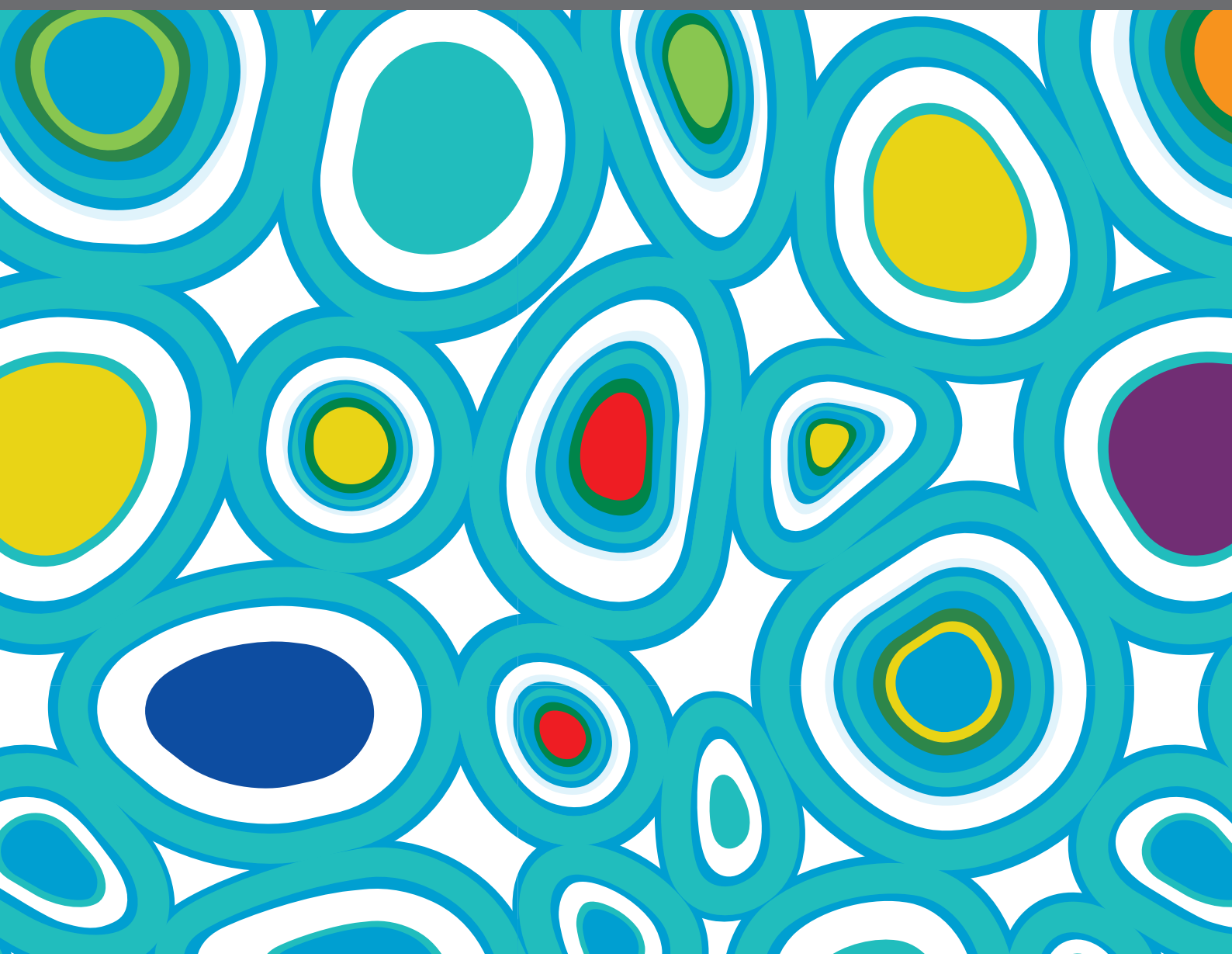
AUTOPHAGY: FROM BIG DATA TO PHYSIOLOGICAL SIGNIFICANCE

EDITED BY: Ioannis Nezis, Sovan Sarkar and Maria Ines Vaccaro
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AUTOPHAGY: FROM BIG DATA TO PHYSIOLOGICAL SIGNIFICANCE

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Table of Contents

04 ***Editorial: Autophagy: From Big Data to Physiological Significance***
Sovan Sarkar, Maria I. Vaccaro and Ioannis P. Nezis

07 ***What We Learned From Big Data for Autophagy Research***
Anne-Claire Jacomin, Lejla Gul, Padhmanand Sudhakar, Tamas Korcsmaros and Ioannis P. Nezis

21 ***Melanoma LAMP-2C Modulates Tumor Growth and Autophagy***
Liliana Pérez, Anthony L. Sinn, George E. Sandusky, Karen E. Pollok and Janice S. Blum

36 ***Autophagy in Neutrophils: From Granulopoiesis to Neutrophil Extracellular Traps***
Panagiotis Skendros, Ioannis Mitroulis and Konstantinos Ritis

48 ***Hypoxia and Selective Autophagy in Cancer Development and Therapy***
Ioanna Daskalaki, Ilias Gkikas and Nektarios Tavernarakis

70 ***The Interplay of Host Autophagy and Eukaryotic Pathogens***
Robert J. Evans, Varadharajan Sundaramurthy and Eva-Maria Frickel

85 ***The Ins and Outs of Autophagy and Metabolism in Hematopoietic and Leukemic Stem Cells: Food for Thought***
Angela Ianniciello, Kevin M. Rattigan and G. Vignir Helgason

100 ***Crosstalk Between Mammalian Autophagy and the Ubiquitin-Proteasome System***
Nur Mehpare Kocaturk and Devrim Gozuacik

127 ***Initial Steps in Mammalian Autophagosome Biogenesis***
Daniel Grasso, Felipe Javier Renna and Maria Ines Vaccaro

137 ***Identification of Kinases and Phosphatases That Regulate ATG4B Activity by siRNA and Small Molecule Screening in Cells***
Niccolo Pengo, Krisna Prak, Joana R. Costa, Christin Luft, Alexander Agrotis, Jamie Freeman, Christina A. Gewinner, A. W. Edith Chan, David L. Selwood, Janos Kriston-Vizi and Robin Ketteler

150 ***Selective Autophagy and Xenophagy in Infection and Disease***
Vartika Sharma, Surbhi Verma, Elena Seranova, Sovan Sarkar and Dhiraj Kumar

167 ***Autophagy-Virus Interplay: From Cell Biology to Human Disease***
Liyana Ahmad, Serge Mostowy and Vanessa Sancho-Shimizu

175 ***Chemical Biology Strategies to Study Autophagy***
Piyush Mishra, Veena Ammanathan and Ravi Manjithaya

185 ***Chemical Screening Approaches Enabling Drug Discovery of Autophagy Modulators for Biomedical Applications in Human Diseases***
Prashanta Kumar Panda, Alexandra Fahrner, Somya Vats, Elena Seranova, Vartika Sharma, Miruna Chipara, Priyal Desai, Jorge Torresi, Tatiana Rosenstock, Dhiraj Kumar and Sovan Sarkar

204 ***Generation and Characterization of Germline-Specific Autophagy and Mitochondrial Reactive Oxygen Species Reporters in* Drosophila**
Kiran Nilangekar, Nidhi Murmu, Govind Sahu and Bhupendra V. Shravage

222 ***Transcriptional Regulation of Autophagy: Mechanisms and Diseases***
Chiara Di Malta, Laura Cinque and Carmine Settembre

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EDITORIAL
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Editorial: Autophagy: From Big Data to Physiological Significance

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Editorial on the Research Topic

Autophagy: From Big Data to Physiological Significance

Autophagy is a fundamental catabolic process where cytoplasmic components are sequestered into double-membrane vesicles called autophagosomes, which then fuse with lysosomes and their content is degraded. Our knowledge about autophagy sharply increased during the last decade. This significant progress helped us to understand better the molecular mechanisms of autophagy and to elucidate its role in health and disease. This special issue contains a collection of three original research papers and 12 review articles covering a broad range of topics highlighting how big data and screening approaches can help toward uncovering the molecular mechanisms of autophagy.

Recent years have witnessed the development of large-scale multi-omics studies on autophagy via genomics, transcriptomics, proteomics, lipidomics, and metabolomics. Jacomin et al. comprehensively describe the omics studies undertaken in the field of autophagy, and the integration of these omics datasets for better understanding of autophagy regulation and the involvement of autophagy in other biological processes. In addition, future approaches involving single-cell analysis, patient-derived samples, and high-content analysis have been suggested. The authors also outlined the web-based resources for studying autophagy, such as for the prediction of Atg8-family interacting proteins, and autophagy network and databases. Overall, the emerging big data and *in silico* tools not only elucidate the global landscape of autophagy but also provide critical resources for further research in this field.

There is a growing interest toward the biomedical exploitation of autophagy modulators for the treatment of myriad human diseases. Two articles comprehensively review the screening methods for the drug discovery of chemical autophagy modulators. The first article by Panda et al. summarizes the *in vitro* chemical screening approaches for identifying autophagy modulators in mammalian cells. These methods that are commonly being used, involve reporters based on the autophagic marker LC3 or specific autophagy substrates like p62 and certain aggregation-prone proteins. The chemical screenings pertaining to the discovery of the pharmacological modulators of autophagy have been described. Of biomedical relevance, the therapeutic benefits of autophagy modulators have been highlighted in animal and iPSC models of selected human diseases, such as in neurodegenerative disorders, cancer, infectious diseases, liver diseases, and myopathies, as well as in lifespan extension. The second article by Mishra et al. primarily focuses on the chemical biology strategies utilizing high-throughput assays to monitor autophagy in yeast and mammalian cells. These assays are based on the growth of yeast cells, fluorescence readouts of LC3 reporters in mammalian cells, and luminescence measurements of autophagic cargo clearance including organelle turnover in both yeast and mammalian cells. Apart from describing the therapeutic

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applications of autophagy modulators, how these compounds act as valuable tools to elucidate the regulation of autophagy have also been highlighted.

For developing novel autophagy modulators, high-throughput screens were undertaken in the research article by Pengo et al. for identifying the regulators of ATG4B activity. The protease ATG4B is a key regulator of the LC3/GABARAP conjugation system essential for autophagosome formation. Inhibition of ATG4B activity has been suggested for cancer treatment. Through chemical and genetic screens utilizing a cellular luciferase-based assay for measuring ATG4B activity, the compound STK683963 and the kinase AKT2 were identified as activators. Although this study focused on the enhancers of ATG4B activity, these regulators could impact on the kinetics of LC3/GABARAP processing and influence autophagy. The datasets of ATG4B modulators arising from the screens have been provided for further investigation.

There is significant development in the understanding of the molecular mechanisms of autophagy regulation, such as the initial steps of autophagosome biogenesis in mammals. The review article by Grasso et al. provides a detailed overview of the early events in mammalian autophagosome formation including their membrane origins and cellular localization. The four major aspects outlined in this article encompass autophagy induction via physiological stressors, autophagy initiation via mTOR and AMPK, initiation of autophagosome formation via the ULK1 complex, and the molecular mechanisms of phagophore generation prior to autophagosome formation.

Although it was initially believed to be a bulk process, it is now well-established that autophagy is a selective process. Xenophagy is a type of selective autophagy and refers to the selective autophagic degradation of invading bacteria and viruses, and is an important aspect of the hosts' innate immune response to protect against infection. Three review articles in this collection highlight the importance of xenophagy in diseases. Depending on the virus, autophagy can restrict or promote viral replication, and play key roles in modulating inflammation and cell survival. Ahmad et al. provide an overview of autophagy-virus interplay highlighting the protective role of autophagy in human infections. They summarize recent discoveries showing the role of autophagy in immunity and inflammation upon viral infection. Finally, they discuss therapeutic implications and potential caveats associated with using autophagy to control viral infections in humans. Sharma et al., focus on bacterial degradation by autophagy. They describe how several bacterial effectors regulate host autophagy during infection and how this affects inflammation. They also present a detailed overview on the role of several selective autophagy receptors and adaptors on bacterial xenophagy. Finally, they describe how ubiquitin ligases and deubiquitinases regulate bacterial xenophagy. Evans et al., provide a comprehensive overview of the interplay between host autophagy and eukaryotic pathogens. They focus on eukaryotic pathogens *Plasmodium*, *Toxoplasma*, *Leishmania,* and the fungal pathogens *Candida albicans*, *Aspergillus fumigatus,* and *Cryptococcus neoformans*.

Neutrophils are effector cells of immune system in humans and are the first cells to respond to tissue inflammation. Skendros et al. review the role of autophagy in the biology of neutrophils. They describe the link between autophagy and regulation of granulopoiesis and neutrophil degranulation. They also describe how autophagy affects net formation, the extracellular chromatin strands carrying various highly active neutrophil-derived granular and cytosolic proteins. Finally, they explore how elements of autophagic machinery could be effective therapeutic targets for the enhancement of antimicrobial defense or the amelioration of neutrophil/NET-driven inflammation and thrombosis.

Ianniciello et al. explore the relationship between autophagy and metabolism in the leukemic stem cells (LSCs). They give an overview of the metabolic features involved in hematopoietic stem cells (HSCs) that utilize glycolysis and fatty acid oxidation as sources of energy. HSCs develop high levels of autophagy; ATG7, ATG5, and the ULK1 complex have been linked with mitophagy in HSCs. Autophagy, which contributes to fuel LSCs energy demand and hypoxic environment, along with mutations and epigenetics modifications driving LSCs expansion, are proposed to be principal contributors in HSCs leukemic transformation. In conclusion, authors highlight the relevance of combining current treatment with the autophagy inhibitor chloroquine in LSCs.

Di Malta et al. focus in the transcriptional regulation of autophagy, particularly on the role of the MiT/TFEB transcription factor family. TFEB activation not only promotes the increment of lysosomal catabolic efficiency but also controls the expression of ATG genes driving the autophagic flux. The description of the opposed role of ZKSCAN3 and TFEB let us understand the nuclear events that control autophagy. Cytosolic TFEB and nuclear ZKSCAN3 inhibit lysosome gene expression under nutrient starvation conditions. Normoxia and hypoxia conditions also regulate ATG genes such as Bnip3 through NFKB and E2F1. Finally, they propose that the modulation of the transcriptional control of autophagy could be considered as possible therapeutic strategies for complex diseases.

Kocaturk and Gozuacik describe the relationship between autophagy and ubiquitin proteasome system (UPS). Both degradative mechanisms are linked by the ubiquitin signaling pathway. Proteins with K48-based ubiquitin chains are directed for UPS and aggregates with K63-based ubiquitin chains are directed for autophagic degradation. Both K48- and K63-linked ubiquitylation were observed in cases of xenophagy, which is an example of coregulation of the UPS and autophagy. In addition, UPS and autophagy act as cooperative mechanisms in mitophagy, peroxiphagy, and ERphagy. Moreover, UPS can regulate degradation of transcription factors involved in autophagy. Eventually, this article discusses the possible role of the cross talking between autophagy and UPS in degenerative diseases and cancer.

Daskalaki et al. present a comprehensive summary of recent findings on selective autophagy in hypoxia and discuss emerging links between these pathways and cancer pathophysiology. In response to hypoxia, HIF-1 is stabilized and translocate to the nucleus to initiate the transcription of multiple genes involved in autophagy, glucose metabolism and mitochondria respiration.

Importantly, HIF-1 regulates essential genes for the assembly and function of the autophagy machinery. This article also focuses in the role of FUN14 domain-containing protein 1(FUNDC1) in the regulation of mitophagy in normoxia vs. hypoxia. Furthermore, hypoxia induces degradation of other organelles by selective autophagy and the components of these selective pathways in cancer are discussed.

In the research article by, Pérez et al. a role of lysosome-associated membrane protein LAMP-2C in the regulation of melanoma growth and survival is presented. They show that melanoma cell expression of LAMP2C mRNA significantly increased in response to pro-inflammatory cytokine interferon-gamma. This increased expression affected macroautophagy and chaperone-mediated autophagy in several human melanoma lines. Melanoma cells with enhanced LAMP-2C expression displayed increased cell cycle arrest, increased expression of Chk1 and p21, and greater apoptosis and necrosis. In addition, human melanoma cell xenografts with increased LAMP-2C expression, displayed reduced growth in immune compromised murine hosts. Melanomas with high LAMP-2C expression showed increased necrosis and reduced cell density upon histological analysis.

Nilangekar et al., developed new genetic tools to study autophagy in the context of gametogenesis and germline stem cell aging. They generated three transgenic lines mCherry-Atg8a, GFP-Ref(2)P, and mito-roGFP2-Orp1 that are specifically expressed in the germline compartment during Drosophila oogenesis. These reporters can be used to monitor and quantify autophagy and the production of reactive oxygen species during oogenesis. They are a valuable tool that can be used in designing genetic screens to identify novel regulators of autophagy and redox homeostasis during oogenesis.

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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REVIEW
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What We Learned From Big Data for Autophagy Research

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Autophagy is the process by which cytoplasmic components are engulfed in double-membraned vesicles before being delivered to the lysosome to be degraded. Defective autophagy has been linked to a vast array of human pathologies. The molecular mechanism of the autophagic machinery is well-described and has been extensively investigated. However, understanding the global organization of the autophagy system and its integration with other cellular processes remains a challenge. To this end, various bioinformatics and network biology approaches have been developed by researchers in the last few years. Recently, large-scale multi-omics approaches (like genomics, transcriptomics, proteomics, lipidomics, and metabolomics) have been developed and carried out specifically focusing on autophagy, and generating multi-scale data on the related components. In this review, we outline recent applications of *in silico* investigations and big data analyses of the autophagy process in various biological systems.

Keywords: autophagy, big data, proteomics, bioinformatics, transcriptomics

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INTRODUCTION

To maintain their homeostasis, cells require an appropriate balance between anabolism and catabolism. There are two main degradative processes for intracellular components in eukaryotic cells: autophagy and the ubiquitin-proteasome system (Dikic, 2017). Whereas the ubiquitin-proteasome system is primarily known for its implication in the turnover of short-lived proteins, autophagy contributes to the degradation of long-lived cytosolic proteins, as well as large protein complexes and organelles (Yin et al., 2016). Autophagy plays a fundamental role in maintaining a healthy cell, and defective autophagy process has been associated with a broad range of pathologies. As such, it is not surprising that more and more studies are focusing on understanding the molecular mechanisms of autophagy. With the advent of the post-genomics era, a growing number of studies make use of the analysis of massive molecular data sets for a more comprehensive understanding of autophagy processes related to basal condition and disease/infection pathologies.

In the present review, we go through examples on the latest research in understanding autophagy mechanisms based on the analysis of a large volume of data from omics studies, and how these findings have been gathered in databases freely available to the scientific community.

AUTOPHAGY: FROM PHYSIOLOGY TO PATHOLOGY

Physiological Roles of Autophagy

Autophagy is a survival mechanism greatly conserved among every eukaryotic organism. Autophagy functions essentially as an adaptive response to stress, particularly in the condition of nutrient deprivation, allowing for cell and organism survival. When nutrient resources are restricted, cells are able to break down and reprocess all sort of macromolecules including proteins, lipids, and carbohydrates which can then be reused as essential building blocks for the synthesis of new macromolecules and the production of energy (Kaur and Debnath, 2015).

Although most of the knowledge on the autophagy process was generated from studies performed in different conditions of stress, it is now broadly acknowledged that constitutive degradation of cytoplasmic contents by basal autophagy under favorable growth conditions also plays an essential role in cell physiology. A basal level of autophagy is essential for the maintenance of cellular homeostasis in post-mitotic cells (for instance, neurons or hepatocytes) that cannot dilute their deleterious components through division. As such, autophagy facilitates the disposal of supernumerary or damaged proteins and organelles before they become toxic to the cell (Pankiv et al., 2007; Kirkin et al., 2009; Okamoto et al., 2009; Richter et al., 2016). A broad range of studies has revealed that basal autophagy decline is often associated with pathologies such as neurodegeneration, cancer and inflammation.

Because of the proficiency of autophagy to target large organelles such as mitochondria for degradation, it is not surprising it is largely exploited by the innate immune system to fight microbial invasion (Gomes and Dikic, 2014; Randow and Youle, 2014). The term xenophagy is used to refer to the autophagic degradation of bacteria, viruses, and parasites which are strict intracellular pathogens. Besides, autophagy has other roles in immunity such as the control of pro-inflammatory responses and antigen presentation by macrophages (Crotzer and Blum, 2009).

The Plurality of Autophagic Processes

Autophagy relates to a set of catabolic processes for the delivery of cytosolic components to the lysosome for degradation. To date, three types of autophagy processes have been described, depending on the manner by which the cargo reaches and is delivered to the lysosome: macroautophagy, (endosomal-) microautophagy, and chaperone-mediated autophagy (**Figure 1**). Initially, autophagy was described to be non-specific and degrading material in bulk with minimal regulation. However, recent evidence described autophagy as a tightly regulated process which makes use of a multitude of accessory proteins in order to identify and transport the cargo to the lysosome (Yu et al., 2018).

In chaperone-mediated autophagy (CMA), cytosolic proteins interact with the chaperone heat shock 70 kDa protein 8 (HSPA8/HSC70) via their pentapeptide KFERQ; the chaperone in turn binds to the lysosomal-associated membrane protein 2A (LAMP2A) (Agarraberes and Dice, 2001). The recognition and binding of the substrate to monomers of LAMP2A induces its multimeric assembly, which is essential for the translocation of unfolded substrate proteins into the lysosome (Gough and Fambrough, 1997; Salvador et al., 2000; Bandyopadhyay et al., 2008). Although the regulation of CMA has not been fully identified yet, it appears that the phosphorylation status of the regulatory protein GFAP contributes to the stability of the CMA translocation complex (Bandyopadhyay et al., 2010; **Figure 1**).

Microautophagy is the newest of the autophagy process sub-type that has been identified and to this date remains poorly understood. Early studies conducted in yeast have suggested that, during microautophagy, cytosolic content is sequestered by invagination of the lysosomal membrane that pinches off into the lumen (Müller et al., 2000). The inability to detect direct invagination of the lysosome has considerably delayed the investigation of microautophagy in higher eukaryotic organisms. It was only recently that studies have demonstrated the existence of a process similar to yeast microautophagy which occurs at the level of late endosomes instead of lysosomes. As such, this process is being referred to as endosomal-microautophagy (eMi) (Sahu et al., 2011; Mukherjee et al., 2016). While eMi seems to contribute mainly to in-bulk degradation of cytosolic substrates, it appears that proteins can be selectively targeted in a similar way than CMA with the implication of the chaperone HSC70 and the presence of KFERQ motifs on substrate proteins (Tekirdag and Cuervo, 2017; **Figure 1**).

Last but not least, the best-characterized form of autophagy is macroautophagy (mostly simply referred to as autophagy; **Figure 1**). It is characterized by the engulfment of intracellular material in a double-membrane vesicle called the autophagosome. Mature autophagosomes are transported along microtubules and ultimately fused with lysosomes leading to the degradation of the autophagosome contents (Yin et al., 2016). Initiation of autophagosome formation depends on the activation of the ULK and PI3K-III complexes. The PI3K-III complex is itself activated by the ULK complex and is responsible for the generation of PI3P (phosphatidylinositol-3-phosphate), essential for autophagy induction (Feng et al., 2014). Although initially thought to be nonselective, the delivery of cargo to the autophagosome occurs in a specific and controlled manner. At the membrane of the autophagosome, ATG8-family proteins serve as anchor points for the recruitment of the cargo and autophagy machinery. ATG8 proteins are bound to the autophagosomal membrane after their conjugation to phosphatidylethanolamine (PE) via two ubiquitin-like conjugation systems that involve several ATG proteins (Nakatogawa et al., 2009). Specialized selective autophagy receptors are required for the proper targeting of the cargo. Many of those autophagy receptors (also known as LIR-containing proteins, LIRCPs) share common features such as a Ubiquitin Binding Domain (UBD) which allows them to bind to polyubiquitinated cargo, or a LC3-interacting region (LIR) motif which allows for the binding of the receptor to ATG8 proteins at the autophagosome membrane (Pankiv et al., 2007; Behrends and Fulda, 2012; Birgisdottir Å et al., 2013; Jacomin et al., 2016; Gatica et al., 2018).

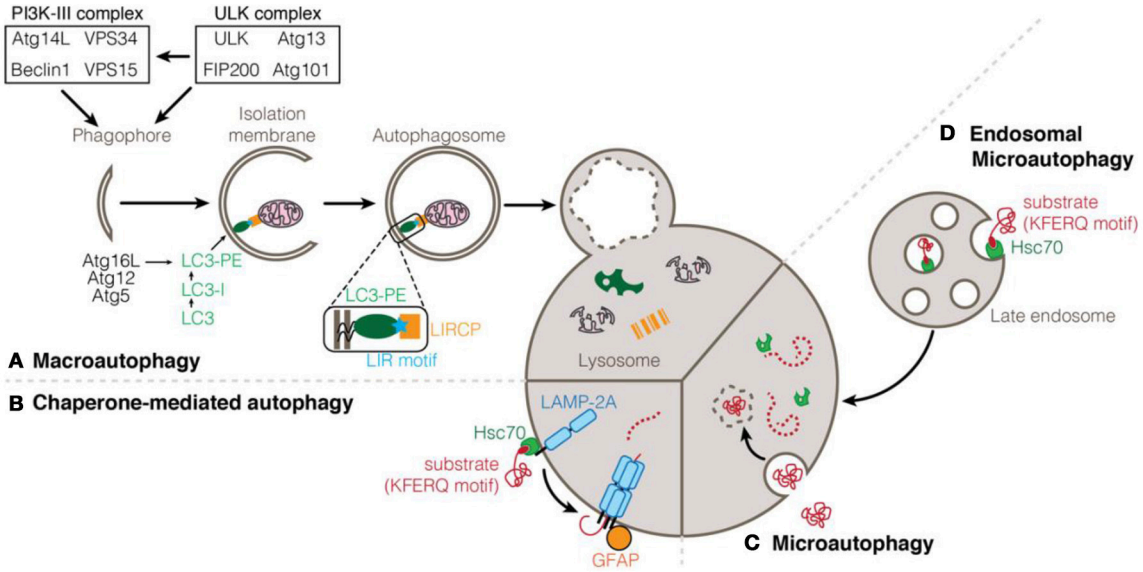


FIGURE 1 | Types of autophagy processes. **(A)** Macroautophagy is induced by the activation of the ULK1 and PI3K-III complexes. Modification of LC3/ATG8 proteins with phosphatidylethanolamine (PE) and anchorage to the membrane of the elongating autophagosome depends on the Atg12-Atg5-Atg16L conjugation system. LC3 can interact with cargoes and selective autophagy receptors via a LIR motif. Enclosed autophagosome eventually fuses with the lysosome for the degradation and recycling of its content. **(B)** Chaperone-mediated autophagy (CMA) consists in the translocation of proteins into the lysosome through pores formed of LAMP-2A protein stabilized by phosphorylated GFAP. **(C)** Microautophagy consists in the internalization on cytoplasmic components into the lysosome by direct invagination of the lysosomal membrane. **(D)** Endosomal-microautophagy depends on the isolation of cytosolic protein in the late endosome before being addressed to the lysosome for degradation. Both CMA and endosomal-microautophagy rely on the chaperone protein Hsc70 that can bind to substrate proteins containing a KFERQ motif.

