR1 can be regulated by tumor-promoting proteins CPSF6 (cleavage and polyadenylation factor-6) and mutant RPL39 (ribosomal protein L39, A14V). CPSF6 interacts with ADAR1 to stabilize its localization and enhance its RNA editing activity (151). Moreover, CPSF6-mediated activation of ADAR1 can be inhibited by prolactin, a mammary differentiation factor. The oncogenic mutant of RPL39 (A14V) induces expression of ADAR1 to promote tumor growth and chemoresistance through the functions of iNOS (inducible nitric oxide synthase) and activated STAT3 (152).

In the brain, where functions of ADAR2 have been extensively studied, a splicing factor SRSF9 (serine and arginine rich splicing factor 9) was found to repress ADAR2-mediated RNA editing. SRSF9 interacts with ADAR2 and its editing targets in the nucleus to disrupt the formation of ADAR2 dimer, which is necessary for the editing of genes involved in controlling cell survival (153, 154). These findings also signaled the importance of the splicing machinery in the regulation of RNA editing (155). In CRC, PKCζ (protein kinase C zeta) phosphorylates ADAR2 to activate its RNA editing activity. Phosphorylated ADAR2 inhibits liver metastasis of CRC by promoting the accumulation of miR-200, potentially through editing of COPA and other targets (54).

Considering the complex relationship between ADAR1- and ADAR2-mediated RNA editing, it is not surprising that mechanisms exist to regulate their RNA-editing functions simultaneously. One recent example of this is the RNA helicase, DHX9 (DEAH box helicase 9). By using an overexpression system in an esophagus carcinoma cell line (EC109), DHX9 was found to preferentially promote and repress ADAR1- and ADAR2-mediated RNA editing, respectively (156). The end result is a strong correlation between DHX9 expression and tumorigenesis. High-throughput screening has been employed to identify endogenous regulators of ADAR-mediated RNA editing (157, 158). Attempts to identify additional enhancers and inhibitors, both intrinsic and extrinsic, of the RNA editing machinery are ongoing.

APOBEC1-mediated C-to-U RNA editing is carried out in a multiprotein "editosome" (159). Many components that are important for the function of this editosome have been identified, including ACF (APOBEC1 complementation factor), HNRNPAB (heterogeneous nuclear ribonucleoprotein A/B; or ABBP-1), DNAJB11 (DnaJ heat shock protein family member B11; or ABBP-2), KSRP (KH-type splicing regulatory protein), CELF2 (CUGBP Elav-like family member 2; or CUGBP2), SYNCRIP (synaptotagmin binding cytoplasmic RNA interacting protein; or GRYRBP), and RBM47 (RNA binding motif protein 47) (160–166). A rare negative regulator of this editosome, BAG4 (Bcl2-associated Athanogene-4), was found to suppress APOBEC1-mediated RNA editing by shuttling APOBEC1 to the cytoplasm (167). Aside from ACF and RBM47, the physiological significance of these regulators remains to be confirmed beyond *in vitro* experiments (166, 168).

Although more investigations are needed to confirm the roles of these APOBEC1 partners in APOBEC1-regulated cancer development, an interesting study using a mouse model of testicular germ cell tumors (TGCTs) hinted strongly at such connections. Using 129/Sv inbred mice that develop spontaneous TGCTs, *Apobec1* deficiency was found to affect TGCT susceptibility either alone or in combination with mutations of *Dnd1* (Deadend1), another TGCT risk factor that shares strong sequence homology with Apobec1-editosome member Acf (169). Pending validation of the involvement of Apobec1-mediated RNA editing in this model, this result suggests that APOBEC1-mediated impact on tumorigenesis is subjected to complex regulatory mechanisms, possibly involving one or more members of the editosome. More interestingly, the effect of Apobec1 deficiency on TGCT susceptibility was influenced by the context of germ-lineage (maternal vs. paternal) and it manifests in a transgenerational manner. It suggests that APOBEC1 regulates heritable epigenetic changes, presumably through RNA-editing, to impact the development of human diseases such as testicular cancer (170).

One major regulator of metabolism, peroxisome proliferator-activated receptor alpha (PPARα), impacts APOBEC1-mediated RNA editing *in vivo*. In mice lacking LDL receptor, PPARα agonist ciprofibrate (a common cholesterol-lowering drug) reduces hepatic RNA editing of apoB by decreasing the expression of Acf (171). The result is increased accumulation of apoB100-associated VLDL and atherosclerosis. It also demonstrated that regulatory mechanisms of RNA editing could impact human diseases by affecting their response to treatments.

Since RNA editing plays a prominent role in cancer development, tumorigenesis-associated pathways and factors could prove to be important regulators of the machinery. For example, tumor suppressor protein TP53 is involved in nearly all aspects of tumorigenesis, but few connections have been made between TP53 and RNA editing (172). So far, TP53 has only been deleted to create viable cell models to study ADAR1-mediated RNA editing in the innate immune response (93). The fact that TP53 status influences the ADAR1-associated phenotype indicates a larger role for TP53 in the world of RNA editing. A recent study identified IFI16 (interferon gamma inducible protein 16) as a common interacting partner of ADAR1 and p53, strengthening this possibility (173).

There are stronger hints for roles of another TP53-related protein, ARF (alternative reading frame; or CDKN2A/p14), in

RNA editing. ARF is well known for its ability to activate TP53 by inhibiting MDM2 (mouse double minute 2), but many TP53-independnet functions of ARF have also been identified (174). One of such functions was recently demonstrated in triple-negative BCs, where ARF collaborates with TP53 to suppress a tumor-promoting inflammatory pathway involving interferon-β and STAT1, which are also important factors in ADAR-mediated RNA editing (90, 93, 132, 175). Combined with the fact that both ARF and ADAR use the nucleolus as a critical hub, it seems to place ARF in close proximity to the center of RNA editing universe (176–178).

As our understanding of RNA editing in cancers and metabolic disorders improves, it is likely that many other connections will be discovered between RNA editing and established factors associated with these human diseases.

Opportunities for Therapeutic Applications

With greater understanding of the relationship between RNA editing and human disease comes the opportunity for innovative therapeutic approaches. RNA-based therapies, which include targeting both RNA itself and its modifications, are becoming viable options to slow down or even reverse the course of human disease (179–181). This warrants further investigation of the mechanisms of RNA-editing and including it as an integral part of RNA-based therapies. Indeed, not only the overall role of RNA editing is being studied in various diseases, but also creative molecular technologies are being developed to identify and verify specific RNA editing events using cell lines or clinical samples (12, 17, 182). High-throughput sequencing and "omics" profiling enable researchers to create comprehensive "maps" of RNA editing in the human transcriptome (158, 183, 184). A recent study integrated genomic, transcriptomic, and proteomic data to pinpoint RNA editing events that are directly responsible for proteomic diversity leading to disease-relevant alterations in cancer samples (15).

Modulation of RNA Editing

In principle, modulating the expression or activity of RNA editases is a reasonable strategy for treating diseases driven by dysregulated RNA editing. In cancer cells with elevated ADAR-mediated RNA editing, such as breast and lung cancers, downregulation of ADAR expression reduces their tumorigenic capacity (17, 39, 185). To counteract apoB48-mediated chylomicron formation and lipid absorption, expression of APOBEC1 was reduced to create transgenic rabbits that are resistant to diet-induced obesity (122). Beyond altering the expression of RNA editases, molecular tools are also being developed to perform selective inhibition of RNA editing. For example, target-specific inhibition of RNA editing has been demonstrated by using either morpholino-based or 2′-O-methyl/locked nucleic acid mixmer antisense oligonucleotides (186, 187).

In situations where the RNA editing level is inversely correlated with disease progression, promoting RNA editing could have beneficial effects. In cancer cells where ADAR2 is downregulated, overexpression of ADAR2 displays tumor-suppressive activity (16, 53). Overexpression of APOBEC1 combined with an endothelial functional modulator, SR-BI (scavenger receptor, class B, type I), was tested in a cell culture model to show anti-atherogenic potential by altering lipoprotein composition and increasing nitric oxide levels (188). To augment the effect of RNA editing, artificial and manipulatable tools have been engineered to control the RNA editing machinery. SNAP-tag technology was used to assemble, through covalent bonding, a RNA-editing complex containing the catalytic domain of ADAR1 and a guide RNA. By integrating a light-sensitive protection molecule between the editase and the guide RNA, the target–specific RNA editing machinery can be switched on and off via light (189).

Application of RNA Editing

The concept of creating a RNA-guided editase has been adopted to attempt target-specific RNA editing. Proof-of-principle studies have been conducted to demonstrate the potential to target disease-relevant genes and restore proper protein function through RNA editing (190, 191). This strategy has also been applied through the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas genomic editing system. By fusing the deaminase domain of ADAR2 with a catalytically inactive Cas13, this complex can be led by a guide RNA to perform specific and robust RNA editing (192, 193). Continuous efforts to increase the efficiency, reduce off-target editing, and promote simultaneous editing of multiple targets, will push these technologies closer to therapeutic application (194, 195).