Pathologies Associated With Autophagy Dysfunctions

Extensive research over the past years has unraveled a central role of autophagy not only for cellular homeostasis but also for various pathological conditions. Autophagy dysfunction has been observed during aging, and several genetic alterations in cancer, neurodegenerative and immune-related diseases have been associated to autophagy and autophagy genes (Zhou and Zhang, 2012; Carroll et al., 2013; White, 2015). While it is widely accepted that autophagy is involved in disease development and progression, its exact roles often appear to be controversial across similar studies, highlighting that its implication is most likely to be context-dependent. For instance, the cytoprotective function of autophagy is believed to have tumor-suppressive potential at the early stages of tumorigenesis, and that loss of autophagy can be associated with increased risk of cancer (Roy and Debnath, 2010). Nonetheless, autophagy has also been shown to allow premalignant cells to escape genotoxic stress and inflammation, thus promoting tumorigenesis (Hu et al., 2012; Kubisch et al., 2013).

During aging, however, activation of autophagy is widely accepted as being beneficial to counteract mechanisms involved in the development of neurodegenerative diseases (Nakamura and Yoshimori, 2018). Current studies are exploring how autophagy induction could constitute a strategy for the prevention and treatment of neurodegenerative diseases. Indeed, in healthy cells, autophagy allows for the elimination of ubiquitinated protein aggregates and non-functional organelles. Accumulation of protein aggregates inside neuronal cells is a hallmark of neurodegenerative and age-associated diseases (including, but not restricted to, Alzheimer's and Parkinson's diseases, ALS, Huntington's disease; Del Roso, 2003; Bartlett et al., 2014; Tóth et al., 2014; Menzies et al., 2017). Moreover, numerous proteins implicated in autophagy or lysosomal function were found to be mutated in neurodegenerative diseases (Menzies et al., 2017).

WEB-BASED RESOURCES RELATED TO AUTOPHAGY

With the increasing interest in the field of autophagy, the past 15 years have seen a rise of publicly available autophagy-related resources. These resources provide access to a broad range of data types and offer functionalities for the identification and characterization of proteins involved in various autophagic processes. Currently, there are several databases containing information on the autophagy components and the molecular players which modulate the process from different regulatory layers (transcriptional, post-transcriptional, translational etc.; **Table 1**, **Figure 2**). The following section aims to highlight the different servers that allow for either the identification of autophagy-related proteins and genes or the characterization of features that could link proteins to autophagy.

Prediction of Atg8-Family Interacting Proteins

Atg8 family proteins are a central component of the autophagic machinery. Their covalent anchorage to lipid membranes is

TABLE 1 | Currently available autophagy-related resources.

Database	URL	Description	References
Autophagy Regulation Network	http://autophagyregulation.org/	Autophagy core proteins and their regulators with experimentally verified and predicted interactions in human.	Türei et al., 2015
The Human Autophagy Database	http://autophagy.lu/	Manually curation of autophagy proteins and their regulators.	
The Autophagy Database	http://www.tanpaku.org/autophagy/	Provides information about proteins related to autophagy, including protein structure data in 41 species.	Homma et al., 2011
The Autophagy Ontology	http://atgo.ucsd.edu/	Ontology of functions related to autophagy, explains and presents the hierarchy of functions in autophagy in yeast and human.	Kramer et al., 2017
ELM	http://elm.eu.org	Annotation and detection of eukaryotic linear motifs (ELMs).	Gouw et al., 2017
Gerontology-Autophagic-MicroRNA Database	http://gamdb.liu-lab.com/index.php	Experimentally validated interactions between miRNA and genes or proteins in gerontology-related disorders manually curated from the literature focusing on autophagy.	Zhang et al., 2016
hfAIM	http://bioinformatics.psb.ugent.be/hfAIM/	Identification of LIR/AIM motifs in protein sequences	Xie et al., 2016
iLIR	http://repeat.biol.ucy.ac.cy/iLIR	Identification of LIR/AIM motifs in protein sequences and providing a PSSM score indicating the significance of the hits.	Kalvari et al., 2014
iLIR database	http://ilir.uk/model	A database listing all the putative LIR-containing proteins from 8 model organisms; GO analysis.	Jacomin et al., 2016
iLIR@viral	http://ilir.uk/virus/	Database of all the putative LIR-containing proteins in viruses.	Jacomin et al., 2017
ncRDeathDB	http://www.rna-society.org/ncrdeathdb	Collection of programmed cell death associated non-coding RNAs (including microRNA, long noncoding RNA/lncRNA and small nucleolar RNA/snoRNA).	Wu et al., 2015
SLiMSearch	http://slim.ucd.ie/ slimsearch/	Search function in given protein(s) for a specific consensus motif for the discovery of novel motif instances.	Krystkowiak and Davey, 2017
THANATOS	http://thanatos.biocuckoo.org/	Manually curated collection of experimentally verified regulators during programmed cell death in eukaryotes.	Deng et al., 2018

crucial for the expansion and closure of the autophagosome. They are also essential for the selective degradation of cargoes. In the past decade, Atg8-family protein interactomes have been extensively studied, and the interaction of a number of proteins with the Atg8 homologs is mediated by a pentapeptide known as LIR/AIM/LRS (LC3-interacting region, Atg8-interacting motif, LC3 recognition sequence; Pankiv et al., 2007; Ichimura et al., 2008; Noda et al., 2010). The presence of such short linear motifs has provided a reliable way to predict the interaction between any given protein of interest and the Atg8-family members. As such, tools have been developed for the identification and prediction of LIR motifs.

The iLIR Server

The iLIR server scans an input protein sequence for the presence of putative LIR motifs (Kalvari et al., 2014). The results are sorted either as an extended LIR-motif (xLIR), or "canonical" LIR motif (WxxL), where "x" can be any amino acid with the only restrictions for W (W/F/Y) and L (L/I/V) positions. Besides, each motif is associated with a position-specific scoring matrix (PSSM) based on experimentally validated LIR motifs; the higher the PSSM score, the higher the confidence in the predicted motif to be involved in the interaction with Atg8-family proteins. Finally, iLIR also overlays the LIR motif results with intrinsically disordered protein regions as predicted by the ANCHOR package. Such protein segments are likely to form stabilizing interactions upon binding. The combination of a high PSSM scoring (>13) xLIR motif that overlaps with an ANCHOR region should provide reliable predictions. The main limitation of this resource is that it cannot predict any non-canonical LIR motifs. The iLIR server is freely available online at the URL *http://repeat.biol.ucy.ac.cy/iLIR* and *http://ilir.warwick.ac.uk/search.php*.

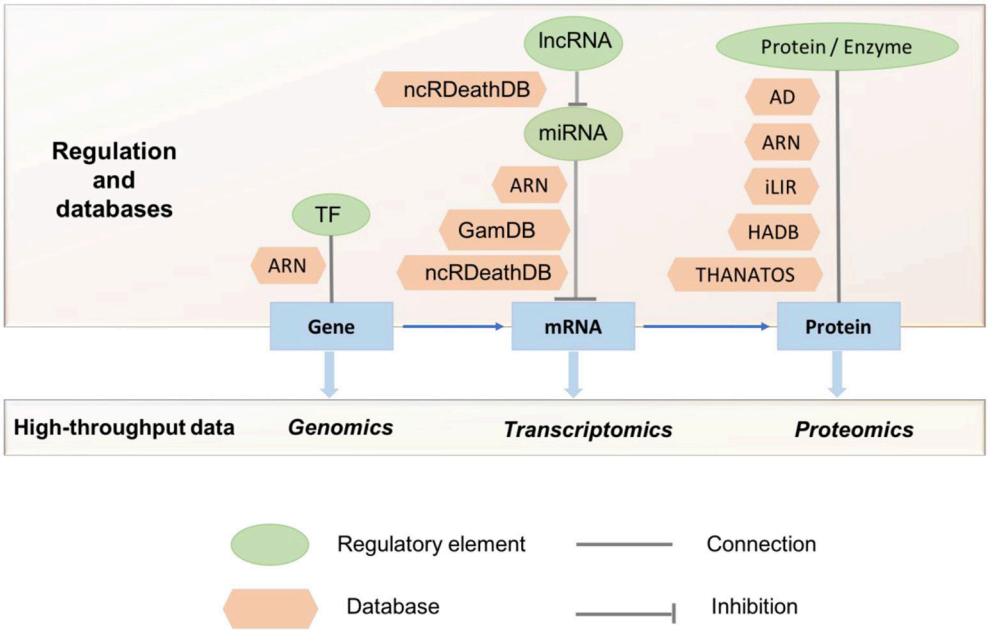


FIGURE 2 | Studies using multi-omics data to understand autophagy and its regulation. Currently available autophagy-related databases highlighting the different stages of regulation and high-throughput data are shown. TF, Transcription Factor; ARN, Autophagy Regulation Network; GamDB, Gerontology-Autophagic-MicroRNA Database; AD, Autophagy Database; HADB, Human Autophagy Database; THANATOS, THe Autophagy; Necrosis, ApopTosis OrchestratorS.

The High-Fidelity AIM System (hfAIM)

The high-fidelity AIM system (hfAIM) is a server used for the prediction of putative LIR/AIM motifs in sequences of interest using five regular expression motifs (Xie et al., 2016). As a proof of concept, the authors have utilized hfAIM to identify potential LIRs in PEX proteins from several model organisms. Using a cell biology approach, they have identified PEX10 as containing at least one functional LIR motif and to interact with Atg8 in the plant model *Arabidopsis thaliana*. The hfAIM resource is available online at the URL: *http://bioinformatics.psb.ugent.be/hfAIM/*

The Eukaryotic Linear Motif Resource (ELM)

The Eukaryotic Linear Motif (ELM) resource is a database, and web server focused on short linear motifs (SLiMs) (Gouw et al., 2017). SLiMs are short protein sequences that can be involved in protein-protein interactions and the modifications of protein sequences. SLiMs are implicated in almost all cellular processes, including cell signaling, trafficking, protein stability, cell-cycle progression. ELM was first released in 2003 and has been regularly updated since then. This resource has incorporated 7 entries related to LIR motifs: 4 ELM classes (added in May 2014) and 3 ELM candidates still being evaluated. ELM lists 24 instances of LIR motifs, including one instance identified in the protozoan parasite *Plasmodium falciparum*, one instance for the nematode *Caenorhabditis elegans* widely used as a model organism, 22 instances are related to human proteins (21 are canonical LIR motifs, 1 correspond to the non-canonical LIR motif from NDP52 necessary for its interaction with LC3C). ELM is maintained by several European groups coordinated by the Gibson group at the European Molecular Biology Laboratory (EMBL). ELM is publicly available online at the URL *http://elm.eu.org*.

SLiMSearch

SLiMSearch is a short, linear motif (SLiM) discovery tool which allows the user to use a motif consensus to search a proteome and discover putative novel motif instances (Krystkowiak and Davey, 2017). Consensus matches are annotated with experimental, proteomic and genomic data; annotations include the description of domains and structures, post-translational modification, single-nucleotide polymorphism, and isoforms. SLiMSearch also provides functional enrichment and evolutionary analysis tools. It is possible to analyse GO terms, keywords and enrichment of interacting partners. SLiMSearch supports a range of species, including bacteria, plants and fungi, and is freely available online at http://slim.ucd.ie/slimsearch/.

Autophagy Networks and Databases

The Autophagy Regulatory Network (ARN)

ARN is a multi-layered molecular interaction database related to autophagy in human, providing users with validated and predicted interaction between proteins, transcription factor and gene, and miRNA and mRNA (Türei et al., 2015). All the interactions in the database have been gathered and extensively manually curated from 26 resources. The interaction network was built around a core of 38 autophagy proteins and gathers over 397,000 interactions. All autophagy components and regulators have been linked to major signaling pathways.

The download functionality gives the user the flexibility to use locally the entire ARN data or a part of it in a broad range of formats (CSV, Cytoscape).

The Autophagy Regulatory Network resource is publicly available online at the URL http://autophagyregulation.org.

iLIR Database and iLIR@viral

The iLIR prediction server has been used to develop two databases: the iLIR database and iLIR@viral. The iLIR database provides a list of all the putative canonical (xLIR and WxxL motifs) LIR-containing proteins identified using the iLIR resource (see before) in the proteomes of 8 model organisms, combined with a Gene Ontology (GO) term analysis (Jacomin et al., 2016). The iLIR@viral database focuses on the identification of putative LIR-containing proteins in viruses known to be linked to autophagy (Jacomin et al., 2017). The databases are accessible online at the URLs http://ilir.uk/model/ and http://ilir.uk/virus/, respectively.

The Thanatos Database

THANATOS (THe Apoptosis, Necrosis, AuTophagy OrchestratorS) is a database that integrates sequence data curated from the literature and related to programmed cell death in eukaryotes (Deng et al., 2018). The database was built based on the manual curation of the literature to identify autophagy-related proteins in the most commonly used models, followed by ortholog searches in 164 eukaryotes. As of the last update in May 2017, the THANATOS database contains information about 191,543 proteins from 164 eukaryotes, which are potentially associated with autophagy and cell death pathways. The web interface allows the user to search the database and retrieve data using keywords and browsing by species and cell death type. Information related to posttranslational modifications on query sequences is also available. The THANATOS database is publicly available online at the URL http://thanatos.biocuckoo.org/.

The Human Autophagy Database (HADb)

The Human Autophagy Database lists over 200 human genes and proteins involved directly or indirectly with autophagy that have been manually collected from the literature. For each entry, HADb provides information on the sequence, transcripts and isoforms. Links to external resources and relevant literature are also available for each entry. HADb is publicly available online at the URL http://autophagy.lu/.

The Autophagy Database

The Autophagy Database is a freely accessible web resource aiming at providing up-to-date information about proteins related to autophagy, including protein structure data (Homma et al., 2011). This resource was last updated in January 2017 and contains information regarding 582 reviewed protein entries. The database also provides additional data regarding orthologous/homologous proteins of the reviewed entries. In addition to offering the possibility to look through the available data, the server also provides the possibility for the user to search the database using keywords and BLAST homology based on query sequences against the database entries. The Autophagy Database is publicly available online at the URL http://www.tanpaku.org/autophagy/.

The ncRNA-Associated Cell Death Database (ncRDeathDB2.0)

The noncoding RNA (ncRNA)-associated cell death database (ncRDeathDB) documents over 4,600 ncRNA-mediated programmed cell death entries (Wu et al., 2015). The ncRDeathDB gathers published data that describe the roles of ncRNAs (including microRNA, long noncoding RNA/lncRNA and small nucleolar RNA/snoRNA) in programmed cell death. The current version of ncRDeathDB summarizes data from 12 species with 4,615 ncRNA-mediated programmed cell death entries: 2,403 entries associated with apoptosis, 2,205 entries associated with autophagy and 7 entries associated with necrosis. The ncRNA-associated cell death interactions resource is publicly available online at the URL http://www.rna-society.org/ncrdeathdb.

AutomiRDB

AutomiRDB is a web resource that combines information related to experimentally identified human miRNAs and their autophagic target genes/proteins in different types of cancers (Chen et al., 2015). An extensive text-mining of the literature was conducted to identify all the known autophagy-related miRNAs. A combination of several miRNA predictive databases was used to predict candidate miRNAs targeting the 49 cancer-related autophagy genes/proteins identified by the authors. The database gives access to 493 miRNAs related to autophagy, 90 targeted autophagic genes or proteins, and 18 types of cancers. Hyperlinks of targets and diseases are provided for easy access to other databases, such as UniProt, OMIM. AtomiRDB is available at the URL http://www.chen-lab.com/index.php

GAMDB

GAMDB (Gerontology-Autophagic-MicroRNA Database) is an open-access knowledge depository which contains 836 microRNAs associated with autophagy, 197 targeted genes or proteins, and 56 diseases related to aging (Zhang et al., 2016). The database was developed based on published articles and public online databases. Experimentally validated autophagy-related miRNA and targeted autophagic genes/proteins in gerontology-related diseases were manually curated from the literature. In the GAMDB website, the user can use microRNAs as keywords to conduct a query and retrieve detailed information. The users also can upload the novel microRNA information through a submission interface. The GAMDB is available at the URL http://gamdb.liu-lab.com/index.php

OMICS STUDIES IN THE AUTOPHAGY FIELD

Read-outs from high-throughput, omics approaches provide us with context-specific and dynamic information on the state and regulation of cellular processes, such as autophagy. Given that "omics" based investigations are a pivotal area of current research to provide a more holistic understanding of biological systems,

such approaches have guided our insights into the regulation of autophagy. In this section, we seek to provide a few examples of studies to illustrate how omics approaches can be used for gaining a better understanding of autophagy processes.

Genomics and Transcriptomics

Genomic approaches including gene mapping and DNA sequencing study the structure and function of the genome, while transcriptomic approaches provide information about the transcriptional changes in the organism on the RNA level. Yang et al. examined autophagy components in 84 species (eukaryotes, eubacteria, and archaebacteria) to discover similarities and differences in terms of the various autophagy phases and the regulatory components involved. Proteome-level data from UniProt was used to identify homologous proteins involved in autophagy across species. Finally, proteins which exist in different taxa were identified with the "hmmsearch" tool in HMMER3 (*http://hmmer.org/)*. Phylogenetic trees using 16S/18S rRNA were reconstructed as a reference to compare the similarity among autophagy genes in different species. Normalized symmetric tree similarity algorithm was used to measure the similarity of the constructed phylogenetic trees. The resulting analysis has revealed that the core autophagy proteins are present in most of the investigated species with the difference being a fewer number of core proteins in plants and protists. The main difference lies in the vesicle elongation, and maturation phase of the autophagy pathway wherein most plants are characterized by a lack of some of the ATG5–ATG12 conjugation-related proteins. Some of the prokaryotic homologs of the core autophagy proteins were also suggested to play different roles in the process upon comparison with the eukaryotic species. For instance, ATG11 has an essential role in selective autophagy regarding the yeast organism, in contrast to Archaebacteria this protein is liable for DNA repair and reorganization of chromosomes. By collating the phylogenetic trees, the most significant similarity was found to be among proteins which are responsible for the autophagosome nucleation and ATG9 cycling. In terms of selective autophagy, the phylogenetic trees were quite different from each other. The analysis also highlighted the fact that parasitic organisms such as *Entamoeba histolytica* and *Plasmodium yoelii* which have relatively fewer ATG proteins still exhibited autophagic activity (Yang et al., 2017).

Foldvari-Nagy et al. published a similar observation when they used both BLAST and HMMER methods to identify autophagy-like proteins in 40 non-unikont parasitic protists (e.g., *Trypanosoma* and *Plasmodium)*. According to a comprehensive computational analysis, *Atg1* and genes encoding its induction complex were found to be lacking in the genomes of all the studied species. As an alternative induction of autophagy, $<$20 species contained Atg6/Beclin1, in the other remaining investigated non-unikont parasites, this protein does not appear in the genome. In this case, there is no evidence which could provide a clue on how autophagy is induced in these species (Foldvari-Nagy et al., 2014).

The relationship between autophagy and the cellular homeostasis in the nervous system is poorly discovered, based on our knowledge malfunction of autophagy causes protein aggregation and neurodegeneration. Lipinski and colleagues investigated the transcriptional level alterations between healthy aging and Alzheimer disease (AD), and they found up-regulated autophagy in brain samples from AD patients (compared to normal brain samples). Based on these observations, it was suggested that the up-regulated autophagy signatures in the AD patients could be a compensatory mechanism in order to remove the accumulated protein aggregates (Lipinski et al., 2010).

Besides its importance in neuronal functions, autophagy also influences the identity and function of myeloid cells as well. Huang et al. examined how the expression pattern of autophagy genes is changing when myeloid cells differentiate to monocytic and granulocytic cells. RNA-Seq data from CD34+ hematopoietic stem and pluripotent cells (HSPCs) exposed to monocytic and granulocytic induction helped discover the relationships between autophagy and the differentiation of HSPCs. Differentially expressed genes involved in the autophagy process were inferred by combining the observed transcriptional changes and annotations/regulator information obtained from the Autophagy Database, Autophagy Regulatory Network (ARN) and Human Autophagy Database (HADb). Based on the analysis of the temporal gene expression data using a standard clustering algorithm, 22 autophagy genes were found to be significantly altered during the monocytic and granulocytic differentiation process of myeloid progenitors into monocytes and granulocytes. The results suggested that autophagy is essential to maintain the balance between different states (quiescence, self-renewal, and differentiation) in myeloid cells (Huang et al., 2018).

Autophagy has also been investigated using multi-omics approaches in the context of its role in host-pathogen interactions. Lu et al. studied how autophagy influences the homeostasis in lungs and resistance to influenza infection by comparing the transcriptional profiles of lung macrophages derived from normal mice and mice deficient in multiple autophagy components including Atg5, Atg7, Atg14, Epg5, and FIP200. RNA-seq analysis revealed the dysregulated pathways in autophagy-deficient macrophage cells. It turned out that *Epg5* is essential for basal autophagy and autophagosome formation and is supported by increased inflammation and lethal influenza virus infection resistance in the lung upon its absence (Lu et al., 2016).

Proteomics

Mass spectrometry (MS)-based proteomics is an invaluable way for studying protein-protein interactions, protein expression, subcellular localization, and post-translational modifications. Given the increased interest in the identification of new players in the regulation of autophagy, it is not surprising that MS-based proteomics approach has been widely used and has successfully contributed to advancing the knowledge on autophagy.

One of the most straightforward applications of MS-based proteomics is the comparison of whole cell proteomes between autophagy-deficient cells and autophagy-competent cells. As such, a study conducted by Zhuo and colleagues in atg7$^{-/-}$ MEFs has identified 66 upregulated and 48 downregulated proteins

(Zhuo et al., 2013). This study led to the identification of F-actin and showed that it plays a role in both basal and starvation-induced autophagy. Analysis of the whole neosynthesized proteome after induction of autophagy was performed by adding an azide methionine mimetic, azidohomoalanine (AHA), into the culture media. AHA is subsequently incorporated into newly synthesized proteins, which can be further enriched by affinity isolation before analysis by LC-MS/MS. This innovative approach has been successfully used in HeLa cells, allowing for the profiling of 711 newly synthesized proteins. Several hits were further validated and characterized; for instance, ATP5B, RACK1, and SLC25A3 proteins were identified as playing a role in the promotion of autophagy (Wang et al., 2016).

The formation of the autophagosome is one of the hallmarks of the autophagy process. Due to its importance, studies focused specifically on the proteome of the autophagosome have been performed. The characterization of proteins associated with the autophagosomes were carried on isolated autophagosomes. Dengjel and colleagues used a density gradient to separate fractions containing the autophagosomes from MCF7 breast cancer cells. Selected fractions were analyzed by LC-MS/MS, and for nonspecific co-purifying proteins, the PCP-SILAC method was applied (Dengjel et al., 2012). A total of 728 putative autophagosome-associated proteins were identified from the analysis of autophagosomes isolated from cells subjected to amino acid starvation or treatment with either the autophagy-inducer rapamycin that inhibits the mTOR complex 1, or the lysosomal inhibitor concanamycin A. Only 94 proteins were common to all stimuli and some of them were previously identified by independent studies that aimed to identify autophagosome-membrane associated proteins (Gao et al., 2010; Øverbye et al., 2014). The poor overlap between these three studies may be due to the difference in stimuli used, cell types, and purification and MS analysis differences.

Another advantage of the MS-based proteomics resides in the high-throughput identification of protein-protein interaction partners. In 2010, an extensive study of the autophagy network by Behrends and colleagues provided a global view of the autophagy interaction landscape in basal condition. In this study, 32 human proteins related to autophagy or vesicle trafficking were used as prey in the 293T cell line, and the immune complexes were analyzed by MS. A total of 751 interactions among 409 candidate interacting proteins were revealed. The study focused on the protein partners of the six ATG8-family proteins. Thirty-eight ATG8-interacting proteins were tested *in vitro* for their binding to ATG8 proteins. Up to 60% of the interactions were reduced or lost when the LIR-docking site on ATG8 proteins was mutated, indicating that a substantial proportion of ATG8-interacting proteins do so through an LDS/LIR-dependent binding (Behrends et al., 2010). More recently, Le Guerroué and colleagues used a state-of-the-art proximity-proteomics-based autophagosome content profiling to identify the interacting partners of the ATG8 proteins. Screening for the interactors of all six ATG8 proteins in mammals, they identified 1,147 proteins with considerable overlap across GABARAP and LC3 family members and among GABARAP and LC3 subfamilies. This approach led them to identify the mitochondrial protein MTX1 that is targeted by LC3C and p62 to maintain basal mitochondrial homeostasis through a piecemeal mitophagy pathway (Le Guerroué et al., 2017).

Metabolomics and Lipidomics

Metabolomics is a recently emerging field aimed at the systemic profiling of the metabolites, which are the small molecule intermediates and products of metabolism. Metabolomics is a powerful approach to complement other "omics" as metabolite profiles and concentration reflect the functional and physiological state of cells/organisms. Studies of the metabolome are based on two key techniques: nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) (Markley et al., 2017). Because autophagy is tightly associated with the cell stress status, it is not surprising that autophagy-related metabolomes will be subject to changes depending on the nature of the stresses happening in the cells (Stryeck et al., 2017).

Autophagy is strongly activated by starvation conditions characterized by low levels of glucose or amino acids. When glucose levels are high, ATP is converted into cAMP and is itself further degraded into AMP. As such, a high AMP:ATP ratio reflect a high glucose level; while a reduced AMP:ATP ratio is typical of starvation conditions when glucose levels are low. Thus, glucose, ATP, cAMP, and AMP are appropriate read-outs used in autophagy-related metabolomics studies (Stryeck et al., 2017).

Mutations in the *RAS* oncogene control tumor growth and RAS-associated tumors heavily rely on autophagy. Because cancer usually depends on high glucose level, Lashinger and colleagues have used a mouse model system to investigate the effect of caloric restriction and autophagy on the development of RAS-driven tumors (Lashinger et al., 2016). They have shown that combining autophagy blockade (Atg5-deficient mice) to caloric restriction was sufficient to reduce the tumor volume significantly. Using NMR, they observed that caloric restriction induced a switch away from glucose metabolism, characterized by a reduction of glucose, amino acids, and tricarboxylic acid cycle intermediates, and upregulation in ketone bodies. Similar observations made by Gaglio et al. wherein blocking autophagy using the inhibitor chloroquine caused massive cell death of NIH-RAS cancer cells *in vitro*. However, using chloroquine *in vivo* did not produce any notable effect on highly aggressive NIH-Ras xenografts. Nevertheless, changes in the metabolome of the tumors were observed after treatment, suggesting that RAS-driven tumors have the ability to adapt to environmental modifications and metabolic stress using metabolic rewiring and alternative pathways (Gaglio et al., 2016).

Another *in vitro* study, conducted by Redmann et al. on cultured primary rat cortical neurons from E18 embryos used HPLC-MS metabolomics approach to investigate the impact of lysosome inhibitors on bioenergetics and metabolism (Redmann et al., 2017). Notably, they showed that autophagy inhibition decreased metabolites of the TCA cycle, essentially downstream

the citrate synthase and those linked to glutaminolysis. Their results implicate that inhibitors of autophagy impact on cellular bioenergetics and metabolism in primary neurons, probably due to decreased mitochondrial quality control.

Lipidomics is a sub-category of metabolomics that focuses on the identification and quantification of cellular lipids. While it has been described that changes in the cellular level of ceramides—a family of lipids—can affect autophagy, little is known about the regulation of these lipids by autophagy itself. A recently published study by Alexaki and colleagues sought to evaluate the implication of autophagy in the regulation of ceramides in the liver, as autophagy is essential in this organ to maintain homeostasis and prevent metabolic diseases. To this end, the authors used HPLC-MS to identify and quantify the ceramide species in the liver from wild-type and *Atg7*$^{-/-}$ mice. They observed that ceramides are significantly increased in autophagy-deficient livers, which is correlated with an increase in serine palmitoyltransferase, the enzyme that catalyzes the *de novo* synthesis of sphingolipids, included ceramides. Based on their observations, the authors suggest that autophagy may contribute to the regulation of serine palmitoyltransferase level by targeting the degradation of the protein subunits or endoplasmic reticulum membranes containing excessive ceramide near serine palmitoyltransferase (Alexaki et al., 2014). In an *in vitro* study, Tharkeshwar and colleagues have focused their interest on the lipid composition of isolated lysosomes in the context of Niemann-Pick disease type C1 (NPC1) deficiency. Niemann-Pick disease type C (NPC) is a severe inherited lysosomal storage disorder, most often originating from the loss-of-function of NPC1. Their lipidomics analysis on lysosomes isolated using superparamagnetic iron oxide nanoparticles (SPIONs) revealed the build-up of several species of glycerophospholipids and other storage lipids in lysosomes of NPC1-deficient cells (Tharkeshwar et al., 2017).

INTEGRATION OF OMICS DATASET TO UNDERSTAND AUTOPHAGY BETTER

Integration of omics datasets with signaling and regulatory networks is used to study biological processes and their regulation on a systems-level. Recent studies supported by advances in experimental omics technologies and computational data integration approaches have shed light on the mechanistic and regulatory aspects of autophagy. Here, we list a few examples from various areas using different omics approaches.

Despite the dramatic increase in the volume of generated information on autophagy such as the identification of core components, their regulators etc., the complexity of the autophagy process itself as well as the functional hierarchy in the biological system makes data representation and analysis a challenging task. Some of these issues were overcome by using Gene Ontology (GO) which assigns genes and their encoded products with specialized ontology terms capturing the entire range of the functional hierarchy. Kramer et al. recently developed a general framework—AtgO (http://atgo.ucsd.edu/index.html), to explain and present the hierarchy of autophagy functions in yeast. Published omics data was combined with a newly generated genetic interaction map targeted at autophagy. It contains almost 500 genes which could be related to the process of autophagy according to the literature and results from experiments. Using protein sequences and structure details, researchers revealed interactions between proteins or genes, co-expression levels and similarities between genes. The AtgO process schema was applied to human data as well which resulted in the Human Autophagy Ontology database (hAtgO) containing 1,452 genes and 1,664 terms describing autophagy based on the expression profile of genes, interactions between proteins, co-localization, etc. (Kramer et al., 2017).

Due to the homeostatic role of autophagy and its dysregulated/deregulated status in many chronic diseases, exploring the connections between cancer and autophagy is a growing research area. While on one hand autophagy suppresses tumorigenesis, cancer cells also activate the process to avoid the stress and up-regulate growth and tumor aggression (Lorente et al., 2018). Autophagy strongly influents cancer so that modulation of this process has been identified as a potential target for cancer therapy (Kubisch et al., 2013). Omics data integration is widely used to investigate the genomic events and their interactions, as well as the potential regulatory mechanisms affected in cancer (Sompairac et al., 2018). In their recent study, Chen et al. focussed on identifying genes related to breast cancer and its multiple subtypes at the genomic level with an integrated bioinformatics approach. They used the Least Absolute Shrinkage and Selection Operator (LASSO) method which is designed for -omics data integration. By bringing together three different data types namely mRNA expression (DNA microarray), DNA methylation (Illumina Methylation Assay), and copy number alteration (GenomeWideSNP_6 array) data provided by The Cancer Genome Atlas database (TGCA—*https://cancergenome.nih.gov/),* they identified the regulators of BECN1, a core autophagy component, which has an inhibitory effect on tumor formation (Chen et al., 2017).

Aneuploidy, an unbalanced karyotype when the cell contains extra chromosomes, is an often discovered disorder during cancer. Stingele et al. used data from comparative genomics, transcriptomics, and proteomics to investigate the molecular manifestations of aneuploidy. In more details, they discovered those proteins and transcripts which are encoded on the extra chromosomes. Focussing on the fate of transcripts and proteins encoded on the extra chromosomes, the gene copy number, mRNA and protein levels were measured with array comparative genomic hybridization, microarray analysis, and mass spectrometry, respectively. The integrative analysis revealed that mRNA levels increased with gene copy numbers, but the relative abundance of proteins was significantly reduced. Interestingly, autophagy and lysosome-mediated degradation were found to be consistently up-regulated suggesting a higher demand for autophagy in aneuploid cells possibly due to an elevated requirement to degrade the extra-coded proteins (Stingele et al., 2013).

Arabidopsis thaliana is a versatile plant model organism in which autophagy plays a key role in various processes from immune responses to environmental adaptation. Autophagy is also activated during leaf senescence and exposure to external stressors such as pathogen attack, starvation etc. Masclaux-Daubresse et al. investigated the effect of autophagy alterations by studying the transcriptomic and metabolomic signatures of autophagy mutants. Gas chromatography-mass spectrometry (GC-MS) was used to measure small sized molecules and liquid chromatography-mass spectrometry (LS-MS) and enzymatic assays for complex molecules. Microarray analysis followed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) measured the transcriptomic level changes. Integration of metabolomics and transcriptomics data from the rosette leaves elucidated the pleiotropic effect of autophagy activity on cellular homeostasis and revealed the dependence on autophagy of various metabolic pathways. Based on the results from the transcriptomics datasets, many genes related to plant stress response were also overrepresented upon autophagy malfunction in the mutants (Masclaux-Daubresse et al., 2014).

PROMISING OMICS APPROACHES—THE FUTURE OF SYSTEMS-LEVEL AUTOPHAGY STUDIES

Single-Cell Analysis in Autophagy Research

Single cell sequencing gives us an inside view at a cellular level by enabling the measurement of genomic and transcriptomic readouts from individual cells. This possibility has opened up investigations into biological processes and functions in different cell types—which has hitherto not been possible at high-throughput. Several studies which look into the role of autophagy in numerous cell-types have also recently been published.

Due to its central and homeostatic role in cellular physiology and metabolism, autophagy interacts with a vast number of other processes in the cell. Filippi-Chiela et al. probed the interplay between autophagy and senescence using human glioma cells which were modified to represent the DNA damage-induced senescence using a relevant model. Using single-cell sequencing, they demonstrated the differences in the correlation between the two processes in glioma cells compared to the cell-type non-specific population. While at the population cell level, autophagy and senescence were negatively correlated, data from the human glioma cells indicated otherwise with a complete absence of such a correlation between the two processes (Filippi-Chiela et al., 2015).

Xu et al. revealed a similar study to investigate the differential dynamics between autophagy and apoptosis using single-cell sequencing in tandem with experimental live microscopy imaging of cells from multiple cell lines. The cells were exposed to various autophagy eliciting stimuli including starvation and mTOR inactivation. Autophagy was induced to different degrees suggesting autophagy to be a pathway with a wide magnitudinal response window. However, under all the tested stimuli, apoptosis also was induced in a binary fashion—either completely induced or absent, thus representing a bimodal response mechanism. Using fluorescent reporters and live cell imaging, the dynamic responses of individual cells were tracked to reveal that autophagy preceded apoptosis. However, in those cells which mounted a very strong autophagic response, there was a time-lag before the activation of the cell death. This was further verified by the upregulation of apoptosis in a cell line deficient in *atg5* which is essential for autophagic activity (Xu et al., 2013).

Hematopoietic stem cells (HSCs) are a lineage of stem cells which are present in both early embryonic and adult hematopoietic organs with the capability to renew themselves and differentiate into multiple lines of blood cells. Due to the essential role of autophagy in self-renewal and differentiation in embryonic hematopoiesis, Hu et al. investigated its role in five populations of mice cells related to HSC formation during the process of mouse embryogenesis by measuring gene expression using single-cell RNA sequencing. The results from the study revealed an increase in the transcriptional level of various autophagy component encoding genes in endothelial cells which were classified as pre-HSCs. This observation was synchronous with the down-regulated Notch signaling thus suggesting that autophagic activity could play a significant role in the formation of HSCs during the gestation period (Zhou et al., 2016; Hu et al., 2017).

Although the single-cell analysis is a rapidly evolving approach, there are two main challenges which could be solved in the future, namely the integration and interpretation of omics data. In general, the single-cell analysis covers various steps (isolation, sorting, library preparation, and sequencing) when the cell is separated from its bulk environment. On one hand, while the examination of the cell in an isolated milieu can lead to data loss, on the other hand, the cell is a dynamic structure where the molecular states will differ temporally across different sampling points. A future goal will be to improve the *in situ* analysis techniques in fixed cells or tissues and develop the analysis of live imaging. The second challenge is to carry out multi-omics profiling which provides insights into cell regulatory mechanisms, such as autophagy. Examining the biological processes by using the techniques mentioned above facilitates to understand the complexity of human health/disease in order to develop more effective therapies (Yuan et al., 2017).

Patient-Derived Samples

Compared to various *in vitro* models, investigations on patient-derived samples are desirable since it provides researchers with the necessary basis to understanding disease mechanisms for translational purposes as well as personalizing treatments. Martínez-Pizarro et al. investigated the role of endoplasmic reticulum (ER) stress and autophagy in fibroblasts derived samples diagnosed with homocystinuria—a disease characterized by defective cysteine metabolism and manifested by neurological and behavioral anomalies. Earlier studies reported upregulated levels of reactive oxygen species and apoptotic activity in fibroblasts derived from homocystinuria patient biopsies. However, the exact mechanisms in terms of the molecular pathologies underlying the disease were not explained. In the

study by Martínez-Pizarro et al. the authors used a combination of techniques such as PCR, Western blotting, microscopy and cytosolic Ca^{2+} imaging followed by *in vitro* assays to first identify and then verify the mechanistic roles of the identified molecular mediators. Gene and protein expression profiling revealed the induction of a number of proteins related to ER stress and calcium signaling. In addition, autophagy was also found to be activated along with mitophagy-mediated degradation of modified mitochondria. Treatment with antioxidative agents inhibited autophagy suggesting that reactive oxygen species affect autophagy which otherwise would impart a protective role under homeostatic conditions (Martínez-Pizarro et al., 2016).

Inflammatory bowel diseases (IBD) represent a group of intestinal disorders which are characterized by chronic inflammation. The most common forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC): while CD affects any part of the gastrointestinal tract, UC results in inflammation in the colon and the rectum. Silverberg and colleagues collected samples from healthy and diseased patients suffering from CD or UC. They extracted RNA from peripheral blood mononuclear cells and examined the post-transcriptional regulation of autophagy. The study revealed that there are differences in miRNA expression between the healthy and diseased samples. miRNAs are small non-coding RNA molecules which bind to mRNAs thereby silencing their transcription. miRWalk 2.0 software was used to predict miRNA-mRNA interactions and thereafter with GO and pathway analysis, the dysregulated pathways were identified and analyzed. As a result, the authors observed that the most of the differentially expressed miRNAs can effect on the autophagy pathway confirm the significant effect of miRNA-mediated modulation on autophagy (Mohammadi et al., 2018).

High Throughput Screening (HTS) and High Content Analysis (HCA)

With interest in identifying new regulators of autophagy in different cell types or different stress conditions, High Throughput Screening (HTS) and extraction of vast amount of data from automated image analysis have gained in popularity over the past decade. Indeed, High Content Analysis (HCA) allows for the unbiased quantitation of phenotypic images through the development of automated image acquisition and analysis. Most autophagy-focused HCA screenings rely on the quantitation of autophagosomes using fluorescent-tagged LC3. In order to identify new autophagy-related genes, He and colleagues used a library of cDNA to investigate the implication of 1,050 genes of unknown function in autophagy. The overexpression of the transmembrane protein TM9SF1 resulted in the accumulation of autophagosomes and general increase of the autophagy flux in HeLa cells. Silencing of TM9SF1 using si-RNA impaired starvation-induced autophagy. In addition, TM9SF1 localized to the autophagosome and lysosome, suggesting a direct implication of this protein in the autophagy process, although its molecular mechanism and function remain unclear (He et al., 2009). Focusing on bacterial effector associated with Crohn's disease a screen was performed by overexpression 224 GFP-fused proteins from Adherent Invasive *E. coli* (AIEC) strain LF82 in HeLa cells and monitoring the induction of autophagy using mCherry-LC3 (Collins and Huett, 2018). The analysis also concentrated on overall cellular and nuclear morphology and actin cytoskeleton. This work did not provide any molecular mechanism, but instead was made available to the scientific community as all the raw images and original analysis files can be downloaded for further analysis (Collins and Huett, 2018). Another recent study focused on the identification of modulators that are specific for p62-mediated selective autophagy (Hale et al., 2016). Instead of using tagged-LC3 in their primary screening, Hale and colleagues used GFP-p62/SQSTM1 and lysosomal marker LAMP2 to screen si-RNA targeting over 12,000 genes in U2OS fibroblast cell line. The mean intensity in GFP-p, as well as GFP-p62 colocalization with LAMP2 were used to assess the induction of autophagy. From the 12,000 genes initially screened, 10 hits were eventually selected and validated to induce an upregulation of the autophagy flux when knocked down (Hale et al., 2016). Altogether, these three studies showed that image-based high content analysis is a robust strategy for the identification of new modulators of autophagy, that can be applied to a broad range of conditions.

Giving the fact that autophagy is related to a broad range of pathologies, targeting autophagy machinery components and regulators could be an appealing alternative to classical chemotherapeutic agents. To this end, in order to fill the gap in the number of autophagy inhibitors and potential therapeutic agents, Peppard and collaborators designed a phenotypic, cell image-based assay for small molecules that affects the accumulation of autophagosomes in starved cells expressing GFP-LC3 (Peppard et al., 2014). Over 240,000 compounds were screened, leading to the identification and qualification of about 400 active molecules.

However, stimulating autophagy may constitute a promising approach to prevent or treat some pathologies in aging-related diseases. Chiang and colleagues sought to identify new autophagy inducers that disrupt the binding between Beclin-1 and Bcl-2 (Chiang et al., 2018). For their primary screening of about 300,000 small molecules, they developed an assay using a split-luciferase to measure the interaction between these two key regulators in cells. Selected compounds were then tested *in vitro* using a Beclin-1/Bcl-2 AlphaLISA assay to measure the interaction between the two proteins. This two-step screen led to the identification of three active molecules that induce autophagy.

OUTLOOK

Autophagy is one of life's fundamental processes. Recent research has indicated roles for autophagy in an increasing number of pathologies, from bacterial and viral infections to cancer, and more recently in neurodegenerative and other age-related diseases. The importance and significance of the autophagy process was highlight very recently with the Nobel Prize award to

Prof. Yoshinori Ohsumi for his pioneering studies revealing the mechanisms of autophagy in baker's yeast 30 years ago (Tsukada and Ohsumi, 1993). Big data and omics approaches provide us with the key to further elucidate the complex autophagy network and its integration with other cellular networks in the context of both health and disease.

AUTHOR CONTRIBUTIONS

A-CJ and LG wrote the manuscript with input from PS. TK and IN edited the manuscript.

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Melanoma LAMP-2C Modulates Tumor Growth and Autophagy

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Autophagy plays critical but diverse roles in cellular quality control and homeostasis potentially checking tumor development by removing mutated or damaged macromolecules, while conversely fostering tumor survival by supplying essential nutrients during cancer progression. This report documents a novel inhibitory role for a lysosome-associated membrane protein, LAMP-2C in modulating autophagy and melanoma cell growth *in vitro* and *in vivo*. Solid tumors such as melanomas encounter a variety of stresses *in vivo* including inflammatory cytokines produced by infiltrating lymphocytes directed at limiting tumor growth and spread. Here, we report that in response to the anti-tumor, pro-inflammatory cytokine interferon-gamma, melanoma cell expression of *LAMP2C* mRNA significantly increased. These results prompted an investigation of whether increased melanoma cell expression of LAMP-2C might represent a mechanism to control or limit human melanoma growth and survival. In this study, enhanced expression of human LAMP-2C in melanoma cells perturbed macroautophagy and chaperone-mediated autophagy in several human melanoma lines. *In vitro* analysis showed increasing LAMP-2C expression in a melanoma cell line, triggered reduced cellular LAMP-2A and LAMP-2B protein expression. Melanoma cells with enhanced LAMP-2C expression displayed increased cell cycle arrest, increased expression of the cell cycle regulators Chk1 and p21, and greater apoptosis and necrosis in several cell lines tested. The increased abundance of Chk1 protein in melanoma cells with increased LAMP-2C expression was not due to higher *CHEK1* mRNA levels, but rather an increase in Chk1 protein abundance including Chk1 molecules phosphorylated at Ser345. Human melanoma cell xenografts with increased LAMP-2C expression, displayed reduced growth in immune compromised murine hosts. Melanomas with high LAMP-2C expression showed increased necrosis and reduced cell density upon histological analysis. These results reveal a novel role for LAMP-2C in negatively regulating melanoma growth and survival.

Keywords: LAMP-2, LAMP-2C, macroautophagy, chaperone-mediated autophagy, melanoma, tumor

Abbreviations: Chk1, checkpoint kinase 1; CMA, chaperone-mediated autophagy; CQ, chloroquine; HSC70, heat shock cognate protein 70; HSP90, heat shock protein 90; IFN-γ, interferon-gamma; LAMP, lysosome-associated membrane protein; LC3, microtubule-associated protein light chain 3; MA, macroautophagy; ROS, reactive oxygen species.

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INTRODUCTION

Basal levels of autophagy are critical to cellular homeostasis by eliminating malfunctioning organelles and long-lived proteins (Levine and Kroemer, 2008). Autophagy increases with nutrient deprivation and hypoxia (Levine and Kroemer, 2008). Defects in autophagy impact several diseases, including cancer (Levine and Kroemer, 2008; Morselli et al., 2009; Choi, 2012). However, the role of autophagy in cancer development is complex. While basal autophagy may function as a tumor suppressor, increased or induced autophagy may contribute to tumor survival during cancer progression (Morselli et al., 2009; Choi, 2012).

Two forms of autophagy, MA and CMA are detectible in human cells and upregulated in many tumors (Morselli et al., 2009; Kon et al., 2011; Choi, 2012). MA increases with cell nutrient stress and temporally wanes as CMA increases and is sustained. During nutrient or growth factor deprivation, MA and CMA are upregulated to promote cell survival by recycling building blocks, modulating bioenergetics, and shifting metabolism. During MA, cytoplasmic macromolecules and organelles are sequestered inside autophagosomes, which fuse with lysosomes to promote content degradation. Basal levels of MA may prevent tumor development by modulating chromosome stability and removing mutated proteins and damaged mitochondria (Morselli et al., 2009; Choi, 2012). However, with tumor progression and exposure to metabolic stresses, MA is induced to recycle nutrients, favor tumor survival and resistance to anti-cancer therapies (Morselli et al., 2009; Choi, 2012). During CMA HSC70 and HSP90, capture cytoplasmic proteins for selective translocation into lysosomes for degradation (Agarraberes and Dice, 2001). CMA is upregulated in many tumors including melanoma, breast, and lung cancers (Kon et al., 2011; Saha, 2012; Zhou et al., 2016). CMA relies on a LAMP-2A to translocate cytoplasmic proteins into lysosomes (Cuervo and Dice, 1996). Hyper-expression of LAMP-2A is observed in tumors, while disrupting LAMP-2A expression slows tumors growth and metastasis (Kon et al., 2011; Zhou et al., 2016).

LAMP-2 is a highly glycosylated protein localized in acidic lysosomal and endosomal compartments. Alternative splicing generates three isoforms LAMP-2A, LAMP-2B, and LAMP-2C, which differ primarily in the sequence of their transmembrane and cytosolic tail (Eskelinen et al., 2005). LAMP-2A and LAMP-2B are constitutively expressed by all cells, while LAMP-2C has a much more limited tissue distribution (Perez et al., 2016). LAMP-2A, the receptor for CMA may modulate aging and tumor growth (Cuervo and Dice, 1996, 2000; Kon et al., 2011; Perez et al., 2016). LAMP-2B is involved in lysosome biogenesis and MA (Nishino et al., 2000). Mutations in LAMP-2B have been reported to disrupt autophagosome maturation. LAMP-2C can facilitate DNA and RNA translocation into lysosomes, while enhanced LAMP-2C expression inhibits CMA in human B lymphoblasts (Fujiwara et al., 2013a,b; Perez et al., 2016). Yet, little is known about LAMP-2C function in tumor cell growth and autophagy.

In vivo, tumors such as melanomas encounter infiltrating immune cells producing pro-inflammatory cytokines, which can induce stress and limit tumor growth. While melanoma cells express relatively low levels of LAMP-2C compared to LAMP-2A and LAMP-2B, as shown here exposure of these cells to the cytokine IFN-γ significantly increased *LAMP2C* mRNA abundance. By contrast, only marginal changes in *LAMP2A* mRNA expression and no difference in *LAMP2B* mRNA abundance were detected in IFN-γ treated melanoma cells. These cytokine-induced changes suggested that LAMP-2C could potentially play a role in regulating tumor cell survival and responses to stress. In this study, we explored the role of LAMP-2C in the growth and survival of human melanoma cells using a rodent xenograft model. Human melanoma cells were transfected to increase LAMP-2C protein expression. In the melanoma cell line DM331, ectopic expression of LAMP-2C resulted in decreased expression of LAMP-2A and LAMP-2B proteins. CMA was diminished in cells with increased LAMP-2C, as indicated by the increased abundance of several proteins typically targeted for degradation by CMA including Chk1, IκBα, and p21 (Cuervo et al., 1998; Park et al., 2015; Zhang et al., 2018). Significant reductions in MA were also detected in melanomas with increased LAMP-2C expression based on analysis of MA flux and autophagosome abundance. Ectopic expression of LAMP-2C altered melanoma cell growth *in vitro* and cell cycle progression with increased apoptosis and necrosis detectable in several melanoma cell lines. These changes in the cell cycle may be related to the greater abundance of Chk1 and phospho-Chk1 as well as p21 in melanomas with increased LAMP-2C. *In vivo*, human melanoma cells with increased LAMP-2C displayed reduced growth and increased necrosis compared with the parental melanoma cell line. This study demonstrates a novel role for LAMP-2C in melanoma growth and offers innovative strategies for targeting subcutaneous melanoma.

MATERIALS AND METHODS

Cell Lines and Transfection

The human melanoma cell line DM331 provided by Dr. V. Engelhard (University of Virginia) was maintained in RPMI-1640 with 5% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1% L-glutamine (Slingluff et al., 2000). The human melanoma cell line SLM2-Mel provided by Dr. W. J. Storkus (University of Pittsburgh School of Medicine) was maintained in the same media with 0.1% β-mercaptoethanol (Haque et al., 2002). Melanoma cell lines were transfected using Xfect Transfection Reagent (Clontech, Mountain View, CA, United States). Control vectors or vectors encoding human *LAMP2C* have been described (Perez et al., 2016).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To detect *LAMP2* or *GAPDH* transcript expression, cellular RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, United States) and cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States). Primers for *LAMP2* and *GAPDH* amplification were described (Perez et al., 2016). *LAMP2* cDNA was amplified using 2X ReddyMix PCR Master Mix (Thermo

Fisher Scientific, Waltham, MA, United States) for 35 cycles. *GAPDH* cDNA was amplified for 30 cycles. PCR products were resolved by agarose gel.

Real-Time Quantitative PCR (qPCR)

qPCR was performed using custom Taqman primers for *LAMP2A*, *LAMP2B*, and *LAMP2C* (Perez et al., 2016) or commercial primers *CDKN1A*, *CHEK1*, *CTSA*, *CTSB*, *CTSD*, *NFKBIA*, *TP53*, *ACTB*, *GAPDH* or *18S*, and the 7500 Fast RT-PCR System from Applied Biosystems. Gene expression was normalized to *ACTB*, *GAPDH* or *18S* mRNA levels and presented as a relative fold change compared with control samples or presented as mRNA expression relative to *18S* mRNA levels. For analysis of fold changes in mRNA, if differences of less than twofold were detected, trends in expression were noted rather than statistical significance.

Western Blotting

Cells were lysed on ice for 30 min with RIPA buffer, protease inhibitor cocktail ± phosphatase inhibitor cocktail. Cell lysate proteins (80 μg) were resolved on SDS-PAGE and transferred to nitrocellulose for western blots. Blots were quantitated by densitometry using ImageJ (NIH, Bethesda, MD, United States) and normalized to cellular actin. Antibodies against LAMP-2A (Cat #ab18528), LAMP-2B (Cat #ab18529), HSP90 (Cat #ab13494), and cathepsin A (Cat #ab79590) were from Abcam (Cambridge, MA, United States). Chk1 (Cat #2360), phospho-Chk1 (Ser345) (Cat #2341), IκBα (Cat #4814), phospho-IκBα (Ser32/36) (Cat #9246), LC3B (Cat #2775), and histone H3 (Cat #3638) were from Cell Signaling Technology (Danvers, MA, United States). LAMP-2 (Cat #H4B4-c) was from DSHB (Iowa City, IA, United States) and HSC70 (Cat #ADI-SPA-815) from Enzo Life Sciences (Farmingdale, NY, United States). Anti-Myc Tag (Cat #05-724) and cathepsin D (Cat # IM03) were from EMD Millipore (Billerica, MA, United States). Cathepsin B (Cat # sc-13985), p53 (Cat # sc-126), and p21 (Cat # sc-756) were from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Actin (Cat # MS-1295-P0) was from Thermo Fisher Scientific.

Interferon-Gamma Treatment

DM331 cells were incubated 24 h at 37°C with 400 or 2000 units (IU) of recombinant human IFN-γ (R&D Systems, Minneapolis, MN, United States). Cells were harvested and *LAMP2* mRNA was measured by qPCR.

MA Analysis

To detect MA flux, cells were incubated for 16 h at 37°C ± 20 μM CQ (Sigma-Aldrich, St. Louis, MO, United States) (Mizushima and Yoshimori, 2007; Mizushima et al., 2010; Klionsky et al., 2012). Western blotting was used to detect cellular LC3I and LC3II. Cellular LC3I and LC3II protein levels were normalized relative to actin protein levels to account for protein sample loading. MA flux was determined by subtracting the relative ratio of LC3II/actin in untreated cells from the relative ratio of LC3II/actin for CQ treated cells. To monitor MA in real time within live cells, melanoma cells were incubated 4 h at 37°C with media ± serum. Vesicles produced during MA in normal or starvation conditions were stained using CYTO-ID Autophagy detection kit (Enzo Life Sciences) and analyzed by flow cytometry (Guo et al., 2015).

Lysosomal Proteases or Calpain Inhibition

To detect changes in LAMP-2A protein levels, cells were incubated 18 h at 37°C ± 20 μM CQ or 10 μM calpeptin (EMD Millipore). Samples were resolved on SDS-PAGE and analyzed by western blotting.

Apoptosis Assay

For detection of apoptotic and necrotic cells, real time analysis of caspase-3 and caspase-7 activity was detected using CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (Invitrogen, Carlsbad, CA, United States). During apoptosis, caspase-3 and caspase-7 are activated and able to cleave a cell permeable fluorogenic substrate DEVD peptide. The bright fluorogenic signal produced by caspase-3 and caspase-7 activity indicates apoptotic cells. Cells positive for AAD dead cell stain help separate live from dead cells. Samples were analyzed by flow cytometry.

Subcellular Fractionation

Cytoplasmic and nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) following manufacturer's recommended instructions. Samples were resolved on SDS-PAGE and analyzed by western blotting.

Cell Cycle Analysis

Cells were fixed with 70% cold ethanol (−20°C) for 1 h at 4°C, washed with ice-cold PBS, incubated 15 min at 37°C with 100 μg/ml RNase A (Sigma-Aldrich), and then stained 30 min at room temperature with 50 μg/ml of propidium iodide (Sigma-Aldrich). Samples were analyzed by flow cytometry.

[^{3}H] Thymidine Incorporation

Cells were incubated with [^{3}H] thymidine for 8 h at 37°C. Thymidine incorporation was quantified using Wallac 1450 Microbeta Plus liquid scintillation counter (Perkin Elmer, Shelton, CT, United States).