Unintended Consequences and Unique Opportunities

As intriguing as the idea to reverse disease conditions by modulating RNA editing levels, it is not without potential drawbacks. Since different human diseases are associated with either elevated or reduced levels of RNA editing, altering it one way or the other poses the risk of undesired consequences. For example, it is theoretically possible to modulate the overall levels of inosine-containing RNA by regulating ribonuclease V (196, 197). But dysregulation of ribonuclease V has been linked to cancers and psychiatric disorders, indicating the potential hazards (198, 199). This concern can be extended to approaches targeting an individual RNA editase, as mutations of ADAR1 and ADAR2 have been linked to devastating genetic diseases (95, 200).

In addition to impacting disease progression, RNA editing could also affect drug response. In MM, ADAR1-mediated RNA editing promotes immunomodulatory drug resistance (26). APOBEC1-mediated RNA editing of apoB influences the liver's response to lipid-lowering drugs like fibrates (171). Moreover, ADAR1 can directly alter the cellular response to a drug by regulating the RNA editing and expression of xenobiotic-metabolizing-related factors, such as AhR (aryl hydrocarbon receptor) and CYP1A1 (Cytochrome P450, family 1, member A1) (201). These studies highlight potential side-effects of both stand-alone and combinatorial therapies involving modulation of RNA editing.

Man-made, RNA-guided, and target-specific RNA editing has the potential to become the next revolution in (epi)genetic

therapy. It offers a unique opportunity to modulate protein functions without altering the sequence and integrity of the genome. Indeed, its unique characteristics haven't gone unnoticed and RNA editing has been incorporated into cutting-edge technologies, such as CRISPR-Cas genome editing (192). Recent studies pointing out the potentially crippling effects of genome editing, however, should serve as a cautionary tale when considering using RNA editing in a similar fashion (202–205). Further studies are needed to ensure the efficacy and safety of this approach before considering clinical applications.

Having mentioned the potential problems, it should be acknowledged that there are tremendous opportunities for RNA-editing-based therapies to treat human diseases. By engaging biological events at the RNA level, RNA-editing could be associated with many-to-one or one-to-many relationships between RNA editases/events and downstream effects. Examples of "many-to-one" include the "see-saw" effect of PODXL editing by ADAR1 and ADAR2 in GC, and the ability of both ADAR1 and ADAR2 to regulate biogenesis of miRNA let-7 (31, 49, 58). It points out common downstream effectors of multiple editases, offering therapeutic targets that are more specifically linked to diseases. There are also "one-to-many" cases, such as the association of ADAR2-mediated editing of miR376 with both glioblastoma and metabolic disorders (51, 105). Intervention strategies targeting "ADAR2-miR376" as a unit thus could have broader range of applications.

The relationship between cancer development and metabolic disorders has been strengthened in recent years, and it appears to be a two-way street. It is well-established that cancers often overcome unwanted stresses by hijacking metabolic pathways, and metabolic disorders like obesity and diabetes are strong predisposing factors to cancers (206, 207). Reversely, cancers can create systematic metabolic imbalances in patients resulting in metabolic diseases such as diabetes or CVD (208, 209). Understanding the cross-talk between cancer and metabolic diseases is one of the most critical challenges for human health, and RNA editing is an important piece of the puzzle.

CONCLUSION

As this review shows, there has been an explosion of information regarding RNA editing in the last 3–5 years. This is indeed an exciting time to study this unique epigenetic phenomenon, with plenty of opportunities and challenges ahead. Some burning questions, as highlighted throughout this review, will need to be answered in the near future. How is new information used to reshape the central dogma of cell biology of "DNA to RNA to protein?" Are there more RNA editases waiting to be discovered? How do we reliably identify RNA editases, and corresponding editing events, in specific human diseases? Can part of the RNA editing machinery be targeted as a monotherapy, or is combining these interventions with other parallel treatments, such as immunotherapy, a better course of action?

Outside the purview of this article, RNA editing also plays significant roles in other physiological conditions, such as infectious, inflammatory/autoimmune, and neurodegenerative diseases (210–213). It will be interesting, in some cases necessary, to investigate the inter- and intra-relationships between the roles of RNA editing in these diseases with those in cancers and metabolic disorders. The ultimate goal is to leverage this information into actionable therapeutic innovations. More than 30 years after its initial discovery, the significance of RNA editing in human disease is being recognized more than ever.

AUTHOR CONTRIBUTIONS

CK and JW conceived of the topic of the manuscript. CK drafted the manuscript and created images with BioRender. CK, LM, and JW revised the manuscript.

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