Reactive Oxygen Species Analysis

Basal ROS were measured by incubating cells with 5 μM CellROX Deep Red Reagent (Thermo Fisher Scientific, Waltham, MA, United States) for 30 min at 37°C. This cell-permeant dye is non-fluorescent while in a reduced state, and fluoresces upon oxidation by ROS. Samples were analyzed by flow cytometry.

Proteasome Assay

Proteasome activity was determined using Proteasome-Glo Chymotrypsin-Like Cell-Based Assay (Promega, Madison, WI, United States) (Moravec et al., 2009). Cells were trypsinized and plated according to the manufacturer's recommended

instructions. Cells were incubated with Proteaseome-Glo Cell Based Reagent to deliver the substrate (Succinyl-LLVY-aminoluciferin) into the cytoplasm of the cells. Aminoluciferin, which is the substrate for luciferase, is released following cleavage of this peptide substrate by the proteasome. Luciferase consumption of aminoluciferin results in a luminescent signal that is proportional to the amount of proteasome activity (Moravec et al., 2009). Luminescence was detected using SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, United States). Studies have indicated the specificity of this assay in multiple cultured cell lines in detecting changes in proteasome activity (Moravec et al., 2009).

Xenograft Studies

Female NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ mice 6–8 weeks of age were obtained from the *In Vivo* Therapeutics Core of the Indiana University and injected in the flanks with 5×10^5 melanoma cells. Animals were monitored two to three times a week following tumor implantation to detect changes in health and weight. Tumor size was measured biweekly as length (mm) × width (mm) × width (mm)/2 to obtain a measure of volume in mm cubed. All animals were terminated 20 days after tumor implantation, and tissues and palpable tumors were collected for analysis.

Ethics Statement

Mice were maintained in specific pathogen-free conditions under conditions approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine and the Guide for the Care and Use of Laboratory Animals.

Tissue Processing and Staining

Tissues were fixed in 10% neutral-buffered formalin at 4°C for 24 h followed by processing and paraffin embedding. Five-micrometer sections were cut and stained for hematoxylin and eosin (HE) or phospho-Histone H3 (EMD Millipore, Cat #06-570).

Assessment of Necrosis and Phospho-Histone H3 (pH3) Positivity

The Aperio ScanScope CS system whole slide digital imaging system (Leica Biosystems, Buffalo Grove, IL, United States) was used for imaging slides at 20X. Necrosis was determined by quantifying cells with nuclear fragmentation in randomly selected fields of primary tumors. As a measure of mitosis, five hot spots were selected per slide and cells positive for pH3 staining were quantified using the Positive Pixel Count V9 algorithm of Aperio ImageScope software (Leica Biosystems, Buffalo Grove, IL, United States). pH3 positivity represents pH3 positively stained cells divided by the total number of cells in the selected areas.

Statistical Analysis

Data were analyzed by two-way ANOVA or by two-tailed, unpaired Student's *t*-test using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, United States). A value of $p < 0.05$ or less was considered significant for all experiments. Error bars indicate SD unless noted otherwise.

RESULTS

Expression of LAMP-2C in Human Melanoma Cells

Therapeutic treatment of many cancers including melanoma with IFN-γ, is well documented (Zaidi and Merlino, 2011). IFN-γ negatively impacts tumor growth and alters the expression of multiple genes (Zaidi and Merlino, 2011). Exposure of tumors to IFN-γ can induce cell stress marked in some cases by increased cellular ROS production, upregulation of the DNA damage response, as well as enhanced cell senescence and death (Hubackova et al., 2016). Prior work had shown that exposure to toll-like receptor ligands, immune mediators often associated with infection, alters *LAMP2* isoforms mRNA expression in human B lymphoblasts (Perez et al., 2016). To address whether differential regulation of *LAMP2* isoforms is observed in human melanomas, we exposed melanoma cells to IFN-γ. A twofold to threefold induction of *LAMP2C* mRNA was observed upon melanoma cells exposure to IFN-γ with very modest changes in the more abundant *LAMP2A* and no induction of *LAMP2B* (**Figure 1A**). These results suggest that *LAMP2C* expression can be upregulated by cytokine stress in human melanomas.

The hierarchy of endogenous *LAMP2* mRNA expression (*LAMP2B* > *LAMP2A* > *LAMP2C*) was consistent among two distinct human melanoma cell lines, DM331 and SLM2-Mel (**Figures 1B,C**). Given the low basal levels of *LAMP2C* mRNA in each melanoma cell line, this isoform was ectopically expressed in each cell line to examine its impact on autophagy, cell growth and survival (**Figure 2**). LAMP-2 isoforms can be detected using commercial antibodies that recognize conserved epitopes, but individual isoform analysis can be challenging given their structural homology. To circumvent the absence of antibodies against LAMP-2C, melanoma cells (DM331 or SLM2-Mel) were transfected with a plasmid encoding C-terminal myc tagged *LAMP2C* yielding DM331 2C myc or SLM2-Mel 2C myc cells (**Figures 2A,B**). As a control, the parental cell lines were transfected with an empty vector to produce DM331 pCMV or SLM2-Mel pCMV cells (**Figures 2A,B**). As an additional control, DM331 cells were also transfected with a distinct empty vector (DM331 zeo) or a plasmid encoding untagged *LAMP2C* (DM331 2C) to ensure the myc tag was not impacting function (**Figure 2C**). Increased *LAMP2C* mRNA was detected in melanoma cells transfected with the *LAMP2C* plasmid (**Figure 2**). Higher levels of ectopic *LAMP2C* mRNA were detected in DM331 cells compared to the SLM2-Mel cells, regardless of myc tag addition (**Figure 2**). While there was no significant change in *LAMP2A* mRNA levels with ectopic LAMP-2C expression in cells, a slight reduction was observed in mRNA levels of *LAMP2B* (**Figure 2**).

Western blot analysis of melanoma cells revealed similar electrophoretic migration of ectopic LAMP-2C and other LAMP-2 isoforms on SDS-PAGE (**Figures 2B,C**, **3**). LAMP-2 isoforms

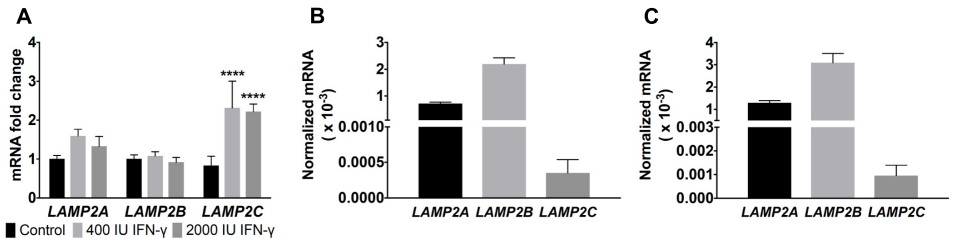


FIGURE 1 | *LAMP2* expression in melanomas during IFN-γ treatment. **(A)** DM331 cells treated for 24 h with 400 or 2000 IU of IFN-γ. Gene expression of *LAMP2A*, *LAMP2B*, and *LAMP2C* were analyzed by qPCR. mRNA levels were normalized to *ACTB* expression with the expression of each isoform set equal to one for control cells without cytokine exposure. **(B)** Endogenous mRNA levels of *LAMP2* isoforms in DM331 cells were quantitated relative to *18S* mRNA levels. **(C)** Endogenous mRNA levels of *LAMP2* isoforms in SLM2-Mel cells. Gene expression was quantitated relative to *18S* mRNA levels. Data were analyzed by two-way ANOVA. $^{****}p < 0.0001$ ($n = 3$).

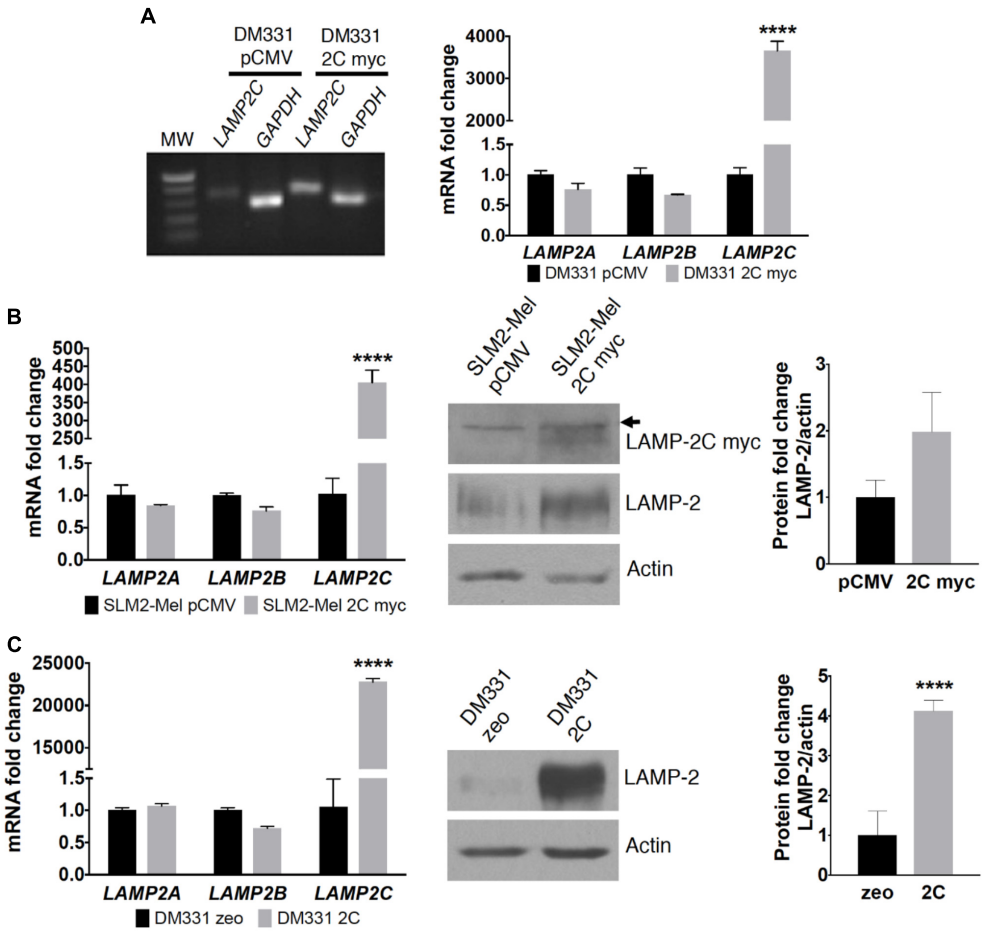


FIGURE 2 | LAMP-2 expression in human melanoma cell lines transfected with LAMP-2C. **(A)** DM331 cells were transfected with an empty vector (pCMV) or a plasmid encoding for C-terminal myc tagged *LAMP2C*. RT-PCR analysis for *LAMP2C* overexpression was detected in an agarose gel. mRNA levels of *LAMP2A*, *LAMP2B*, and *LAMP2C* transcripts were analyzed by qPCR and normalized to *18S* expression. To detect relative changes in the expression of each isoform, the normalized expression of each isoform was set equal to one in DM331 pCMV cells. **(B)** SLM2-Mel cells were transfected with an empty vector (pCMV) or a plasmid encoding for C-terminal myc tagged *LAMP2C*. mRNA levels of *LAMP2A*, *LAMP2B*, and *LAMP2C* in these cells were analyzed by qPCR and normalized to *ACTB* expression. The relative expression of each isoform was set equal to one for SLM2-Mel pCMV control cells. Cell lysates were probed for the c-myc tagged LAMP2C or total LAMP2 protein with actin used as a control for sample loading. Arrow indicates non-specific protein band detected with anti-myc antibody. **(C)** DM331 cells were transfected with an empty vector (zeo) or a plasmid encoding for *LAMP2C* with no tag sequence. mRNA levels of *LAMP2A*, *LAMP2B*, and *LAMP2C* in these cells were analyzed by qPCR and normalized to *GAPDH* expression. To examine relative changes in each isoform, the expression of individual isoforms was set to one for the DM331 zeo control cells. Cell lysates were probed for total LAMP2 protein with actin used as a control for sample loading. Data were analyzed by two-way ANOVA or by two-tailed, unpaired Student's *t*-test. $^{****}p < 0.0001$ ($n = 2$–3).

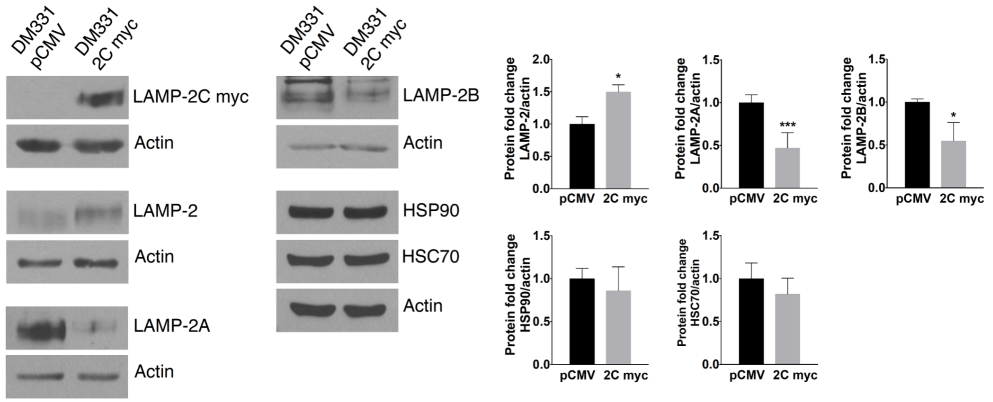


FIGURE 3 | LAMP-2C ectopic expression in melanoma cells altered LAMP-2A and LAMP-2B protein expression. LAMP-2C myc, total LAMP-2, LAMP-2A, LAMP-2B, HSC70, HSP90, and actin were detected in DM331 pCMV and DM331 2C myc cells. The normalized expression of each protein was set equal to one for DM331 pCMV cells for relative comparison with protein levels in DM331 2C myc cells. Data were analyzed by two-tailed, unpaired Student's *t*-test. $*p < 0.05$ and $***p < 0.001$ ($n = 3$).

are translated as polypeptides of approximately 42 kDa, with glycosylation of these isoforms yielding proteins which migrate as diffuse bands on SDS-PAGE with an apparent molecular mass of 120 kDa. The diffuse appearance and similar electrophoretic migration of LAMP-2C ectopically expressed with or without a myc tag in melanomas, was consistent with a high degree of glycosylation observed with other LAMP-2 isoforms. Cellular levels of total LAMP-2, detected with an antibody recognizing all isoforms, were increased 1.5- to 4-fold in melanoma cells likely due to the increase in *LAMP2C* mRNA (**Figures 2B,C**, **3**). Notably, the expression of both LAMP-2A and LAMP-2B proteins was reduced about 50% in cells with increased LAMP-2C (**Figure 3**). Cellular levels of CMA chaperones HSC70 and HSP90 were unperturbed by increased LAMP-2C (**Figure 3**). These findings suggest that increased LAMP-2C expression in melanoma cells may affect cellular levels of LAMP-2A and LAMP-2B proteins.

Tumors have been manipulated using molecular approaches to reduce constitutive *LAMP2A* mRNA expression to impact cell growth (Kon et al., 2011; Saha, 2012; Zhou et al., 2016). Here, the reduction in LAMP-2A protein abundance with ectopic LAMP-2C expression suggested post-translational regulation of this isoform's expression. A lysosomal serine protease cathepsin A and a cytoplasmic cysteine protease calpain I regulate LAMP-2A protein stability and turnover (Cuervo et al., 2003; Villalpando Rodriguez and Torriglia, 2013). To examine whether melanoma cell LAMP-2C expression impacts proteolytic turnover of LAMP-2A, DM331 2C myc cells were incubated with CQ, a weak base which prevents cathepsin A activation in acidic organelles, or with calpeptin, a cell permeable calpain inhibitor. The addition of these agents to control DM331 pCMV cells, with low endogenous LAMP-2C, slightly increased steady state LAMP-2A protein abundance (**Figures 4A,B**). Yet in melanoma cells with high LAMP-2C expression, treatment with these inhibitors unexpectedly promoted an even greater reduction in cellular LAMP-2A protein levels. CQ treatment neutralizes lysosome, endosome, and autophagosome pH, reducing the activity of multiple enzymes including proteases functional at low pH. We examined several lysosomal cathepsins to determine if LAMP-2C expression increased the abundance and maturation of these enzymes to active proteases, possibly explaining the observed decrease in melanoma cell levels of LAMP-2A protein with ectopic LAMP-2C expression. Cellular levels of mature and precursor forms of lysosomal proteases cathepsin A and cathepsin B were unchanged in melanoma cells by ectopic LAMP-2C. Expression of the mature cathepsin D (30 kDa) protein was also not statistically different with ectopic LAMP-2C expression in cells, while cathepsin D immature precursors (46 kDa and 52 kDa forms) were significantly decreased in cells with high LAMP-2C expression (**Figure 4C**). The 30 kDa and 46 kDa forms of cathepsin D are functional aspartyl proteases. Quantitative analysis of transcripts for cathepsin genes *CTSA*, *CTSB*, and *CTSD* corroborated that ectopic expression of LAMP-2C in melanoma cells did not increase the expression of these lysosomal enzyme mRNAs (**Figure 4C**). Rather a slight decrease in *CTSA* and *CTSD* mRNA was detected in cells with ectopic LAMP-2C. Thus, the decreased abundance of LAMP-2A observed in melanoma cells with high LAMP-2C expression, was not linked to an increased cellular accumulation of these three cathepsin proteases. Together, these results suggest increased LAMP-2C expression in melanoma cells perturbs steady state levels of LAMP-2A and LAMP-2B, each of which has been implicated in regulating autophagy pathways.

LAMP-2C Expression Impacts CMA and MA

Impaired CMA can alter intracellular accumulation of select cytoplasmic proteins targeted for degradation by this pathway. Steady state levels of two well-described CMA protein substrates, the cell cycle regulator Chk1 and the inhibitor of NF-κB signaling pathway IκBα, were examined in melanoma cells with ectopic LAMP-2C expression (Cuervo et al., 1998; Park et al., 2015).

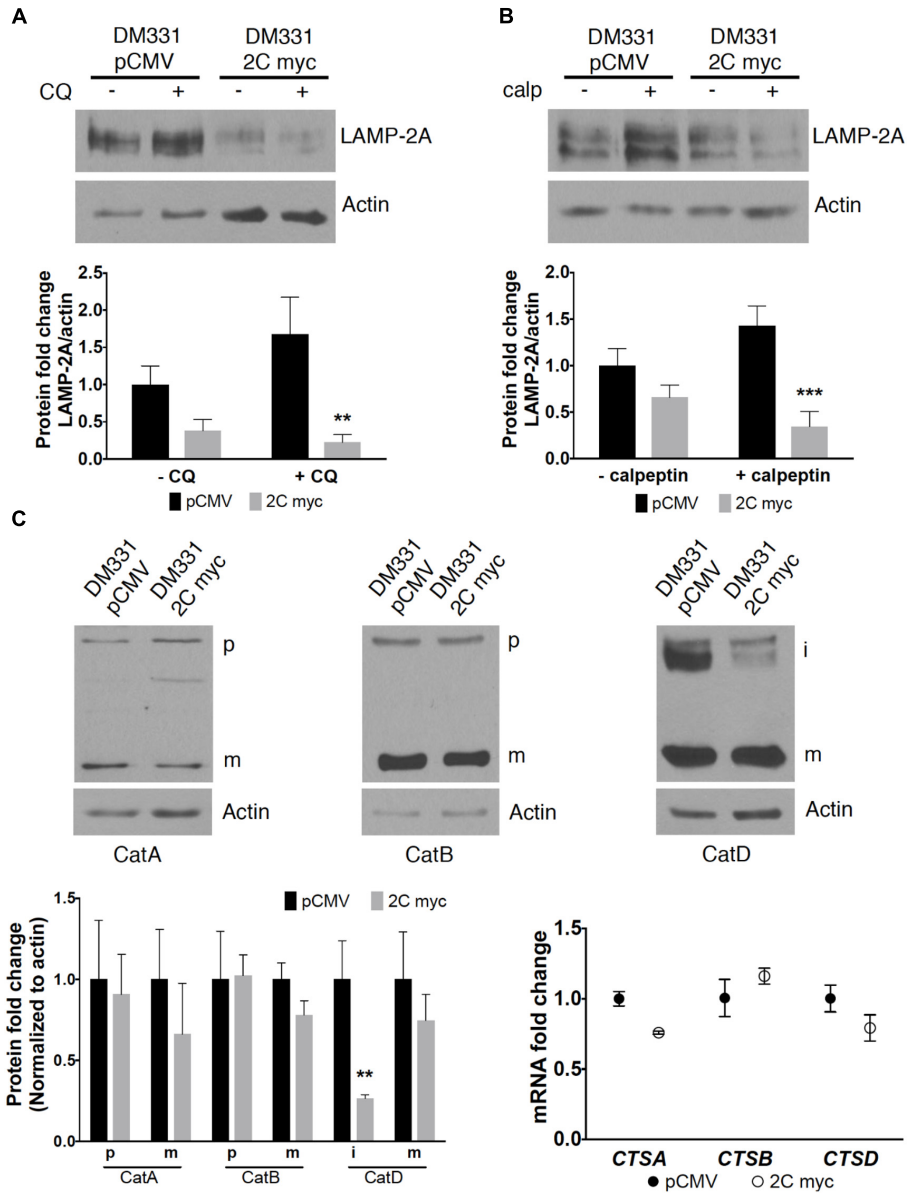


FIGURE 4 | Changes in LAMP-2A protein expression were not due to increased cathepsin or calpain abundance in melanomas expressing LAMP-2C. DM331 pCMV and DM331 2C myc cells were incubated overnight at 37°C with ±20 μM CQ **(A)** or 10 μM calpeptin (calp) **(B)** to inhibit lysosome proteases or calpain activity, respectively. LAMP-2A levels were detected by western blotting, evaluated by densitometry, and normalized to actin protein levels. LAMP-2A levels were calculated relative to DM331 pCMV cells cultured without CQ or calpeptin. **(C)** Maturation and gene expression of lysosome proteases cathepsin A (CTSA), cathepsin B (CTSB), and cathepsin D (CTSD) was evaluated in cells overexpressing LAMP-2C. Lysates were resolved by SDS-PAGE and probed to detect the precursor (p), intermediate (i), or mature (m) form of cathepsin A, cathepsin B, and cathepsin D. Protein expression was quantified by densitometry and levels were normalized to actin levels. mRNA levels of *CTSA*, *CTSB*, and *CTSD* transcripts were analyzed by qPCR and normalized to *18S* expression. Measurements in **(A–C)** are relative values calculated by setting the results obtained for DM331 pCMV cells equal to one for comparison to DM331 2C myc cells. Data were analyzed by two-way ANOVA. $^{**}p < 0.01$, and $^{***}p < 0.001$ ($n = 3$).

Elevated levels of Chk1 and total or phosphorylated IκBα were observed in DM331 2C myc melanoma cells, suggesting disruptions in the proteolytic turnover of these proteins via CMA (**Figure 5A**). Increased cellular expression of Chk1 and IκBα was not due to higher *CHEK1* and *NFKBIA* mRNA transcripts, again consistent with CMA disruption in melanoma cells with high LAMP-2C expression (**Figure 5B**). Changes in autophagy can impact cytoplasmic protein degradation by the proteasome (Park and Cuervo, 2013). CMA substrates Chk1 and IκBα can be diverted to the proteasome in some cell types (Alkalay et al., 1995; Zhang et al., 2005). We quantitated proteasome proteolytic activity in DM331 2C myc cells using a specific proteasome substrate, succinyl-LLVY-aminoluciferin, delivered selectively into the cytoplasm of melanoma cells. Proteasome activity was

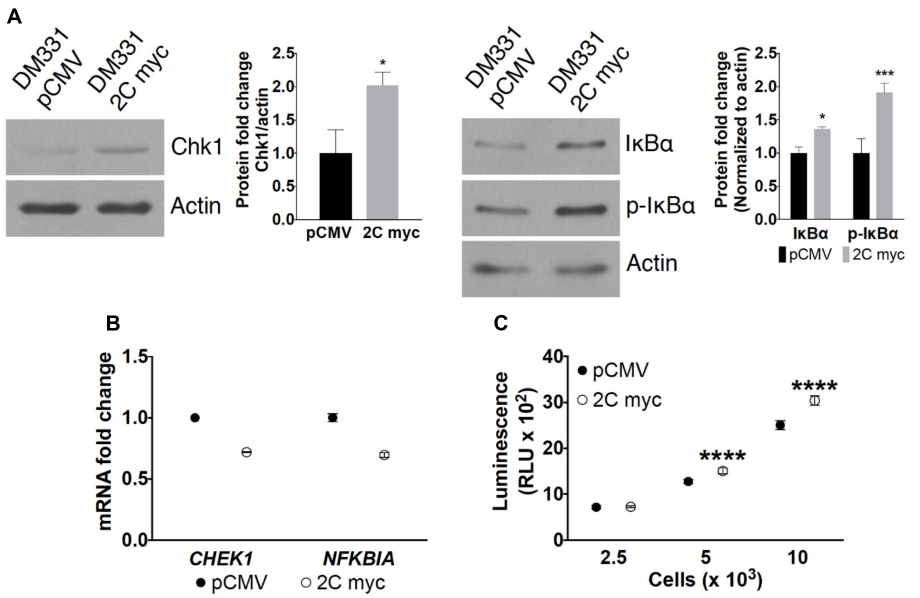


FIGURE 5 | Effect of LAMP-2C expression on CMA substrates. **(A)** Cellular levels of CMA substrates Chk1, IκBα, and p-IκBα in DM331 pCMV and DM331 2C myc cells were examined by western blotting. Relative protein levels were calculated by setting the normalized expression to one for DM331 pCMV cells. **(B)** mRNA levels of *CHEK1* and *NFKBIA* transcripts were analyzed by qPCR and normalized to *ACTB* expression. mRNA levels in DM331 pCMV cells were normalized and set to one. **(C)** Proteasome activity was measured using the Proteasome-Glo Chymotrypsin-Like Cell-Based Assay. Cells were incubated with a substrate succinyl-LLVY-aminoluciferin which penetrates into the cytoplasm. This substrate is cleaved by the proteasome to release aminoluciferin which is released from cells. Luciferase is added to these cells, cleaving aminoluciferin to a luminescent product detectable using a luminometer. Data were analyzed by two-way ANOVA or by two-tailed, unpaired Student's *t*-test. $*p < 0.05$, $***p < 0.001$, and $****p < 0.0001$ ($n = 3$).

not reduced in cells with ectopic LAMP-2C expression but rather slightly increased compared to control cells (**Figure 5C**). These data suggest that LAMP-2C myc expression in melanoma cells disrupts CMA and increased cellular protein levels of several CMA substrates.

LAMP-2B is required for efficient cellular MA, thus changes in MA were examined in melanoma cells with increased LAMP-2C (Nishino et al., 2000). The intracellular abundance and stability of LC3 I and II are used to monitor MA (Mizushima and Yoshimori, 2007; Mizushima et al., 2010; Klionsky et al., 2012). During MA, cytoplasmic LC3I is lipidated, and converted to LC3II, which associates with autophagosomes. LC3II is then proteolyzed upon autophagosome maturation marking a full cycle of MA. An accurate measure of this autophagy pathway can be obtained through analysis of MA progression or flux (Klionsky et al., 2012). Changes in LC3I and LC3II protein levels were detected in each of the melanoma cells with ectopic LAMP-2C in the presence or absence of CQ, the latter which neutralizes autophagosome acidification to slow LC3II degradation during MA. Monitoring the relative LC3II (LC3II/actin) levels in cells treated with CQ and subtracting the relative LC3II (LC3II/actin) abundance in cells without CQ, offers a measure of MA progression or flux (Klionsky et al., 2012). MA flux was diminished in DM331 cells with increased LAMP-2C myc (**Figure 6A**). Decreased MA flux was observed in DM331 cells expressing untagged LAMP-2C and a distinct cell line, SLM2-Mel 2C myc (**Figures 6B,C**). Consistent with the flux analysis suggesting disruptions in MA in melanoma cells with ectopic LAMP-2C, the relative levels of LC3I (basal LC3I/actin) were increased in untreated melanoma cells. While relative LC3I abundance in cells does not measure MA, the detected accumulation of LC3I may suggest a slowing or disruption in early stages of MA in the context of reduced flux. As an alternate approach to evaluate cellular MA in these melanoma cells, DM331 2C myc were treated with a dye CYTO-ID that fluoresces upon delivery into autophagosomes. Cellular stresses, such as nutrient starvation, promote an increase in CYTO-ID accumulation in newly forming autophagosomes (Guo et al., 2015). DM331 pCMV and DM331 2C myc cells were incubated ± serum and autophagosomes stained using CYTO-ID to evaluate MA. MA was reduced in melanoma cells with LAMP-2C cultured in serum as detected by flow cytometry (**Figure 6D**). Reductions in MA were apparent in serum nutrient starved DM331 cells with increased LAMP-2C expression compared to control cells (**Figure 6D**). Tumor cells may encounter a variety of stresses *in vivo* including limitations in nutrient availability, oxygen deficiency, and inflammatory mediators. Experiments here suggest that ectopic LAMP-2C expression in melanoma cells reduces MA under basal and stress conditions. Thus, increased LAMP-2C expression in melanoma cells results in disruptions in cellular MA.

Ectopic LAMP-2C Expression Perturbs Cell Cycle and Survival

Autophagy pathways control a variety of cellular processes and have been linked to cell cycle regulation and survival

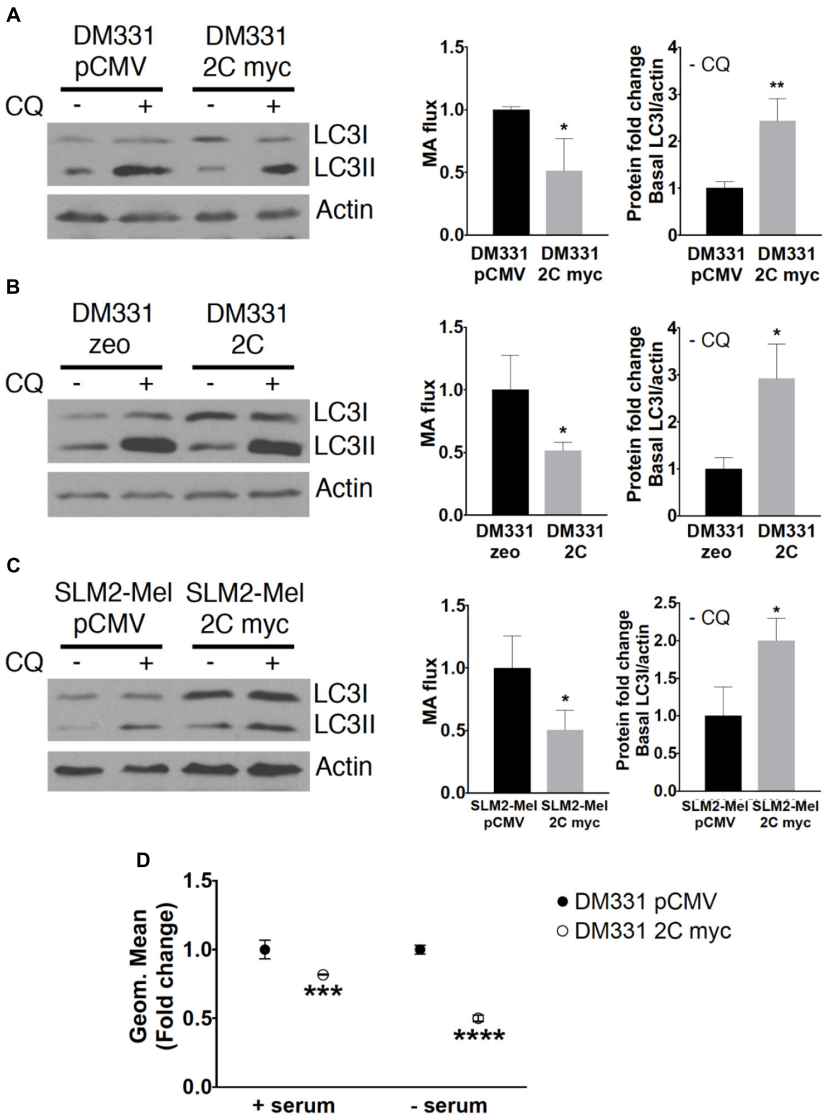


FIGURE 6 | MA was diminished in melanomas with increased LAMP-2C. **(A–C)** DM331 or SLM2-Mel cells were incubated overnight with ±20 μM CQ, an inhibitor of lysosome acidification, to monitor autophagosome formation and turnover. To measure MA flux, the conversion of LC3I to LC3II was detected and normalized to cellular actin levels. MA flux was quantified to detect changes in cellular LC3II levels ± CQ using the equation MA flux = LC3II/actin in CQ treated cells – LC3II/actin in control cells. The relative levels of LC3I/actin in each cell line grown without CQ provides a measure of basal LC3I protein accumulation prior to its enzymatic conversion to LC3II during MA. **(D)** DM331 pCMV and DM331 2C myc cells were incubated with media ± serum, stained with CYTO-ID, and MA monitored by flow cytometry. The geometric mean was set equal to one for DM331 pCMV cells for relative comparison to the geometric mean in DM331 2C myc cells. Data were analyzed by two-way ANOVA or by two-tailed, unpaired Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ ($n = 3$).

(Levine and Kroemer, 2008). Previous reports have shown alterations in cell proliferation and apoptosis of distinct tumors after blocking CMA by LAMP-2A silencing (Kon et al., 2011; Saha, 2012; Zhou et al., 2016). Given LAMP-2A protein levels were reduced in melanoma cells expressing LAMP-2C, this led us to question whether cell proliferation or apoptosis was perturbed in these cells. DM331 2C myc cells exhibited alterations in the cell cycle distribution as monitored by flow cytometric analysis of cellular DNA content (**Figure 7A**). While the percentage of melanoma cells in G_0/G_1 phase decreased with ectopic LAMP-2C expression, an increase was detected in the percentage of these cells in G_2/M phase (**Figure 7B**). A reduction in thymidine incorporation by DM331 2C myc cells was also detected compared to this cell transfected with vector alone (**Figure 7B**). Similarly, fewer DM331 cells expressing untagged LAMP-2C and SLM2-Mel 2C myc cells were at the G_0/G_1 stage, with these melanoma cells displaying more G_2/M phase cells (**Figure 7C**). While differences in cell distributions in S phase were observed with altered LAMP-2C expression, these changes were variable among the different melanoma cells. These data suggest that LAMP-2C expression in these melanoma cells may disturb cell division via cell cycle arrest. To complement these

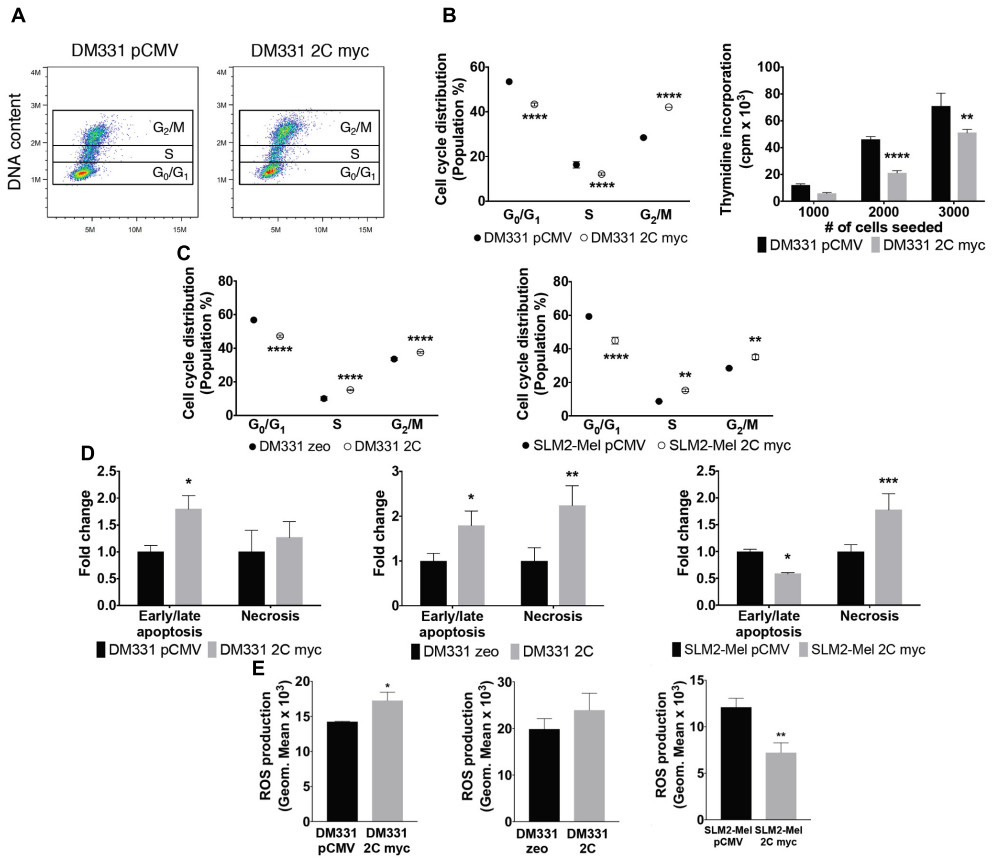


FIGURE 7 | Reduced survival and altered cell cycle in melanomas expressing LAMP-2C. **(A)** Apoptosis and necrosis were examined by incubating DM331 pCMV and DM331 2C myc cells with a fluorogenic substrate specific for activated caspase-3 and caspase-7 in apoptotic cells and AAD dead cell stain to detect necrotic cells. Apoptotic and necrotic cells were detected by flow cytometry. **(B)** Representative dot plot graph of the cell cycle distribution of DM331 pCMV and DM331 2C myc cells. Cell cycle distribution was evaluated by staining DNA content of DM331 pCMV and DM331 2C myc cells with propidium iodide and quantified by flow cytometry. **(C)** Cell cycle distribution of DM331 zeo and DM331 2C was evaluated by staining DNA content with propidium iodide and quantified by flow cytometry. **(D)** Cell cycle distribution was analyzed by staining the DNA content of SLM2-Mel pCMV and SLM2-Mel 2C myc with propidium iodide and detected by flow cytometry. **(E)** To examine basal ROS production, melanomas with and without ectopic LAMP-2C expression were incubated for 30 min at 37°C with 5 μM CellROX Deep Red Reagent and monitored by flow cytometry. Data were analyzed by two-way ANOVA or by two-tailed, unpaired Student's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ ($n = 2$–3).

studies, an analysis of melanoma cell death and necrosis was carried out using melanoma cells with and without ectopic LAMP-2C expression. Levels of apoptosis and/or necrosis were increased in each melanoma cell line with ectopic LAMP-2C expression compared to cells transfected with vector alone (**Figure 7D**). ROS generated by tumor cells can impact cellular autophagy pathways and growth (Poillet-Perez et al., 2015). ROS production was evaluated in melanoma cells with ectopic LAMP-2C and compared to the control melanoma cells with vector alone. There was no consistent increase or decrease in cellular ROS among the three pairs of tumor cell lines tested. Although a slight increase in ROS production was detected with DM331 2C myc cells compared to vector transfected cells, and a reduction in ROS production was observed for SLM2-Mel 2C myc cells compared to the vector transfected cells (**Figure 7E**). These results suggest that increased LAMP-2C expression in melanoma cells perturbs cell cycle progression as well as apoptosis and necrosis.

Chaperone-mediated autophagy substrate Chk1, a key regulator during DNA replication and DNA damage responses, contributes to all cell cycle checkpoints, including G_1/S, intra-S-phase, G_2/M, and the mitotic spindle checkpoint (Patil et al., 2013). In response to genotoxic stress, Chk1 is phosphorylated and activates DNA damage responses to bring about cell cycle arrest, activate DNA repair pathways, and induce apoptosis when DNA damage is severe (Patil et al., 2013). Chk1 Ser345 phosphorylation is critical for this activation and function in response to DNA damage (Patil et al., 2013; Goto et al., 2015). Higher cellular levels of Chk1 Ser345 phosphorylation were detected in DM331 2C myc cells compared to control cells, suggesting increased activation of Chk1 in melanoma cells with high LAMP-2C expression (**Figure 8A**). Although Chk1 is mainly expressed in the nucleus, following activation Chk1 shuttles between the nucleus and cytoplasm (Patil et al., 2013; Goto et al., 2015). Consistent with the increased phosphorylation of Chk1 in cells with ectopic

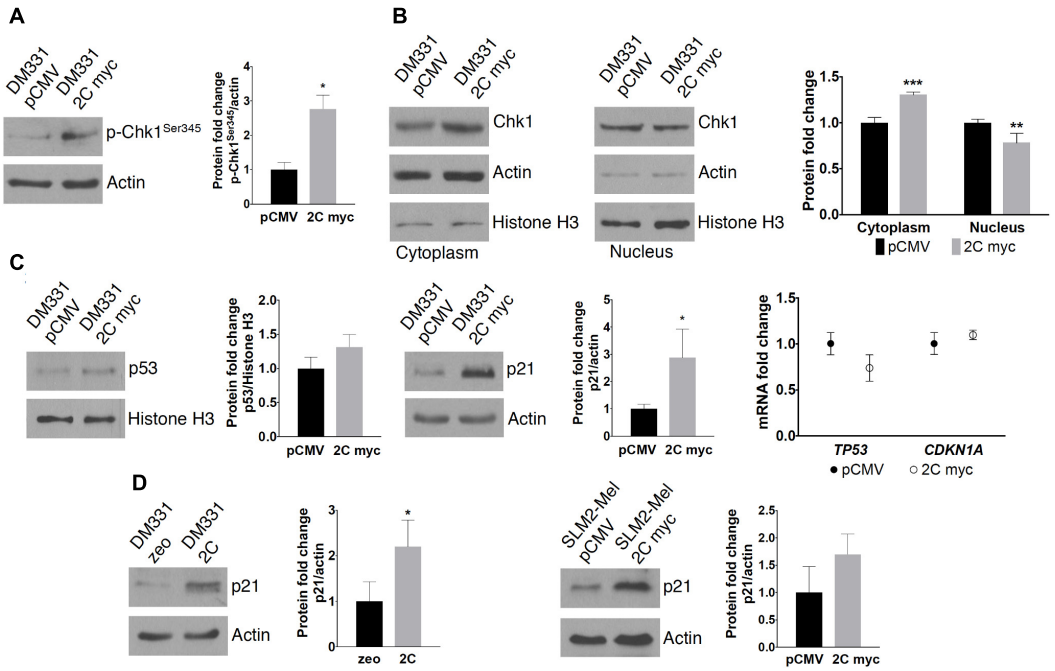


FIGURE 8 | Effect of LAMP-2C on cell cycle regulators. **(A)** Chk1 activation was examined by detecting cellular levels of Chk1 phosphorylation at Ser345. **(B)** Chk1 subcellular localization was determined by extracting cytoplasmic and nuclear proteins from DM331 pCMV and DM331 2C myc cells. Protein levels were detected by western blotting. **(C)** Protein and mRNA levels of cell cycle regulators in DM331 pCMV and DM331 2C myc, p53 and p21, were examined by western blotting and qPCR. **(D)** Lysates from DM331 zeo and DM331 2C were resolved by SDS-PAGE and probed to detect p21 levels. Protein expression was quantified by densitometry and levels were normalized to actin levels. **(E)** Protein levels of p21 in SLM2-Mel pCMV and SLM2-Mel 2C myc were detected by western blotting. Protein expression was quantified by densitometry and levels were normalized to actin levels. Measurements in **(A–E)** represent relative values calculated by setting the results obtained for cells transfected with an empty vector equal to one for comparison to cells with ectopic LAMP-2C. Data were analyzed by two-way ANOVA or by two-tailed, unpaired Student's *t*-test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ ($n = 3$).

LAMP-2C, slightly more Chk1 protein was detected in the cytoplasm of these cells (**Figure 8B**). The tumor suppressor protein p53 and the cyclin-dependent kinase inhibitor p21 play important roles in G_1 and G_2 checkpoints (Giono and Manfredi, 2006; Karimian et al., 2016). Furthermore, increased cellular levels of p53 and p21 have been observed in cancer cells with LAMP-2A downregulation (Kon et al., 2011; Zhou et al., 2016). While p53 protein levels were slightly increased compared to control melanoma cells, cellular levels of p21 were markedly increased in DM331 cells with increased LAMP-2C expression (**Figure 8C**). Protein levels of p21 were also increased in DM331 cells expressing untagged LAMP-2C and SLM2-Mel 2C myc cells (**Figures 8D,E**). Changes in cellular levels of p53 and p21 were not a direct result of altered levels of p53 and p21 mRNA transcripts in DM331 cells with ectopic LAMP-2C myc expression (**Figure 8C**). Thus, enhanced LAMP-2C expression induces cell cycle arrest and affects survival by altering the abundance and activation of key cell cycle regulators.

LAMP-2C Expression Reduces Melanoma Cells Tumorigenic Potential

LAMP-2A knockdown in cancerous cells has been documented to reduce tumorigenic capability and metastatic capacity (Kon et al., 2011; Zhou et al., 2016). Given that *in vitro* studies here showed changes in the cell cycle of melanoma cells, the tumorigenicity of melanoma cells with enhanced LAMP-2C expression was examined *in vivo*. Previous reports have demonstrated NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mouse model provide an excellent *in vivo* system to assess human melanoma metastasis without the complication of host immune responses to tumor (Quintana et al., 2012). Here, NSG mice were injected in the flanks with DM331 cells with or without ectopic LAMP-2C myc, and animals monitored for tumor growth followed by sacrifice 20 days post tumor implant. Subcutaneous xenografts growth was reduced for tumors with high LAMP-2C myc (**Figure 9A**). Histology of primary tumors established differences in anatomy (**Figure 9B**). While melanoma cells from control tumor were spindle-shaped, the LAMP-2C myc tumor cells were epithelial-shaped and loosely joined together (**Figure 9B**). In addition, HE staining revealed necrotic areas in LAMP-2C myc tumors were three times greater than control tumors (**Figure 9C**). To examine tumor cell mitosis, tissue sections from palpable tumors where stained to detect phospho-Histone H3 (**Figure 9D**). While differences in cell density were again observed in comparing tumors with ectopic LAMP-2C or vector alone, no significant difference was detected in phospho-Histone H3 staining. Together these results revealed a novel role for LAMP-2C in diminishing melanoma growth *in vivo*.

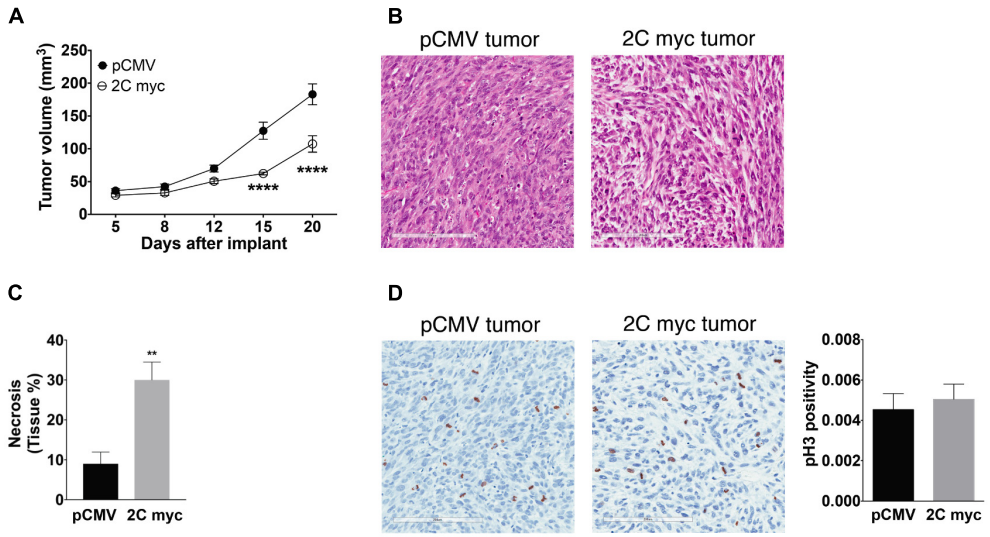


FIGURE 9 | Ectopic expression of LAMP-2C decreased tumor growth in a xenograft mouse model. NSG mice were implanted subcutaneously in the flanks with 5×10^5 DM331 cells with or without ectopic LAMP-2C myc expression. **(A)** Tumor growth was monitored biweekly (n = 13 per group). **(B)** Representative HE staining of primary control tumor or primary tumor with high ectopic expression of LAMP-2C myc. **(C)** Percentage of necrotic areas of primary tumors were evaluated by HE staining (n = 5 per group). **(D)** Representative pH3 staining of primary tumor with or without LAMP-2C myc expression. To determine changes in mitosis, pH3 positive cells were quantified in five hot spots areas of primary tumors. pH3 positivity was quantified dividing the number of pH3 positively stained cells by the total number of cells in the selected areas (n = 6 per group). Data were analyzed by two-way ANOVA or by two-tailed, unpaired Student's t-test. Error bars indicate mean ± SEM. $^{**}p < 0.01$ and $^{****}p < 0.0001$.

DISCUSSION

Deregulation of autophagy pathways has been associated with melanoma development and progression. While immunochemistry of normal human melanocytes revealed low expression of LC3 protein, a histological MA marker, focal staining of LC3 molecules increased in spreading subcutaneous melanoma consistent with increased tumor MA (Checinska and Soengas, 2011; Corazzari et al., 2013). Immunohistochemical analysis of early and late stage melanomas revealed that late stage tumors associated with poor prognosis, expressed reduced levels of p62, a protein whose turnover is linked to enhanced MA (Ellis et al., 2014). High levels of LAMP-2A, a marker for CMA were detected in human melanoma biopsies compared with healthy skin, and reductions in LAMP-2A expression slowed murine melanoma growth *in vitro* (Kon et al., 2011). Such results suggest that pathways or proteins linked to autophagy may influence melanoma cell growth and tumor progression.

Here, studies examined the role of a lysosomal membrane protein, LAMP-2C in modulating autophagy as well as cell cycle and growth in several human melanoma cell lines. LAMP-2C is highly homologous to LAMP-2A and LAMP-2B, which regulate CMA and MA respectively (Eskelinen et al., 2005). While the three LAMP-2 isoforms are derived from a common precursor mRNA, differential expression of these isoforms has been observed. LAMP-2A expression levels and basal CMA activity were increased in a variety of human solid tumors, including melanoma, lung, breast, and gastric cancers (Kon et al., 2011; Saha, 2012; Zhou et al., 2016). Inhibition of the proteasome or MA, has been reported to increase LAMP-2A expression in neural cells (Yang et al., 2013). In contrast with *LAMP2A* and *LAMP2B* mRNA which are broadly expressed in different tissues, *LAMP2C* mRNA has a more limited tissue distribution (Perez et al., 2016). The mRNA for all three *LAMP2* isoforms increased in B lymphoblasts exposed to toll receptor ligands, which are associated with microbial infection (Perez et al., 2016). In the current study, treatment of melanoma cells with the pro-inflammatory cytokine IFN-γ significantly increased *LAMP2C* mRNA abundance with only marginal or no change in *LAMP2A* and *LAMP2B* mRNA. This may be due to an initial increase in the abundance of the *LAMP2* precursor mRNA with selective regulation of mRNA splicing or preferential mRNA stabilization to yield increased *LAMP2C* mRNA. The results with interferon-treated cells are also consistent with tissue or cell type specific differences in LAMP-2 isoform expression. The molecular mechanisms which control the expression of individual *LAMP2* mRNAs have not been well examined. As discussed below, post-transcriptional events can also regulate LAMP-2 protein expression and function.

Ectopic expression of LAMP-2C in melanomas disrupted CMA, as indicated by the accumulation of several proteins typically degraded by CMA including Chk1, IκBα, and p21 (Cuervo et al., 1998; Park et al., 2015; Zhang et al., 2018). Studies have described an intricate cross-communication and compensatory mechanisms among the different autophagic pathways and the proteasome (Park and Cuervo, 2013). Furthermore, several CMA protein substrates, including Chk1 and IκBα, can also be targeted for proteasome degradation in some cell types (Alkalay et al., 1995; Zhang et al., 2005). The current study examined whether cellular proteasome activity

was decreased with increased LAMP-2C expression in tumors. Proteasome activity analysis revealed a slight increase in the activity of this enzyme in melanoma cells with increased LAMP-2C compared to control cells. Thus, it does not appear that increasing melanoma cell LAMP-2C expression, disrupts proteasome function. These findings are also consistent with previous reports demonstrating upregulation of proteasome activity in cancer cells with compromised CMA (Kon et al., 2011). Decreased LAMP-2A and LAMP-2B protein levels were observed in melanoma cells with ectopic LAMP-2C expression in the current study. Work by others has shown that reductions in cellular LAMP-2A levels blocks CMA and promotes accumulation of CMA substrates (Zhou et al., 2005, 2016; Kon et al., 2011). Levels of *LAMP2A* mRNA were unchanged in melanoma cells with ectopic LAMP-2C, suggesting alterations in post-transcriptional regulation of LAMP-2A molecules. Studies of several lysosomal and cytoplasmic proteases known to function in the turnover of LAMP-2A, failed to reveal a clear change in these enzymes that might account for the reduction in cellular LAMP-2A. Instead, attempts to stabilize LAMP-2A using protease inhibitors in cells with ectopic LAMP-2C, resulted in greater reductions in LAMP-2A abundance. While not previously linked to LAMP-2 stability, proteasome activity did increase in melanomas with ectopic LAMP-2C. LAMP-2A molecules also form oligomers in lysosomes which regulate CMA, and it is possible that increased LAMP-2C expression may perturb oligomer formation. Attempts to detect a physical association between LAMP-2A and LAMP-2C in melanoma cells, have not been successful to date. Post-translational modifications of LAMP-2 isoforms including glycosylation and phosphorylation have been reported (Tan et al., 2016; Li et al., 2017), and such modifications could be altered in cells with high levels of LAMP-2C. The SDS-PAGE mobility of LAMP-2A protein from cells with or without ectopic LAMP-2C was similar. Further studies will be necessary to examine the mechanisms influencing LAMP-2A protein abundance and structure in melanomas with increased LAMP-2C.

Increased expression of LAMP-2C in human melanomas also disrupted basal levels of MA as assessed by reduced autophagic flux and autophagosome abundance. Shifts in cancer cell metabolism coupled with changes in the tumor microenvironment can lead to increased hypoxia, nutrient and growth factor deprivation which induce MA (Morselli et al., 2009; Choi, 2012). Melanomas with increased LAMP-2C expression displayed reduced MA induction compared to control cells in response to serum starvation, a form of nutrient stress which typically upregulates MA in tumors to promote survival. As indicated, ectopic expression of LAMP-2C in melanomas reduced cellular levels of LAMP-2B protein with very modest decreases in *LAMP2B* mRNA. Little is known regarding the stability, post-translational modification, or turnover of LAMP-2B. Mutations in LAMP-2B were found in patients with Danon disease and associated with disruptions in MA flux (Crotzer et al., 2010). Results in the current study suggest that manipulating melanoma LAMP-2C expression may offer a novel means to disrupt basal and induced MA as well as CMA in melanomas.

A common feature in many human cancers is disruption of target genes involved in cell cycle progression and apoptosis. Lung and gastric cancer cells with compromised CMA activity exhibited increased levels of cell senescence regulators, such as p53 and p21 (Kon et al., 2011; Zhou et al., 2016). While reduced cell proliferation in lung cancer cells was not linked to cell cycle arrest, gastric cells with LAMP-2A knockdown displayed cell cycle arrest (Kon et al., 2011; Zhou et al., 2016). For murine LAMP-2A deficient fibroblasts cell cycling appeared unchanged, yet inducing DNA damage in these cells with etoposide or irradiation increased the percentage of cells in G_1 and G_2 while reducing cells in S phase (Park et al., 2015). In the current study, increased LAMP-2C levels in human melanomas cells induced cell cycle checkpoint and DNA damage responses as suggested by changes in cell cycle distribution (increased G_2 and reduced G_1 phase cells) with elevated cellular levels of p21 and activated phospho-Chk1 (Ser345). In melanoma cells with ectopic LAMP-2C, Chk1 protein abundance increased twofold while phospho-Chk1 levels were nearly threefold higher compared with cells transfected with vector alone. This may reflect the importance of CMA in the turnover of Chk1 in melanoma cells coupled with stress induced activation of Chk1. By contrast, induction of DNA damage in murine embryonic fibroblasts from Atg7- or Atg5-deficient animals with impaired MA, revealed an increase in proteasome activity, no change in total Chk1 protein levels, and a significant reduction in phospho-Chk1 (Ser345) (Liu et al., 2015). The cell cycle regulator p53 is well known to induce the expression of p21 (Giono and Manfredi, 2006), yet only a slight increase in p53 protein levels was seen in cells with LAMP-2C expression. p53 is targeted for degradation by the proteasome and CMA, dependent on p53 structure and mutations as well as levels of cellular CMA (Vakifahmetoglu-Norberg et al., 2013). Whether elevated protein levels of p21 are induced by a p53-dependent or -independent manner in these melanomas remains to be determined and is beyond the scope of the current study. The detection of increased phospho-Chk1 and p21 in melanoma cells with ectopic LAMP-2C was consistent with increased cell stress, potentially associated with activation of ROS production and/or DNA repair mechanisms. Measurements of ROS levels in melanomas with ectopic LAMP-2C did not reveal a consistent change compared to control cells. In response to DNA damage, Chk1 is phosphorylated at Ser345/Ser317. This activated phospho-Chk1 shifts its localization within the nucleus with some molecules moving into the cytoplasm (Wang et al., 2012). Consistent with this, experiments here revealed increased Chk1 in the cytoplasm of cells with ectopic LAMP-2C compared to the parental melanoma cells. Phospho-Chk1 in the nucleus as well as the cytoplasm appears to modulate distinct cell checkpoint events. Studies by Wang et al. (2012) demonstrated diminished cell viability for Chk1 mutant proteins with increased cytoplasmic residence.

Xenograft studies revealed LAMP-2C expression in melanoma cells reduced melanoma growth *in vivo*. Melanoma xenografts with high LAMP-2C cellular levels also displayed increased necrosis, changes in cell morphology, and less cell density in palpable tumors in stained tissue sections. The increased necrosis

detected *in vivo* in tumors expressing LAMP-2C, was consistent with increased necrosis and apoptosis observed in melanoma cells with ectopic LAMP-2C *in vitro*. Immunohistochemistry was used to examine levels of mitosis *in vivo* for tumor cells with and without ectopic LAMP-2C. While no difference in phospho-Histone H3 was detected in this analysis, differences in tumor cell morphology and density were again apparent. An analysis of the effects of LAMP-2A knockdown on lung tumor cells did not reveal consistent increases in cellular apoptosis compared to LAMP-2A sufficient cells *in vitro*, however necrosis and reduced cell proliferation were noted for xenografts of human lung tumors with LAMP-2A knockdown (Kon et al., 2011). Together, the results in this report demonstrate ectopic expression of LAMP-2C in melanomas disrupted multiple cellular autophagy pathways, as well as cell cycle progression and survival. While the reductions in cell growth and increased p21 levels in these melanoma cells were consistent with decreased CMA and reduced expression of LAMP-2A, the melanoma cells with increased LAMP-2C did exhibit some unique differences. These include minimal changes in p53 protein levels, reduced LAMP-2B expression, reduced MA, cell cycle arrest, and high levels of Chk1 and phospho-Chk1. Increased apoptosis and necrosis were detected for melanomas with increased LAMP-2C expression *in vitro* and *in vivo*. In pilot studies, a highly aggressive triple negative breast tumor line TMD-231 was also transfected to increased LAMP-2C expression. No changes in the cell cycle or thymidine incorporation were observed with the breast tumor line with or without ectopic LAMP-2C. Thus, additional studies will be necessary to determine if LAMP-2C expression can modulate tumor growth and survival beyond melanoma lines. Given the complexity of cellular changes associated with LAMP-2C, it may be difficult to definitively pinpoint whether disruptions in autophagy pathways were linked to alterations in cell cycle and survival. These studies do, however, highlight a potential role for LAMP-2C as a tumor suppressor, which might be exploited to halt melanoma progression.

AUTHOR CONTRIBUTIONS

LP, GS, KP, and JB designed the experiments and interpreted the data. LP, AS, and GS performed the experiments. LP and JB wrote the manuscript. All the authors read and approved the manuscript.

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REVIEW
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Autophagy in Neutrophils: From Granulopoiesis to Neutrophil Extracellular Traps

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Autophagy is an evolutionarily conserved intracellular degradation system aiming to maintain cell homeostasis in response to cellular stress. At physiological states, basal or constitutive level of autophagy activity is usually low; however, it is markedly up-regulated in response to oxidative stress, nutrient starvation, and various immunological stimuli including pathogens. Many studies over the last years have indicated the implication of autophagy in a plethora of cell populations and functions. In this review, we focus on the role of autophagy in the biology of neutrophils. Early studies provided a link between autophagy and neutrophil cell death, a process essential for resolution of inflammation. Since then, several lines of evidence both in the human system and in murine models propose a critical role for autophagy in neutrophil-driven inflammation and defense against pathogens. Autophagy is essential for major neutrophil functions, including degranulation, reactive oxygen species production, and release of neutrophil extracellular traps. Going back to neutrophil generation in the bone marrow, autophagy plays a critical role in myelopoiesis, driving the differentiation of progenitor cells of the myeloid lineage toward neutrophils. Taken together, in this review we discuss the functional role of autophagy in neutrophils throughout their life, from their production in the bone marrow to inflammatory responses and NETotic cell death.

Keywords: autophagy, neutrophil, granulopoiesis, phagocytosis, degranulation, neutrophil extracellular traps, inflammation

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INTRODUCTION

Macroautophagy (hereafter called autophagy) is an intracellular homeostatic mechanism of eukaryotic cells, which is essential for the cellular response to starvation and other types of cell stress including hypoxia, oxidative burst, DNA damage, and infection (Levine et al., 2011). During autophagy, cytosolic constituents are enclosed in double-membrane vesicles, called autophagosomes, and subsequently delivered to lysosomes for degradation (autolysosomes). This dynamic, tightly regulated, biological process protects cell by sensing and clearing damaged cellular elements or intracellular pathogens, providing nutrient supply through recycling of cytosolic macromolecules and organelles (Sil et al., 2018).

Over the last years, a large body of evidence implicates autophagy in several host immune functions, such as phagocytosis, elimination of intracellular pathogens, antigen presentation, thymic selection, maintenance of lymphocyte homeostasis, and regulation of cytokine production (Skendros and Mitroulis, 2012; Deretic et al., 2015; Sil et al., 2018). On the other hand, aberrant or uncontrolled autophagy may lead to autophagy-dependent cell death (Galluzzi et al., 2018). Thus, autophagy is implicated in both cell survival and death, depending on the cell type and stress conditions. Dysregulated autophagy has been associated with a wide range of diseases, including inflammatory diseases, neurodegenerative disorders, and cancer (Levine et al., 2011; Galluzzi et al., 2015; Menzies et al., 2015; Martinez et al., 2016).

Neutrophils represent the most abundant effector cells of immune system in humans and are the first to migrate from bloodstream to sites of tissue inflammation, in response to invading pathogens or host-derived mediators (Mayadas et al., 2014). They are short-lived cells, with a circulating half-life varying from 6–8 h to few days. At steady-state conditions renewal of neutrophils is ensured by constant bone marrow granulopoiesis (Cowland and Borregaard, 2016). However, during severe, systemic, inflammatory settings, a reprogramming of haematopoietic response is commenced, leading to *de novo* generation of high numbers of neutrophils from myeloid progenitors and their mobilization to circulation, in a process called emergency granulopoiesis (Manz and Boettcher, 2014).

Currently, the traditional concept that neutrophils comprise terminally differentiated cells with limited plasticity and highly conserved function, due to their low transcriptional activity, has been revised. Neutrophils express a wide variety of surface receptors that gives them the ability to respond quickly according to disease environmental cues and undergo transcriptional reprogramming leading to *de novo* synthesis of cytokines (Tamassia et al., 2018). This adaption makes neutrophils a phenotypically and functionally heterogeneous cell population (Scapini et al., 2016; Silvestre-Roig et al., 2016; Jablonska and Granot, 2017). Accordingly, upon activation, neutrophils are able to exert their antimicrobial and pro-inflammatory functions by using three distinct mechanisms: phagocytosis, degranulation and the most recently described formation and release of neutrophil extracellular traps (NETs) (Mayadas et al., 2014; Mitsios et al., 2016).

The first evidence that autophagy occurs in human neutrophils provided by Mitroulis et al. (2010) indicated the induction of autophagy machinery in a both phagocytosis-dependent (*Escherichia coli*) and phagocytosis-independent manner (such as IL-1β, TLR agonists, rapamycin, and PMA) (Mitroulis et al., 2010). Since then, many studies have indicated that autophagy is crucially involved in neutrophil biology and its effector functions. This review summarizes the biological role of autophagy in the regulation of granulopoiesis and neutrophil/NETs-driven antimicrobial defense and inflammation, by discussing recent evidence derived from experimental and clinical studies, as well as, potential, autophagy-based, therapeutic strategies against neutrophil-mediated diseases.

ESSENTIALS OF THE MOLECULAR MACHINERY OF AUTOPHAGY

The term autophagy was first introduced by Christian de Duve in 1963 to characterize the ability of lysosomes in self-eating (Levine and Klionsky, 2017). In 1990s, Y. Ohsumi and co-workers identified in yeast the genes that govern the autophagy-related pathways and showed that they are conserved from yeast to mammalian cells, paving the way for the study of autophagy in human health and disease (Takeshige et al., 1992; Tsukada and Ohsumi, 1993; Mizushima et al., 1998).

Today, the functional complexes of autophagy-related (ATG) proteins and many of the molecular events that underline the sequential steps of autophagy, from the initiation of autophagosome formation to fusion with lysosome and formation of autolysosome, have been extensively investigated and well described (Yu et al., 2018). In brief, upon autophagy-related stimuli ATG proteins are activated and recruited to begin the formation of autophagosome as an isolation membrane (phagophore), deriving from rough endoplasmic reticulum subdomain, the omegasome (Levine et al., 2011; Hurley and Young, 2017). In mammals, autophagosome initiation is executed in cooperation with two cardinal protein complexes: the serine threonine kinase complex unc51-like autophagy activating kinase 1 (ULK1) composed of ULK1, ATG13, FAK family kinase-interacting protein (FIP)200 and ATG101, and the downstream class III PI 3-kinase complex I (PI3KC3–C1) that includes PI3KC3/VPS34, PIK3R4/VPS15, Beclin 1, ATG14, and nuclear receptor binding factor 2 (NRBF2) (Itakura et al., 2008; Backer, 2016; Lin and Hurley, 2016). Consequently, PI3KC3–C1 catalyzes the production of phosphatidylinositol 3-phosphate (PI3P), leading to the recruitment of two key ubiquitin-like conjugation systems, the ATG5-12 and the microtubule-associated protein 1 light chain 3 beta (LC3B). ATG5-12 system induces the LC3B I lipidation to generate LC3B II. LC3B-II lipidated protein is translocated at nascent autophagosomal membrane and facilitates growth, elongation, and curvature of the forming autophagosomes (Ichimura et al., 2000; Wild et al., 2014).

Maturation of autophagosome to autolysosome is the final, degradative, step of autophagic molecular machinery. Autophagosomes loose the inner of the two membranes upon fusion with lysosomes to form autolysosomes, assigned to degrade sequestered cytoplasmic cargo by hydrolases. Autophagosome maturation is directed by the molecular complex hVPS34-Beclin 1 in association with hVPS38 (UVRAG) (Liang et al., 2008; Backer, 2016).

Induction of autophagy machinery is regulated by the energy sensing system of AMP-activated kinase (AMPK)/mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) (Laplante and Sabatini, 2012; Sil et al., 2018). During nutrient/energy starvation ATP levels decrease and AMPK is activated, whereas mTORC1 is inactivated. AMPK promotes autophagy by directly activating the pre-initiation complex ULK1 through phosphorylation, and inhibition of mTORC1 permits activated ULK1 complex translocation at early autophagosomal structures to exert its inductive effect (Egan et al., 2011; Kim et al., 2011).

Therefore, inactivation of mTORC1 is a major trigger for autophagy (Kim and Guan, 2015; Sil et al., 2018).

AUTOPHAGY AND REGULATION OF GRANULOPOIESIS

Granulopoiesis, i.e., the generation of granulocytes at steady state conditions or upon hematopoietic stress, including myeloablation or systemic inflammation, is a tightly regulated cascade of events that involves not only committed precursors of this specific lineage, but also hematopoietic stem and progenitor cell (Mitroulis et al., 2018a). Several lines of evidence suggest that cellular metabolism is critical in the regulation of the balance between maintenance of hematopoietic stem cells (HSC) and lineage differentiation (Suda et al., 2011; Mitroulis et al., 2018b). HSCs depend on glycolysis to meet their needs for energy production in the highly hypoxic bone marrow microenvironment (Suda et al., 2011; Takubo et al., 2013; Wang et al., 2014). A switch in their metabolic status from glycolysis to mitochondrial metabolism and oxidative phosphorylation (OXPHOS) has been shown to result in the loss of stemness and the differentiation of hematopoietic progenitors, mainly due to the production of intracellular reactive oxygen species (ROS) (Suda et al., 2011; Maryanovich et al., 2015). Fatty acid oxidation (FAO) has been also shown to negatively regulate HSC maintenance and result in their differentiation to multipotent and lineage committed progenitor cells (Ito et al., 2012).

Several lines of evidence propose an important role for autophagy as a regulator of cellular metabolism in HSC, and as a result, in the regulation of quiescence and differentiation of these cells (Kohli and Passegué, 2014; García-Prat et al., 2017). The clearance of damaged mitochondria by mitophagy prevents the accumulation of ROS in HSCs, which leads to their damage and, finally, apoptosis (Joshi and Kundu, 2013). Loss of *Atg7* in HSC results in impaired HSC function, probably due to the accumulation of damaged mitochondria and production of ROS (Mortensen et al., 2011). The same group also demonstrated that hematopoietic deletion of *Atg7* resulted in robust myeloproliferation with features resembling acute myeloid leukemia (Mortensen et al., 2011). An other study by Warr et al. (2013) demonstrated that forkhead box O3 (FOXO3A)-mediated induction of autophagy is protective for HSC, enabling their survival upon metabolic stress. The homeostatic role of autophagy in hematopoietic progenitor function is also supported by a recent study showing that hyperactive mitophagy due to deletion of the gene encoding the AAA+-ATPase Atad3a has a detrimental effect on HSCs homeostasis, skewing differentiation to myeloid lineage (Jin et al., 2018). Using several genetic mouse models, Ho et al. (2017) further demonstrated that the critical interplay between autophagy and cell metabolism in HSCs leads to epigenetic changes and loss of stemness. Disruption of autophagy due to *Atg12* deficiency resulted in metabolic reprogramming of HSC toward OXPHOS and myeloid lineage bias, resembling the phenotype of activated HSC (Ho et al., 2017). Interestingly, autophagic activity in a subset of HSC during aging is linked with protection against the expected with functional decline of hematopoietic progenitors (Ho et al., 2017), being in line with the well-established role of autophagy in the maintenance of cellular health (He and Klionsky, 2009). Taken together, autophagy has a major involvement in the regulation of early progenitors of hematopoietic system (**Figure 1**).

Even though the contribution of autophagy in HSCs is well established, its role in the progression of later stages of progenitors of myeloid lineage is less studied. Myeloid cell

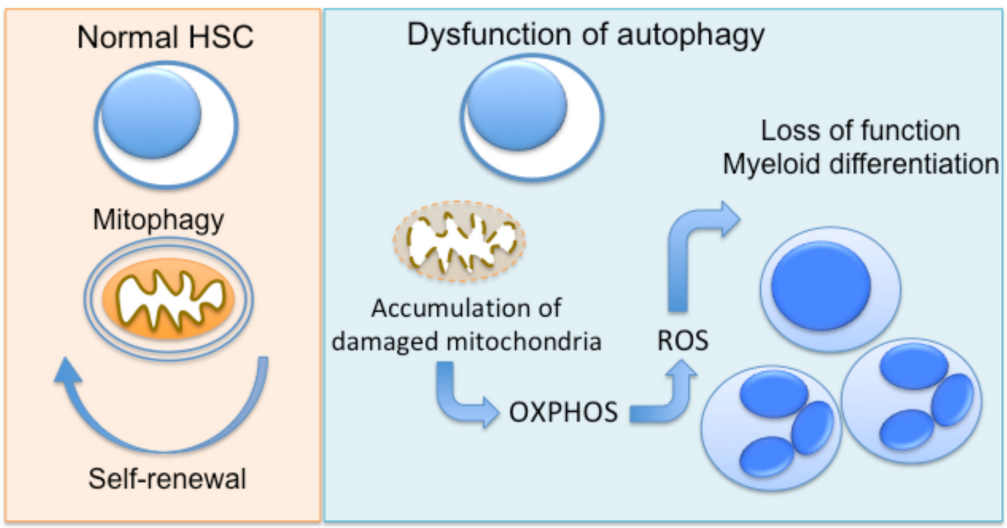


FIGURE 1 | Autophagy regulates hematopoietic progenitor function. In normal hematopoietic stem cells (HSC), autophagy enables the clearance of damaged mitochondria (mitophagy), promoting the maintenance of HSC function. Dysfunctional autophagy, due to deficiency of autophagy genes *Atg7* or *Atg12* in mice has been linked to accumulation of mitochondria and metabolic reprogramming toward OXPHOS. This results in functional decline and differentiation toward myeloid lineage.

specific deletion of *Atg5* has been shown to positively regulate the proliferation rate of neutrophil precursors without being essential for granulopoiesis, leading to accumulation of neutrophils in the bone marrow, blood and spleen, without affecting the functionality of neutrophils in terms of effector functions, apoptosis and migration (Rožman et al., 2015). A seminal study by Riffelmacher et al. (2017) further reinforced the importance of autophagy in granulopoiesis. In this study, it was shown that the degradation of lipid droplets by autophagy is necessary to fuel OXPHOS with fatty acids, resulting in a swift from glycolysis to OXPHOS, a process necessary for the late stages of neutrophil differentiation (Riffelmacher et al., 2017). Finally, a recent study by Huang et al. (2018) reported the differential expression of 22 autophagy-related genes between monocytic and granulocytic differentiation, proposing a role for autophagy in the late stages of differentiation of myeloid precursors toward granulocytes and monocytes.

INTERACTION BETWEEN AUTOPHAGY AND PHAGOCYTOSIS

Phagocytosis and production of ROS are pivotal mechanisms of microbial killing in neutrophils. Several studies have indicated the dynamic interplay between autophagy and phagocytosis in host defense in macrophages (Sanjuan et al., 2007; Gong et al., 2011; Martinez et al., 2015). Autophagy is able to detect and eliminate intracellular pathogens that escape from endocytic compartments of phagocytosis. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-containing protein (NOD)1/2, and the ubiquitin-binding protein p62/SQSTM1 become activated by sensing various pathogen-associated molecular patterns (PAMPs), either on cellular membrane, or in cytosol inducing a kind of selective autophagy that is called xenophagy (Deretic, 2011). Additionally, the autophagic machinery can be actively recruited upon phagocytosis of a pathogen *via* sensing and signaling by an extracellular PRR. In this context, a novel form of selective autophagy, termed LC3-associated phagocytosis (LAP) has been described in murine macrophages (Sanjuan et al., 2007). In LAP, autophagic protein LC3 is conjugated to the traditional, single-membrane, phagosome facilitating the induction of phagolysosome formation and maturation, and enhancing phagocytosis. This translocation is triggered upon TLR engagement by pathogens during phagocytosis and is dependent on common components of autophagic machinery such as PI3KC3–C1-associated proteins, ATG5 and ATG7 (Sanjuan et al., 2007; Martinez et al., 2015).

Apart from the elimination of phagocytosed pathogens, LAP is also associated with the uptake and clearance of apoptotic/necrotic dead cells or immune complexes by using phosphatidylserine or Fc receptors, respectively. Therefore, LAP pathway may protect from aberrant inflammatory responses and mediate immune tolerance (Martinez et al., 2011, 2016).

Although the vast majority of studies regarding autophagy as defense mechanism focus on macrophages, the first observation in neutrophils came from *in vitro* rickettsia-infected guinea pig peritoneal neutrophils, in 1984 (Rikihisa, 1984). Many years ago, the crucial *in vivo* role of autophagy in defense against pathogens has been demonstrated in mice knockout of autophagic factor *Atg5* in monocytes/macrophages, which have been found to be susceptible to infection with *Listeria monocytogenes* and *Toxoplasma gondii* (Zhao et al., 2008). Later on, an autophagy-independent *in vivo* role of *Atg5* in protection against experimental *Mycobacterium tuberculosis* by preventing neutrophil-mediated immunopathology in lungs has been suggested (Kimmey et al., 2015).

Evidence that autophagic machinery operates in human neutrophils was presented in 2010 (Mitroulis et al., 2010). Later on, transmission electron microscopic analysis of bacteria-containing autophagosomes and chemical inhibition of autophagy with 3-methyladenine (3-MA) or bafilomycin A1 (although non-specific) imply that xenophagy may play an antibacterial role in human neutrophils. (Itoh et al., 2015; Ramachandran et al., 2015; Rinchai et al., 2015).

Similar to macrophages, an interconnection between phagocytosis and autophagy pathway has been described in neutrophils. Phagocytosis of *Escherichia coli* triggers the autophagic machinery in neutrophils (Mitroulis et al., 2010). It has been also demonstrated that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and generation of ROS are required for the presence of LC3B in phagosomes of murine and human neutrophils (Huang et al., 2009; Mitroulis et al., 2010). Recently, it has been shown that human neutrophils undergo autophagy following *in vitro* infection with *Streptococcus pneumoniae* that depends on type III PI3K and ATG5 and enhances the rate of neutrophil phagocytosis of bacteria. ATG5 dependence was demonstrated by employing siRNA transfected neutrophils followed by incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Ullah et al., 2017).

Many pathogens have been shown to evade or exploit autophagy in macrophages, aiming to establish an intracellular niche for long-term survival and replication (Skendros and Mitroulis, 2012). Subversion of autophagy by microbes in neutrophils is far less studied. Previously, it has been demonstrated that adherent-invasive *Escherichia coli* strain (AIEC) isolated from Crohn's disease patients can invade human neutrophils triggering the autophagic machinery. However, AIEC was able to escape killing by neutrophil-like PLB cells by disturbing autophagic flux at the autolysosomal step, which permitted intracellular survival of bacteria (Chargui et al., 2012).

Taken together, neutrophils probably use autophagy and phagocytosis both to kill pathogenic microbes and clear cellular debris, and a functional interrelationship between these two defense mechanisms could exist.

AUTOPHAGY AND NEUTROPHIL DEGRANULATION

Upon activation neutrophils release into phagosomes or secrete preformed antimicrobial and inflammatory proteins packed in cytoplasmic granules, in a process known as degranulation. There

are four different types of granules in neutrophils: (a) primary or azurophilic granules (b) secondary or specific granules, (c) tertiary granules, and (d) secretory vesicles. Primary granules constitute the storage site of elastase, myeloperoxidase (MPO), cathepsins, and defensins, secondary granules contain mainly NADPH oxidase, lactoferrin, and matrix metalloprotease 9 (gelatinase), tertiary granules are enriched in gelatinase, but lack lactoferrin, while secretory vesicles are abundant in alkaline phosphatase and various cell membrane and plasma proteins derived from endocytosis (Cowland and Borregaard, 2016; Yin and Heit, 2018).

Notably, granule-derived proteins are required for the major neutrophil functions, including chemotaxis, antimicrobial function and NET release. Elastase and MPO do not only decorate NETs, but are also necessary for NET formation. Accordingly, various, highly concentrated, components of cytoplasmic granules are externalized at affected tissues *via* NET scaffold (Papayannopoulos et al., 2010; Metzler et al., 2011, 2014). Hence, degranulation and NET release are interconnected and share complementary roles during neutrophil activation.

The importance of autophagy in the regulation of neutrophil degranulation has been demonstrated in a study using myeloid-specific autophagy-deficient inflammatory mice models. Autophagy deficiency in neutrophils significantly reduced degranulation *in vitro* and *in vivo* (Bhattacharya et al., 2015). In the same study, ROS generation was also reduced in autophagy-deficient neutrophils, and inhibition of NADPH oxidase diminished neutrophil degranulation, suggesting that NADPH oxidase mediates the effects of autophagy on degranulation (Bhattacharya et al., 2015).

AUTOPHAGY AND NET FORMATION

In 2004, the group of A. Zychlinsky discovered a novel mechanism of neutrophil microbicidal activity, the release of NETs. NETs are a network of fibers that entraps and kills extracellular microbes (Brinkmann et al., 2004). NETs are extracellular chromatin strands carrying various highly active neutrophil-derived granular and cytosolic proteins. Notably, the effectiveness of neutrophil-derived mediators is significantly amplified due to their dense concentration in the fibrous network of NETs (Mitsios et al., 2016; Jorch and Kubes, 2017).

In contrast to apoptosis and necrosis, during NET-mediated cell death (NETosis) chromatin decondenses, the nuclear membrane disintegrates and the plasma membrane ruptures to release NETs (Remijsen et al., 2011a; Galluzzi et al., 2018). This process is also called suicidal NETosis, in contrast to vital NETosis, in which neutrophils are proposed to release NETs without losing their nuclear or plasma membrane, not undergoing cellular death (Pilsczek et al., 2010; Pieterse et al., 2016).

Besides the antimicrobial action of NETs, accumulating evidence highlighted their fundamental role in the pathogenesis of numerous non-infectious inflammatory disorders (Mitsios et al., 2016; Jorch and Kubes, 2017; Sollberger et al., 2018). Moreover, recent clinical and experimental studies suggest that in the context of different diseases, neutrophils release NETs that are qualitatively different and express disease-related bioactive proteins, determined by the disease inflammatory environment. For example, IL-1β-bearing NETs characterize inflammatory flares of typical autoinflammatory diseases such familial Mediterranean fever (FMF) and Still's disease (Apostolidou et al., 2016; Skendros et al., 2017; Angelidou et al., 2018). Autoantigens in NETs have been associated with autoimmune diseases such as lupus, rheumatoid arthritis and ANCA-associated vasculitis (Lande et al., 2011; Khandpur et al., 2013; Tang et al., 2015; Lood et al., 2016), and exposure of thrombogenic tissue factor (TF) through NETs drives several thromboinflammatory conditions (Kambas et al., 2012a,b, 2014; von Brühl et al., 2012; Stakos et al., 2015; Chrysanthopoulou et al., 2017).

In order to better explain the variable protein load and action of NETs in different disorders, the "two-hit" model has been proposed. According to this, the inflammatory environment of each disease leads to transcriptional reprogramming in neutrophils inducing the expression of disease-related proteins (first-hit), and an additional stimulus (second-hit) enables NETs formation and extracellular exposure of these proteins via NETs (Mitsios et al., 2016; Skendros et al., 2017). One the other hand, NETs degradation by DNase I and phagocytic removal of NETs by macrophages, represent regulatory anti-inflammatory mechanisms aiming to balance excessive NETosis and limit tissue injury (Hakkim et al., 2010; Farrera and Fadeel, 2013).

Over the last years, emerging evidence indicates that autophagy is tightly associated with NET formation, although the molecular mechanisms linking autophagy with NETosis are not clearly defined. Recent studies suggest that autophagy may represent the "second/NETotic-hit" (as mentioned above) leading to the extracellular delivery of NET-bound bioactive proteins (**Figure 2**; Stakos et al., 2015; Apostolidou et al., 2016; Skendros et al., 2017)

Neutrophil extracellular trap formation is triggered by many pathogenic agents and several proinflammatory stimuli, such as, cytokines (IL-8, TNFα), and interferon (IFN)α, whereas granular enzymes (MPO, elastase), and ROS positively regulate NET release (Mitsios et al., 2016; Papayannopoulos, 2018). Notably, there is a close interdependence between ROS production and autophagy, two major regulators of NETosis. ROS burst induce autophagy, which in turn is required to maintain efficient ROS production (Bhattacharya et al., 2015; Filomeni et al., 2015).

First, Remijsen et al. (2011b) showed that a combination of autophagy and ROS production is necessary for efficient PMA-induced-NET formation in human neutrophils. Inhibition of either autophagy or NADPH oxidase prevented the chromatin decondensation that is critical for NETosis, leading to apoptotic cell death. Furthermore, neutrophils isolated from patients with chronic granulomatous disease that lacking NADPH oxidase activity are incapable to generate NETs (Remijsen et al., 2011b). In parallel, our group demonstrated that neutrophils from patients with acute gouty arthritis exhibit autophagy-mediated spontaneous NET release, linking for the first time autophagy-associated NETosis with sterile inflammation (Mitroulis et al., 2011a). Subsequently, it has been indicated that mTOR and cytoskeletal machinery play a key role

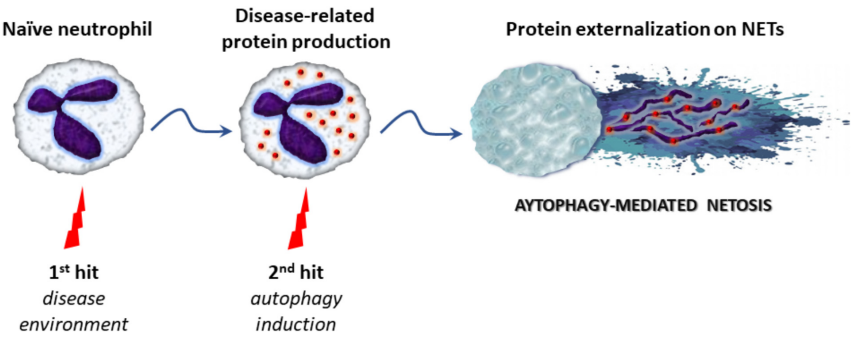


FIGURE 2 | The proposed "two-hit" model in autophagy-mediated NETosis. Initially, various stimuli derived from the inflammatory microenvironment stimulate the production of disease-related proteins in naïve neutrophils (first-hit). Subsequently, triggering of autophagy (second-hit) leads to NET formation and the extracellular release of proteins *via* NET scaffold, contributing to antimicrobial capacity or inflammatory potential of the cell.

in regulating autophagy-mediated NET formation in human neutrophils. Pharmacological inhibition of the mTOR pathway significantly promoted autophagosome formation and histone citrullination facilitating NET release in response to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), whereas blockade of cytoskeletal dynamics abrogated mTOR/autophagy-mediated NETosis (Itakura and McCarty, 2013). Moreover, silencing of *ATG5* in AIEC-infected neutrophil-like PLB human cell line blocked NET formation (Chargui et al., 2012). Recently, it has also been shown that diminished expression levels of Atg5 contributed to reduced capacity of neutrophils to form NETs upon TLR2 ligand stimulation in aged mice, suggesting an important role of autophagy in maintaining the mechanism of NETs (Xu et al., 2017). Interesting, *in vitro* NET generation is impaired in older adults in response to LPS and IL-8 (Hazeldine et al., 2014), and reduced *ATG5* gene expression (Vieira da Silva Pellegrina et al., 2015; Xu et al., 2017). Consistent with this, inhibition of autophagy by pharmacological inhibitors or by small interfering RNA against *ATG7* attenuated LC3 autophagy formation and significantly decreased NET generation in promyelocytes (Ma et al., 2016). Furthermore, knockdown of the inhibitor of PI3K/AKT/mTOR pathway, autophagy inducer, PTEN in HL-60 differentiated neutrophil-like cells resulted in diminished generation of NETs upon stimulation with PMA (Teimourian and Moghanloo, 2015).

On the other hand, conflicting data have also been reported regarding the contribution of autophagy in NET release. In particular, *Atg5*-knockout mouse neutrophils, that exhibit reduced autophagic activity, preserved the capacity to release extracellular DNA. Furthermore, although PI3K inhibition prevented NET formation by human neutrophils, inhibition of late autophagy with bafilomycin A1 and chloroquine did not (Germic et al., 2017). This suggests that an autophagy-independent NETosis pathway may also exist (Pieterse et al., 2016; Germic et al., 2017).

Autophagy/NET-Driven Response in Infection and Sterile Inflammation

Several studies have associated autophagy with the induction of NETs against various microbial agents *in vitro* and *in vivo* (Kenno et al., 2016; Sharma et al., 2017; Ullah et al., 2017). Autophagic machinery is also implicated in the induction of NETosis in experimental and human sepsis (Kambas et al., 2012a; Park et al., 2017). Interestingly, neutrophils isolated from non-surviving septic patients characterized by both impaired autophagy and decreased NET formation. Moreover, induction of autophagy protected mice from lethal sepsis in a NET-dependent fashion (Park et al., 2017).

Increasing evidence indicates the pathogenic role of autophagy-mediated NETs in various clinical models of acute or chronic sterile inflammation, including common IL-1β-mediated autoinflammatory diseases (Mitroulis et al., 2011a; Apostolidou et al., 2016; Papagoras et al., 2017; Skendros et al., 2017), ANCA-associated vasculitis (Kambas et al., 2014; Tang et al., 2015; Sha et al., 2016), active ulcerative colitis (Angelidou et al., 2018), severe asthma (Pham et al., 2017), cancer inflammation (Boone et al., 2015), and IL-17-mediated disorders such as fibrosis (Chrysanthopoulou et al., 2014) and epidermal hyperplasia (Suzuki et al., 2016).

The study of NETosis in IL-1β-mediated autoinflammatory diseases, such as FMF, provided novel mechanistic insights for the role of autophagy in the regulation of IL-1β inflammation (Apostolidou et al., 2016; Akdis and Ballas, 2017; Skendros et al., 2017). FMF is associated with mutations in the Mediterranean fever (MEFV) gene encoding protein pyrin, and is characterized by recurrent inflammatory attacks, often provoked by physical or psychological stress (Ozen et al., 2016).

Previously, it has been demonstrated that alterations in the levels of basal autophagy in neutrophils derived from FMF patients affect their inflammatory potential (Mitroulis et al., 2011b). It has been recently reported that induction of autophagic machinery is linked to the release of NETs-carrying IL-1β during FMF attacks, providing evidence for the involvement of neutrophil autophagy and NET formation in the regulation IL-1β-dependent response (Apostolidou et al., 2016).

Consisting with this, whole transcriptome analysis in neutrophils derived from FMF patients uncovered the role of autophagy-related protein regulated in development and DNA damage responses 1 (REDD1) as a key regulator linking environmental stress with autophagy-mediated NETosis and

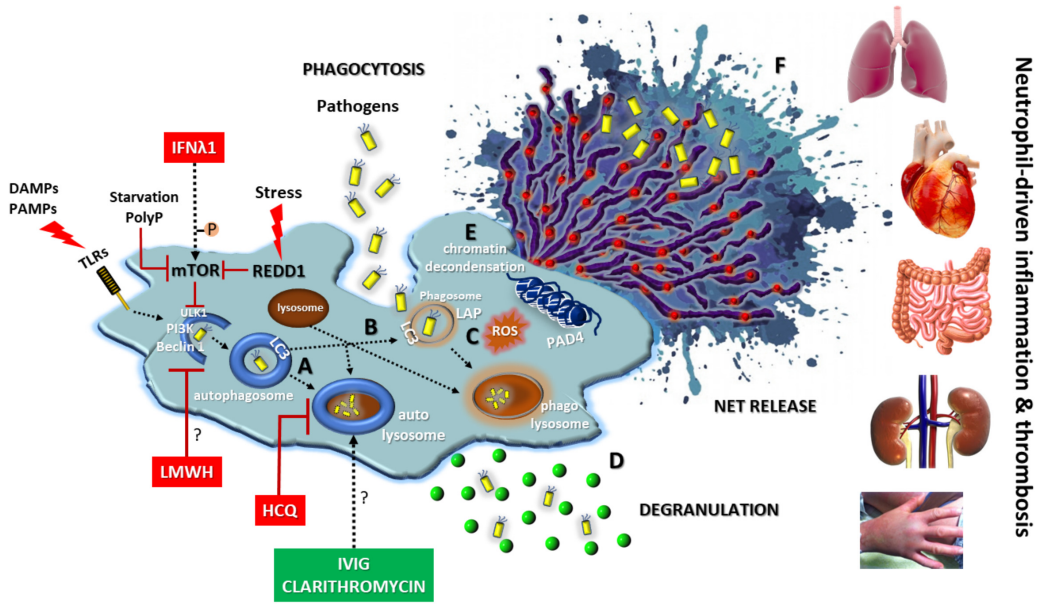


FIGURE 3 | Autophagy in neutrophil biology and disease. Autophagy has a central key role in neutrophil biology by **(A)** promoting intracellular elimination of pathogens (xenophagy), **(B)** accelerating phagocytosis (LAP), **(C)** interplaying with ROS, **(D)** regulating degranulation, **(E)** facilitating the resolution of inflammation *via* NETotic cell death, and **(F)** leading to externalization of various NET-bound bioactive proteins (depicted as red spherical spots). The enhancement of autophagic machinery (e.g., IVIG, clarithromycin) increases the antimicrobial capacity of neutrophils. On the contrary, blocking autophagy pathway at initial (e.g., IFNλ1, LMWH) or late steps (e.g., HCQ) could be beneficial for neutrophil-driven inflammatory or thrombotic diseases. REDD1/mTOR pathway is a main regulator of autophagy in neutrophils. DAMPS, damage-associated molecular patterns; PAMPS, pathogen-associated molecular patterns; P, phosphorylation; PolyP, inorganic polyphosphate; REDD1, regulated in development and DNA damage responses 1; LAP, LC3-associated phagocytosis; ROS, reactive oxygen species; HCQ, hydroxychloroquine; LMWH, low molecular weight heparin; IVIG, intravenous immunoglobulin; Symbol "?" denotes the possible target of the anti-autophagic action.

NET-associated IL-1β autoinflammation (Skendros et al., 2017). REDD1 is a key component of energy homeostasis and inflammation upregulated by various stressors such as glucocorticoids, adrenaline, DNA damage and hypoxia. It has been correlated with the regulation of autophagy through mTOR inactivation or oxidative stress (DeYoung et al., 2008; Qiao et al., 2015; Pastor et al., 2017). Apart from being a regulator of neutrophil-driven IL-1β response, it seems that also affects IL-1β maturation as demonstrated by the colocalization of REDD1 in autolysosomes containing pyrin and NALP3. MEFV mutations prevent localization of pyrin and NALP3 in REDD1 autolysosomes, enhancing IL-1β maturation and release through NETs (Skendros et al., 2017).

REDD1/mTOR/autophagy/NETosis pathway has been also associated with the IL-1β response in active ulcerative colitis supporting the autoinflammatory nature of this inflammatory bowel disease. Notably, in contrast to active Crohn's disease, REDD1 or Beclin-1 expression in colonic neutrophils, and NETosis are diminished according to the distance from the inflamed intestinal area, suggesting that neutrophil autophagy could be a candidate diagnostic and disease severity target in ulcerative colitis (Angelidou et al., 2018).

Furthermore, recently it has been suggested that an unconventional secretory autophagy mechanism is also involved in the secretion of IL-1β by human neutrophils. Pharmaceutical inhibition of autophagy in primary neutrophils or knockdown of *ATG5* in neutrophil-differentiated PLB985 cells markedly reduced IL-1β secretion in culture supernatants after LPS and ATP stimulation. However, NET formation was not investigated in this study (Iula et al., 2018).

Thromboinflammation

The concept of thromboinflammation or immunothrombosis, namely the dynamic cross-talk of innate inflammatory response with thrombosis is extensively studied in many experimental and clinical settings today (Gaertner and Massberg, 2016; Vazquez-Garza et al., 2017). In this context, autophagy emerges as a novel player linking proinflammatory NETs with the initiation and propagation of thrombosis.

In fact, autophagy was shown to mediate on NETs the delivery of functionally active TF, the main initiator of blood coagulation *in vivo*, arming neutrophils with potent thrombogenic capacity. This may be occurred either systemically, such as during the thrombophilic state that characterizes human sepsis, ANCA-associated vasculitis or severe ulcerative colitis (Kambas et al., 2012a, 2014; Angelidou et al., 2018), or locally at the affected coronary branch of myocardial infarction (Stakos et al., 2015; Chrysanthopoulou et al., 2017).

In a recent study utilizing *ex vivo* human system and *in vivo* mice model of arterial thrombosis, it has been indicated that activated platelets of acute ST-segment elevation myocardial infarction (STEMI) patients release inorganic polyphosphate (polyP) in a thrombin-dependent manner, which subsequently induce NET formation in TF-expressed

neutrophils. This mechanism is fine-tuned by autophagy involving the phosphorylation status of mTOR. Importantly, antiviral interferon IFN-λ1/IL-29 emerged as a novel naturally occurring agent that exerts a strong inhibitory effect on NET formation by balancing the action of polyP on mTOR/autophagy pathway (Chrysanthopoulou et al., 2017).

Additionally, an important role for platelet-exposed high mobility group box 1 (HMGB1) in activating autophagy-mediated NET generation has been suggested in a study using thrombi biopsies from acute myocardial infarction patients, pharmacologic and genetic tools (Maugeri et al., 2014).

Taken together, neutrophil autophagy is proposed as a central rheostat of NET-driven tromboinflammation in acute coronary syndrome, and probably other related thrombotic conditions.

TARGETING AUTOPHAGY IN INFECTIONS AND NEUTROPHIL-MEDIATED DISEASES

The above described key role of autophagy in neutrophil biology denotes that elements of autophagic machinery could be effective therapeutic targets for the enhancement of antimicrobial defense or the amelioration of neutrophil/NET-driven inflammation and thrombosis, either as a monotherapy or in combination with classical regimens (**Figure 3**). Combining drugs that act on different targets within the regulatory network of autophagy could be more efficacious than one drug (Iyengar, 2013).

According to the above, intravenous immunoglobulin (IVIG) preparations enhanced *in vitro* both the bactericidal activity and phagocytosis-mediated autophagy of neutrophils isolated from healthy donors, as well as from immunocompromised patients after HSC transplantation, against multidrug-resistant or drug sensitive *Escherichia coli* and *Pseudomonas aeruginosa* strains (Itoh et al., 2015; Matsuo et al., 2015). In another human study, macrolide antibiotic clarithromycin was found to induce *ex vivo* and *in vitro* the release of NETs decorated with the potent antimicrobial peptide cathelicidin LL-37. LL-37-bearing NETs exhibited strong *in vitro* inhibitory activity against multi-drug resistant *Acinetobacter baumannii* growth and biofilm formation (Konstantinidis et al., 2016). Together, these observations imply that targeting autophagy-promoted NETs may present a novel therapeutic strategy to improve infection defense in the aged or immunocompromised individuals.

On the other hand, blocking autophagy might be beneficial in several devastating neutrophil-mediated inflammatory diseases. For example, hydroxychloroquine (HCQ), an old, low-toxicity and low-cost anti-rheumatic drug, is an inhibitor of autophagy impairing both the autophagosome-lysosome fusion and the degradation of the autophagosome contents (Rockel and Kapoor, 2016). In accordance with this, HCQ administration was associated with inhibition of autophagy-mediated NET release preventing disease relapses and reducing the needs for glucocorticoids and anti-IL-1 agents in a case of difficult-to-treat adult-onset Still's disease (Papagoras et al., 2017). In addition, HCQ is a mainstay treatment in systemic lupus erythematosus, a well-defined NET-mediated autoimmune disease (Bosch, 2011; Rockel and Kapoor, 2016).

Recently, it has been reported a novel, autophagy-based, anti-inflammatory action of low molecular weight heparins (LMWH) in peripheral blood neutrophils. Treatment of healthy volunteers with prophylactic doses of LMWHs hindered the ability of neutrophils to activate autophagy and to generate NETs in response to inflammatory stimuli, such as IL-8, PMA, and HMGB1 (Manfredi et al., 2017).

Experimental evidence also suggests that repositioning of IFN-λ1/IL-29 may provide a novel anti-autophagic therapeutic strategy against thromboinflammation that do not interfere with normal hemostasis (Chrysanthopoulou et al., 2017).

CONCLUSION

Autophagy is a key mechanism that is implicated in quite all aspects of neutrophil biology and pathophysiology. The balance of autophagic response in neutrophils is critical for cellular homeostasis and host health. According to environmental danger, autophagy behaves as a double-edged sword for the host neutrophils. It is beneficial by fighting various pathogens and preventing their growth and chronic parasitism. Instead, it is harmful by inducing potent inflammatory responses, including NET formation on systemic and tissue-level. This encourages the design of novel therapeutic agents and/or the repositioning of old drugs targeting autophagic machinery in diseases with crucial involvement of neutrophils/NETs in their pathogenesis. To this end, analysis of big data provided by system biology approaches are urgently needed today.

AUTHOR CONTRIBUTIONS

PS and IM wrote the manuscript and created the figures. KR critically reviewed the manuscript.

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Hypoxia and Selective Autophagy in Cancer Development and Therapy

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Low oxygen availability, a condition known as hypoxia, is a common feature of various pathologies including stroke, ischemic heart disease, and cancer. Hypoxia adaptation requires coordination of intricate pathways and mechanisms such as hypoxia-inducible factors (HIFs), the unfolded protein response (UPR), mTOR, and autophagy. Recently, great effort has been invested toward elucidating the interplay between hypoxia-induced autophagy and cancer cell metabolism. Although novel types of selective autophagy have been identified, including mitophagy, pexophagy, lipophagy, ERphagy and nucleophagy among others, their potential interface with hypoxia response mechanisms remains poorly understood. Autophagy activation facilitates the removal of damaged cellular compartments and recycles components, thus promoting cell survival. Importantly, tumor cells rely on autophagy to support self-proliferation and metastasis; characteristics related to poor disease prognosis. Therefore, a deeper understanding of the molecular crosstalk between hypoxia response mechanisms and autophagy could provide important insights with relevance to cancer and hypoxia-related pathologies. Here, we survey recent findings implicating selective autophagy in hypoxic responses, and discuss emerging links between these pathways and cancer pathophysiology.

Keywords: autophagy, cancer, ERphagy, HIFs, hypoxia, mitophagy, mTOR, pexophagy

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INTRODUCTION

Maintenance of oxygen homeostasis is essential for cellular and organismal survival. Insufficient oxygen availability or hypoxia, represents a common feature of several pathologic as well as physiologic processes. While naturally occurring hypoxia is indispensable for the early onset of mammalian embryonic development, it also contributes to the pathogenesis of several diseases such as stroke, heart failure, and cancer. In any case, evolutionary conserved cellular and systemic responses to oxygen limitation have been developed in organisms as diverse as the nematode *C. elegans* and humans. Such responses attempt to restore tissue oxygenation through sustaining the vascular system and increasing cardiac output. To cope with oxygen deprivation, cells respond by adjusting their metabolic and bioenergetic demands through a number of oxygen-sensing pathways including the hypoxia-inducible factors (HIFs) family of transcription factors -dependent and -independent responses. HIFs belong to the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) family of proteins, which form specific heterodimeric complexes between the HIFα and HIFβ subunits. Specifically, HIF-1α, HIF-2α, and HIF-3α isoforms comprise an oxygen-sensitive alpha subunit which is heterodimerized with the constitutively expressed beta subunit of HIF-1β (Majmundar et al., 2010; Schito and Semenza, 2016). While most of the hypoxia-responsive genes rely on

HIF-1α and HIF-2α heterodimerization with HIF-1β in the nucleus, little is known about HIF-3α regulation and function upon hypoxia. Structural differences as well as tissue-specific expression indicate functional discrepancies between the three isoforms (Koh et al., 2011; Masson and Ratcliffe, 2014; Soni and Padwad, 2017). When oxygen is abundant HIF-1α is rapidly targeted for proteasomal degradation. During this process, prolyl hydroxylases (PHDs) catalyze the hydroxylation of conserved prolyl residues in HIF-1α promoting its ubiquitination through von Hippel Lindau (pVHL) protein and ultimately its degradation from the proteasome. Low oxygen levels inhibit PHDs activity allowing stabilization and nuclear translocation of HIF-1α which is subsequently heterodimerized with HIF-1β (Eales et al., 2016). Hereafter, heterodimerized HIF-1α with HIF-1β will be referred as HIF-1.

Notably, HIF-1-dependent responses are extensively studied, whereas a role of HIF-1-independent responses to hypoxia has just emerged. Particularly, unfolded protein response (UPR) and the mechanistic or mammalian target of rapamycin (mTOR) can act in parallel with, or even substitute HIF-1 activity (Wouters and Koritzinsky, 2008). Based on the severity and duration of hypoxia, each HIF-1- and non-HIF-1-mediated response can involve multiple alternative pathways such as apoptosis and autophagy among others, to promote hypoxia resistance. A balancing act of such responses heavily relies on the coordinated regulation of autophagy by HIF-1, UPR, and mTOR in response to hypoxia (Fang et al., 2015). Deregulation of both mTORC1 and mTORC2 complexes of mTOR signaling represents a common feature of various human solid tumors (Kim et al., 2017). Growing body of evidence shows that inhibition of mTOR protein kinase results in autophagy activation which in turn can be either beneficial or detrimental for tumor survival (Brugarolas et al., 2004; Levy et al., 2017; Paquette et al., 2018; Singh et al., 2018). Similarly, autophagy can be stimulated by UPR induction in response to endoplasmic reticulum (ER) stress and hypoxia (Senft and Ronai, 2015). Equivalently, it appears that HIF-1 possesses diverse regulatory roles in autophagy activation (Mazure and Pouyssegur, 2010). Interestingly rather than being regulated by HIF-1, autophagy *per se*, can regulate HIF-1 stability (DePavia et al., 2016). This reciprocal regulation of autophagy and HIF-1 activity can account for opposing roles of autophagy activation in various human tumors.

Depending on the type of stimulus and cellular damage, mTOR, UPR, and HIF-1 constitute protective responses converging on autophagy. While the molecular mechanism underlying autophagy process has been extensively reviewed elsewhere, little it is known about the role of HIF-1, UPR, and mTOR in hypoxia-induced autophagy (Kaur and Debnath, 2015; Farré and Subramani, 2016; Dikic and Elazar, 2018). Compelling evidence suggests that autophagic degradation of cellular components is triggered in response to hypoxic stress. Coordination of cellular energy releasing and consuming processes such as mitochondrial oxidative phosphorylation (OXPHOS), glycolysis and protein synthesis upon hypoxia has been assigned to autophagy (Mazure and Pouyssegur, 2010; Eales et al., 2016). Toward this direction, proteins, lipids and whole organelles are targeted for degradation, not only to replenish cell with new "building material" but also to readjust cellular function. Specifically, organelles such as mitochondria, peroxisomes and endoplasmic reticulum (ER) among others, are highly targeted by selective autophagy processes named mitophagy, pexophagy, and ERphagy/reticulophagy, respectively. Interestingly, selective degradation of such organelles can be specifically and differentially regulated upon hypoxia when compared to induction of the same processes by other stresses. The existence of specialized mechanisms for selective autophagy induction upon hypoxia highlights the significance of such mechanisms for hypoxic adaptation. Latest findings related to the aforementioned types of selective autophagy triggered upon hypoxia/HIF-1 induction in mammalian systems, is going to be the focus of this manuscript.

HYPOXIA RESPONSE MECHANISMS CONVERGE ON AUTOPHAGY

Due to the multitude of intracellular and environmental stimuli (such as oxidative stress, unfolded proteins, nutrient availability, radiation, heat sock, hypoxia etc.) that an organism has to cope with, it is imperative to maintain the robustness and specificity of cellular protective mechanisms. Among these stimuli, hypoxia and nutrient deprivation represent a common feature of the tumor microenvironment. Adaptation and survival of tumor cells in such a heterogenic microenvironment requires the coordination of several stress response pathways including HIF-1, mTOR, UPR, and autophagy. Of particular importance is the role of hypoxia-induced autophagy in tumor progression. Emerging evidence suggests that various signaling pathways converge on autophagy in response to hypoxia. In this regard, recent progress has demonstrated that autophagy plays an essential role in hypoxic reprogramming of tumor cells conferring resistance to chemotherapy drugs and fostering tumor survival. While hypoxia affects many aspects of tumor biology, the degree to which HIF-1, mTOR, and UPR pathways converge on autophagy to promote survival remains unclear (**Figure 1**).

HIF-1 and Autophagy

In response to hypoxia, activated HIF-1 regulates the transcription of numerous hypoxia-responsive genes, most of which are implicated in energy and oxygen homeostasis (such as glucose metabolism and oxidative phosphorylation etc.). Conversely, glucose deprivation as well as mitochondrial damage can also activate HIF-1, suggesting its feedback regulation by a set of interrelated signaling events possibly through mTOR, UPR, and autophagy. Despite the complexity of HIF-1 regulation, the role of HIF-1 in tumor progression through autophagy has long been appreciated (Masoud and Li, 2015). Recent data suggest that HIF-1-dependent regulation of both selective and bulk autophagy is mediated by changes in the expression of numerous of its target genes. Importantly, core autophagic machinery components have been shown to lie among HIF-1 targets. To this direction, HIF-1-dependent regulation of BCL2 and adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), BNIP3-like (BNIP3L)/NIX,

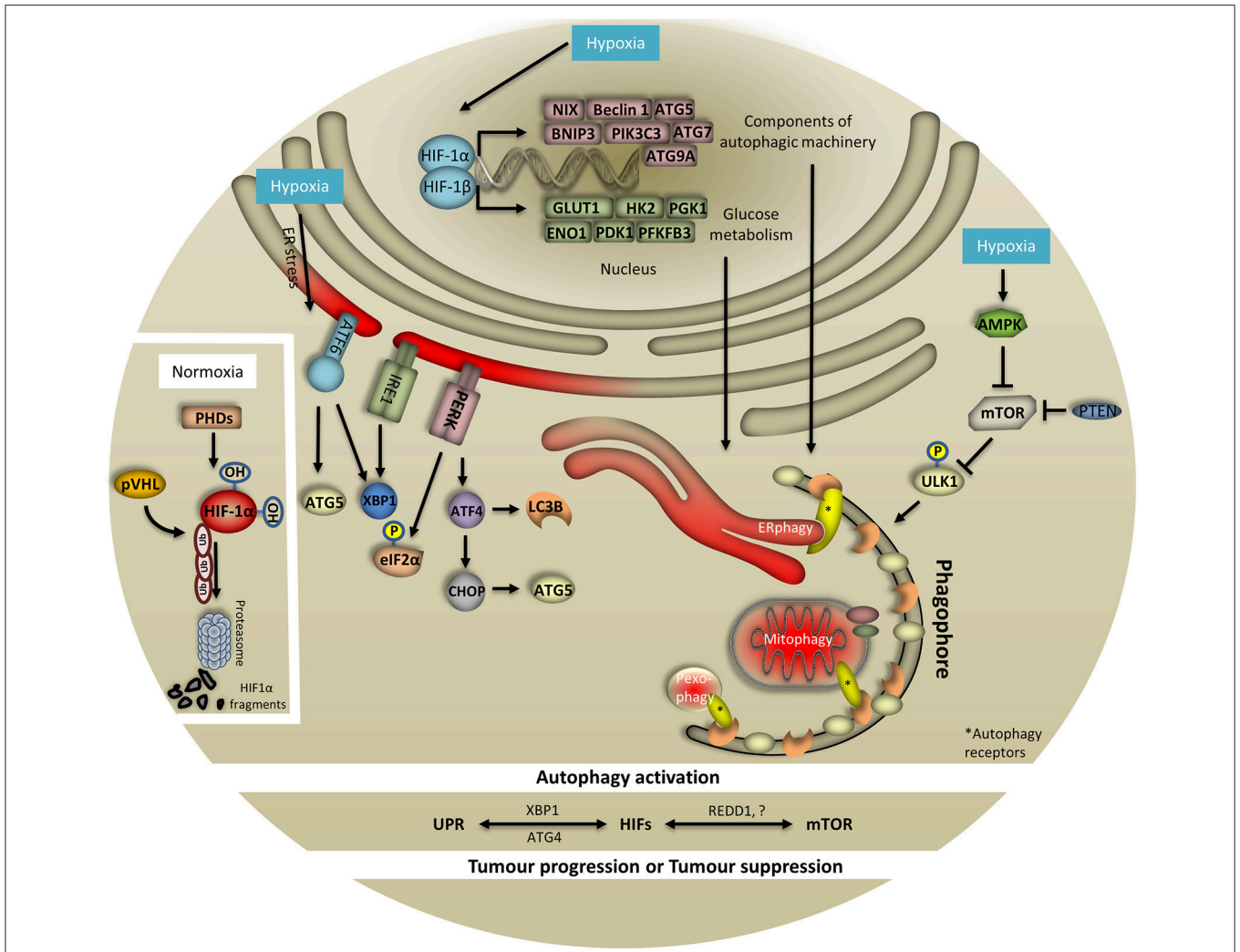


FIGURE 1 | HIF-1, UPR, and mTOR coordinate hypoxia-induced autophagy. To cope with low oxygen levels, cells evoke different oxygen-sensing pathways such as HIF-1, UPR, and mTOR which are tightly coordinated and regularly converge on autophagy. When oxygen is abundant HIF-1 is hydroxylated by prolyls hydroxylases (PHDs) and ubiquitinated by von Hippel Lindau (pVHL) protein. These combined actions result in HIF-1 degradation from the proteasome. In response to hypoxia, HIF-1 is stabilized and translocates to the nucleus to initiate the transcription of multiple genes involved in autophagy, glucose metabolism and mitochondria respiration, among others. Importantly, HIF-1 regulates essential genes for the assembly and function of the autophagy machinery. Particularly, expression of NIX, Beclin 1, ATG5, BNIP3, PIK3C3, ATG7, and ATG9A has been documented to be HIF-1-dependent. Notably, the expression of key glycolytic enzymes that are direct targets of HIF-1 has also been associated with the autophagy process. Specifically, expression of GLUT1, HK2, PGK1, ENO1, PDK1, and PFKFB3 has been reported to be HIF-1-dependent. Surprisingly, in response to oxygen and/or glucose deprivation, expression of HIF-1 targets is linked with the autophagy process. In this respect, hypoxia-induced AMPK negatively regulates mTOR signaling which in turn drives autophagy initiation through ULK1 phosphorylation (bulk and selective autophagy i.e., ERphagy, mitophagy, pexophagy shown here). Similarly, PTEN can inhibit mTOR upon hypoxia and subsequently control autophagy. To this direction, during periods of oxygen limitation cells respond by activating UPR which requires the action of three signaling proteins comprising PERK, IRE1, and ATF6. In the course of PERK-mediated response, eIF2α is phosphorylated to prevent mRNA translation. Moreover, upon hypoxia PERK controls regulation of ATF4 and CHOP which in turn orchestrate the activity of autophagy-related proteins. The contribution of IRE1/XBP1 and ATF6 arms of UPR in autophagy upon hypoxia has just emerged. Although the exact associations with and within UPR, mTOR, HIF-1, and autophagy remain elusive upon hypoxia, identification of XBP1, ATG4, and REDD-1 among others, further supports this notion and highlights the complexity of the system.

Beclin 1, Phosphatidylinositol 3 kinase catalytic subunit type 3 (PIK3C3), ATG9A, ATG5, and ATG7 has already been documented (Zhang and Ney, 2009; Azad and Gibson, 2010; Cerrada et al., 2013; Gui et al., 2016; Abdul Rahim et al., 2017; Zhou et al., 2018). Rather than directly targeting autophagic components, HIF-1 can also regulate autophagy by altering glucose metabolism. In this respect, HIF-1 promotes glucose metabolism through the regulation of glucose transporters−1/3 (GLUT1/3), hexokinases (HK1/2), lactate dehydrogenase (LDHA), phosphoglycerate kinase 1 (PGK1), pyruvate dehydrogenase kinase 1(PDK1), enolase 1 (ENO1), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) among others, although the contribution of each of them to the autophagy process remains elusive (Schofield and

Ratcliffe, 2004; Papandreou et al., 2006; Denko, 2008; Masoud and Li, 2015).

Interestingly, the association with and within HIF-1-related glycolytic enzymes and autophagy has just emerged. During periods of oxygen limitation, autophagy activation controls glucose uptake through GLUT1 activity and its plasma membrane expression (Roy et al., 2017). Recent findings suggest that PGK1 plays a crucial role in autophagy activation through direct binding to VPS34/Beclin1/ATGL14 complex upon glutamate and oxygen deprivation (Qian et al., 2017a). This interaction relies in part, on the protein kinase activity of PGK-1 which phosphorylates Beclin at S30. Compelling evidence indicates that PGK-1 reciprocally regulates glycolysis and autophagy during tumorigenesis (Li X. et al., 2016; Qian et al., 2017b). In line with this, it has been shown that human T cells lacking PFKFB3 redirect their metabolism from glycolysis to the pentose phosphate pathway (PPP) resulting in high NADPH production and low ROS levels which in turn block autophagy (Yang et al., 2013). On the contrary, either genetic or pharmacological inhibition of PFKFB3 constrains the ability of HCT-116 colon adenocarcinoma cells to uptake glucose, accompanied by autophagy induction (Klarer et al., 2014; Shi et al., 2017). Of note, the association of PDK1 with unc-51-like autophagy-activating kinase 1 (ULK1) was shown to regulate autophagy in acute myeloid leukemia (AML) cell lines. Specifically, chemical inhibition of PDK1 with dichloroacetophenone was sufficient to prevent this interaction and subsequently suppress autophagy (Qin et al., 2016). Contrary to the previous study, hypoxia-mediated recruitment of AKT to mitochondria increases PDK1 activity through its phosphorylation on Thr346, which in turn inhibits autophagy in tumor cells (Chae et al., 2016).Culminating effects of multiple factors confound the regulatory role of PFKFB3 and PDK1 in autophagy. Thus, further investigation is required in both a cell-specific and condition-dependent fashion. Furthermore, cancer cells lacking ENO1 enter a catabolic state with increased tricarboxylic acid (TCA), fatty acid oxidation (FAO), and OXPHOS, followed by ROS-induced autophagy (Capello et al., 2016). Recently, it has also been shown that glucose starvation in neonatal rat ventricular myocytes (NRVMs) stimulates autophagy through HK2-mediated inhibition of TORC1 (Roberts et al., 2014). Since autophagy induction by hypoxia and glucose deprivation share common factors including HIF-1 and mTOR, the contribution of each factor to the autophagy process remains enigmatic. Recent findings suggest that mTOR /P70S6K (P70S6-kinase) signaling axis phosphorylates PHD2 at Ser125 and potentiates its activity. On the contrary, PP2A/B55α dephosphorylates PHD2 at Ser125 and reduces its activity. These combined actions control PHD2 enzymatic activity conferring autophagy-mediated hypoxia adaptation of colorectal cancer cells (CRC) in a HIF-1-dependent manner (Di Conza et al., 2017). Collectively, these findings strongly suggest the existence of an unexplored interconnection between mTOR and HIF-1 target genes which impinge on glucose metabolism and in turn control the autophagy process.

Hypoxia and mTOR Regulation of Autophagy

The mTOR signaling pathway plays an essential role in maintaining protein synthesis and metabolic homeostasis in response to low energy production as well as hypoxia and nutrient deprivation. As previously mentioned, cells reduce mitochondrial OXPHOS and favor glycolysis to keep pace with energy supply and demand at low oxygen levels. Loss of energy as well as nutrient balance activate AMP-activated protein kinase (AMPK) and negatively regulate mTOR signaling, which in turn results in autophagy induction through ULK1 phosphorylation at Ser317 and Ser777 (Jung et al., 2010; Kim et al., 2011). While hypoxia-induced mTOR inhibition has been largely appreciated, the extent to which mTOR signals to autophagy in response to hypoxia is poorly understood (Vadysirisack and Ellisen, 2012; Fang et al., 2015). Recent findings showed that sustaining cardiac function upon hypoxia/reoxygenation (H/R) injury relies on autophagy and apoptosis inhibition, in part through the Akt/mTOR signaling axis and miR-21 (Huang et al., 2017). Similarly, a cardioprotective role after H/R injury has been proposed for miR-221 which inhibits autophagy through mTOR signaling (Chen Q. et al., 2016). Interestingly, patient with Crohn's disease exhibit diminished inflammatory response and mTOR signaling followed by induction of autophagy in response to hypoxia (Cosin-Roger et al., 2017).

Questionably, a growing body of evidence focuses on hypoxia-mediated regulation of mTOR signaling in several pathological conditions. For instance, in human prostate cancer cells PTEN-deficiency, which leads to a constituvely active mTOR, reduces hypoxia tolerance. Additionally, loss of eukaryotic initiation factor 4E binding proteins 1/2 (4E-BP1/2) enhances tumorigenesis in a prostate cancer mouse model which is accompanied by increased vascularization and reduced number of hypoxic cells. These findings point toward the notion that 4E-BPs can be targeted for efficient tumor therapy of PTEN-deficient cancer cells (Ding M. et al., 2018). Next, lymphocytes exposed to hypoxia dampen lipogenesis and promote lipid oxidation through mTOR signaling (Yin et al., 2017). Notably, hypoxia-induced cellular acidification as a consequence of imposed metabolic adaptation restrains the circadian clock through mTOR inhibition (Walton et al., 2018). In addition, tuberous sclerosis complex 1 and 2 (TSC1/TSC2) and regulated in development and DNA damage response 1 (REDD1) proteins are not required for mTOR inhibition in hepatocytes exposed to hypoxia (Wolff et al., 2011). While it is well accepted that mTOR signaling regulates autophagy, direct evidence showing the contribution of autophagy to such pathologies remains elusive.

Hypoxia and UPR Regulation of Autophagy

Apart from HIF-1 and mTOR, autophagy acts as an essential node regularly integrated by UPR in response to ER and hypoxic stress (Bi et al., 2005; Fang et al., 2015). Of note, hypoxic stress prevents the formation of disulphide bonds and suppresses proper protein folding in the ER (Rozpedek et al., 2016). To cope with hypoxia-induced proteotoxicity, cells elicit increased UPR which relies

on the action of three established signaling proteins including inositol-requiring protein 1 (IRE1), protein kinase RNA(PKR)-like ER kinase (PERK), and activation transcription factor 6 (ATF6) (Urra et al., 2016). However, the link between UPR and autophagy during periods of limited oxygen availability is poorly understood. In the course of PERK-mediated responses, loss of BiP association with PERK evokes phosphorylation of eukaryotic initiation factor 2α (eIF2α) at Ser51 and subsequently inhibition of mRNA translation (Rozpedek et al., 2016). Importantly, tumor cells lacking eIF2α exhibit increased sensitivity to hypoxia-induced ROS production (Rouschop et al., 2013). Similarly, survival of hypoxic tumor cells has been attributed to autophagy induction through PERK-regulated activation of transcription factor 4 (ATF4) and CCAAT-enhancer-binding protein homologous protein (CHOP). Both ATF4 and CHOP transcription factors control the activity of autophagy-related proteins such as microtubule-associated protein1 light chain 3β (MAP1LC3B/LC3B) and autophagy related gene 5 (ATG5) (Rouschop et al., 2010). Previous studies have shown that hypoxia-induced expression of lysosomal-associated membrane protein 3 (LAMP3) in human tumor cell lines evolves activation of the PERK arm of the UPR (Mujcic et al., 2009). Accordingly, the activity of LAMP3 has been linked with tumor metastasis and poor prognosis independently of HIF-1 (Mujcic et al., 2013; Nagelkerke et al., 2013). In parallel, an autophagy-related cytoprotective role of IRE1 and its downstream target X-box binding protein 1 (XBP1) against hypoxia and tumor growth has only recently emerged (Hetz et al., 2009; Margariti et al., 2013; Chen X. et al., 2014; Fang et al., 2015).

Previous studies have shown that breast cancer cell lines lacking XBP1 exhibit attenuated tumorigenesis due to impaired assembly of XBP1/HIF-1 transcriptional complex and substantial inhibition of downstream hypoxia-responsive genes expression (Chen X. et al., 2014). In addition, it has been reported that co-occupancy of the promoter region of vascular endothelial growth factor A (VEGFA) by ATF4, XBP1, and HIF-1 is indispensable for its expression (Pereira et al., 2014). Given the significance of tumor vascularization for its growth and relapse, it is appealing to further study HIF-1 and UPR co-responsiveness in tumorigenesis. Whether autophagy and XBP1/HIF-1 transcriptional co-occurrence are interrelated with tumorigenesis under the conditions studied, remains to be determined. In this context, interactions between autophagy and ATF6-dependent expression of CHOP and XBP1 have also been documented (Mei et al., 2013). Arguably, the IRE1/XBP1 and ATF6 arms of UPR-induced autophagy are the least studied, therefore further investigation is required to clarify the role of these arms in hypoxia-induced autophagy (Yan et al., 2015).

HYPOXIA-INDUCED SELECTIVE AUTOPHAGY

Mitochondrial number, function and overall homeostasis are widely affected by hypoxia. This can be explained by the fact that oxygen deficiency causes a major metabolic switch: OXPHOS dampens and glycolytic pathways are active, in turn. Under aerobic conditions, the main production source of adenosine triphosphate (ATP) is oxidative phosphorylation which is performed by the electron transport chain (ETC) components inside mitochondria. Oppositely, oxygen shortage under hypoxia, renders ETC dysfunctional, thus unable to produce adequate amounts of ATP. Toward this direction, anaerobic glycolysis is prompted to replenish cellular ATP demands. Except for ATP, reactive oxygen species (ROS) are also generated mainly through the ETC. Interestingly it was shown that increased ROS levels produced upon hypoxia play the major role in the signaling cascade that mediates HIF-1 nuclear translocation and stabilization. On the other hand, excessive ROS cause cellular damage and ultimately cell death. To cope with hypoxia-induced mitochondrial damage, cells evoke increased mitophagy rates to keep a healthy mitochondrial pool. Lowering mitochondrial mass upon hypoxic conditions not only protects against excessive ROS production but also tears apart inactive/useless organelles and recycles their constituents, providing necessary building blocks for other cellular processes.

Selective elimination of mitochondria, known as mitophagy, occurs through the activation of various pathways/mechanisms, such as the phosphatase and tensin homolog-induced kinase 1 (PINK1)/PARKIN pathway and the chaperone-, receptor- and lipid-mediated mitophagy (Ploumi et al., 2017). To date, accumulating evidence shows that receptor-mediated mitophagy is the main type of mitophagy activated upon hypoxia. Several proteins participate in this process; however, the components that function as receptors have the most important regulatory role. Therefore identification of specific mitophagy receptors is a crucial step toward understanding the underlying molecular mechanisms. To date, Bnip3-like/NIP3-like protein X (BNIP3L/NIX), Bcl-2/Adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), and FUN14 domain-containing protein 1 (FUNDC1) are the mitophagy receptors reported to be activated under hypoxic conditions in mammals (Sowter et al., 2001; Bellot et al., 2009; Liu et al., 2012).

FUNDC1-Mediated Mitophagy

FUNDC1 is expressed in all higher eukaryotes and in almost every tissue. Localization studies revealed that it is an outer mitochondrial membrane (OMM) protein which contains three α-helix transmembrane domains. Its N- terminus is exposed to the cytoplasm whereas the C-terminus lies in the intermembrane space (IMS) of mitochondria (Liu et al., 2012). FUNDC1 is enriched in the mitochondria-associated membrane (MAM) upon hypoxia. Interestingly, small amounts of the protein are also found in the ER-mitochondria contact sites under normoxic conditions. Interestingly, functional-domain analysis revealed an LC3-interacting region (LIR) motif in the cytoplasmic N′ terminus of FUNDC1. This domain mediates the FUNDC1-light chain 3 (LC3) associations in a non-canonical conformation and is indispensable for mitophagy induction upon hypoxia. The function of FUNDC1 as a mitophagy receptor under hypoxia is PINK1/Parkin independent and highly specific. This is evident by the fact that depletion of FUNDC1 did not affect either general autophagy or mitophagy induction upon hypoxia-irrelevant stressors such as starvation (Liu et al., 2012).

Detailed mechanistic insight revealed that, under normoxia, FUNDC1 is phosphorylated on its LIR motif by both the proto-oncogene tyrosine-protein kinase Src (Src) and casein kinase 2 (CK2) kinases at Tyr18 and Ser13, respectively. FUNDC1 phosphorylation at these sites and especially at Tyr18 inhibits its association with LC3 (Kuang et al., 2016). These phosphorylation events alter the stereochemical properties of FUNDC1 and decrease its binding affinity for LC3 whereas increase its affinity for binding on other targets (Lv et al., 2017).

On the other hand, upon hypoxia induction, the aforementioned kinases are both dissociated from FUNDC1 through yet not fully understood mechanisms and the levels of phosphorylated FUNDC1 is highly reduced (Chen G. et al., 2014). The inactivation of Src under hypoxic conditions is mediated by a single phosphorylation event, taking place at Tyr416. As a result, phosphorylation on this site blocks FUNDC1 phosphorylation at Tyr18 (Ozkirimli and Post, 2006; Mishra et al., 2009). Importantly, inactivation of both Src and CK2 kinases is mandatory before mitophagy is activated. This inactivation is necessary as: first, only the fully dephosphorylated form of FUNDC1 is the one that binds LC3-II and induces mitophagy and second, the two kinases exhibit functional compensation. Upon hypoxia, FUNDC1 dephosphorylation is promoted by its preferential association with a mitochondrial phosphatase, phosphoglycerate mutase family member 5 (PGAM5). PGAM5 interacts with FUNDC1 and triggers its dephosphorylation at Ser13 as was recently shown in Hela cells (Chen G. et al., 2014). Dephosphorylation of FUNDC1 at this site triggers its association with LC3, followed by mitophagy activation (Wei et al., 2015). PGAM5 and subsequently PGAM5-FUNDC1 associations are multiply regulated. Both in the presence and absence of oxygen, PGAM5 activity is dynamically regulated by Bcl-2-like 1 (BCL2L1/BCL-xL). Under normoxia, BCL2L1/ BCL-xL, which is also an OMM protein, does not physically associate with FUNDC1 but binds PGAM5 through its BH3 domain. This direct binding of BCL2L1/ BCL-xL on PGAM5, renders it inactive, thus unable to dephosphorylate FUNDC1 at Ser13. Furthermore, BCL2L1/BCL-xL by tethering PGAM5 also decreases its availability, thus the interaction of the second with FUNDC1. As a result, FUNDC1-mediated mitophagy is inhibited as evidenced by the decreased association of FUNDC1 with LC3. This function of BCL2L1/ BCL-xL is independent of Beclin 1(BECN1) (Wu H. et al., 2014). Detailed analysis showed that the Bcl-2 homology 3 (BH3) domain of BCL2L1/ BCL-xL is needed but is not sufficient to induce mitophagy. Under hypoxia, on the other hand, BCL2L1/BCL-xL is degraded and PGAM5 is released. Unbound PGAM5 is prone to physically interact with FUNDC1 and trigger mitophagy, as previously described. The involvement of BCL2L1/ BCL-xL in FUNDC1-mediated mitophagy control upon hypoxia is a unique feature of this protein and does not account for every anti-apoptotic component, such as B-cell lymphoma 2 (BCL2) (Chen G. et al., 2014; Wu H. et al., 2014).

In vitro analysis has also revealed that phosphorylation of FUNDC1 at Ser17 increases the interaction of the protein with LC3-II by about three-fold (Lv et al., 2017). This phosphorylation is performed by ULK1 which directly interacts with FUNDC1 and is critical for mitophagy induction under hypoxia. Despite the fact that modifications at Ser17 and Tyr18 are very adjacent, still, they oppositely affect mitophagy induction. Thorough analysis of this phenomenon revealed that SRC-mediated phosphorylation is dominant to and suppresses ULK1 phosphorylation at Ser17 when both events are present (Wu W. et al., 2014). All these phosphorylation events in the cytoplasmic region of FUNDC1 highlight the importance of post-translational modifications and relevance to FUNDC1-mediated mitophagy control.

Apart from the post-translational modifications that regulate FUNDC1-mediated mitophagy upon hypoxia, the receptor is additionally regulated at the post-transcriptional and pre-translational level. This type of regulation is mainly under the control of miRNAs and more specifically of miR-137 which is expressed mostly in the brain. miR-137 binds on the 3′ UTR of FUNDC1, thus post-transcriptionally represses its expression. Subsequently, reduced FUNDC1 protein levels lower the number of FUNDC1-LC3 associations and decrease mitophagy rates. Interestingly, this effect was reversed when a FUNDC1 variant, containing a mutation on the miR-137 binding site on its 3′ untranslated region (UTR), was overexpressed. Upon hypoxia, miR-137 expression is decreased compared to normoxia, allowing mitophagy to be induced (Li et al., 2014). Strikingly, a few studies support that the protein levels of FUNDC1 initially drop upon hypoxia induction (Liu et al., 2012). Despite the fact that not much is known about FUNDC1 transcriptional regulation yet, the notion that FUNDC1 is not regulated transcriptionally, in contrast to other mitophagy receptors such as BNIP3/NIX, prevails (Wei et al., 2015; Williams and Ding, 2015). Since FUNDC1 is not transcriptionally regulated upon hypoxia and its protein levels are reduced, it is possible that miR-137 only partially regulates FUNDC1 expression. The controversial findings regarding miR-137 downregulation and FUNDC1 levels drop in the initiation of hypoxia suggest that additional mechanisms regulate FUNDC1 mRNA stability and expression upon hypoxia, other than the miRs.

A recently identified mechanism could explain this paradox. In this respect, FUNDC1 is targeted by a mitochondrial E3 ubiquitin ligase, membrane-associated ring finger (C3HC4) 5 (MARCH5), for ubiquitination and subsequent degradation. As initially perceived, the levels of FUNDC1 quickly declined upon hypoxia and this effect could be reversed upon treatment with either the proteasomal inhibitor, MG132, or an autophagic flux inhibitor, chloroquine (Chen Z. et al., 2017b). Interestingly, at the initial steps of the hypoxic response, MARCH5 homo-oligomers decrease and MARCH5 shifts toward forming associations with FUNDC1, thus degrading it. A deeper understanding of the MARCH5-dependent ubiquitination and targeted degradation of FUNDC1 revealed K119R as the main ubiquitination site on FUNDC1. MARCH5 physically interacts with FUNDC1 through residues that belong to the cytoplasmic compartments of both proteins. This interaction mediates FUNDC1 ubiquitination, as previously described (Chen Z. et al., 2017a). Furthermore, it is shown that MARCH5-dependent degradation of FUNDC1 is independent of Parkin and precedes the dephosphorylation

events at Tyr18 which activate FUNDC1. This implies that mitophagy is decreased at the onset of hypoxia, allowing cells to maintain their mitochondrial mass at a quite high level. However, if hypoxia is prolonged or becomes more severe, mitophagy escalates and mitochondrial mass drops. The signaling cascade and the hypoxic duration required to regulate these pathways remain elusive (Chen Z. et al., 2017b).

Additionally, MARCH5 ubiquitinates proteins such as Dynamin-1-like protein (DNM1L)/ dynamin-related protein 1(Drp1) which participate in mitochondrial fission (Chen Z. et al., 2017b). Mitochondrial fission is a prerequisite for mitophagy events to take place, at least during Pink1/Parkin-mediated mitophagy which is mainly induced upon mitochondrial depolarization (Twig and Shirihai, 2011; Palikaras et al., 2015). Moreover, FUNDC1 has been linked to enhanced mitochondrial recruitment of DNM1L as well as to higher fission rates. This was found to be dependent on both the presence of FUNDC1 in the MAM and the associations it forms with calnexin (CNX). This observation can be explained by data showing that FUNDC1 accumulates in the MAM upon hypoxia and forms indirect associations with the ER protein CNX through its N- terminus. Besides, indications point toward the view that the associations between FUNDC1 and CNX are important for driving the subcellular localization of FUNDC1 on the MAM (Wu et al., 2016a,b). Even though the components that mediate such an association have not been revealed yet, FUNDC1 is not enriched in this region when CNX is absent. Next, if the hypoxic stress persists, FUNDC1 disassociates from CNX and preferentially binds to DNM1L directly, thus triggering mitochondrial fission. Interestingly, not only depletion of either FUNDC1 or DNM1L is detrimental for fission as expected, but also depletion of CNX. Following these events, FUNDC1 binds to LC3 and promotes mitophagy. Although partially understood, this newly identified (CNX-FUNDC1-DNM1L) axis gives a satisfactory understanding of fission and mitophagy coupling upon hypoxia (Wu et al., 2016b). Complementary studies show that FUNDC1 directly interacts with DNM1L through its cytoplasmic end in a LIR-independent fashion. Interestingly, FUNDC1 can also directly associate with the inner mitochondrial membrane and intermembrane space protein optic atrophy 1 (OPA1). OPA1 regulates mitochondrial fusion and was found to interact with FUNDC1 on K70 residue lying in the intermembrane space. The association between OPA1 and FUNDC1 can also regulate mitochondrial fission, as under stress conditions, such as FCCP treatment, FUNDC1-OPA1 association attenuates, in contrast to the FUNDC1-DNM1L association. The phosphorylation status of FUNDC1 can become the decisive point in regulating the balance of the formed associations. The mechanism which regulates whether FUNDC1 associates with either OPA1 or DNM1L and the balance between the two different associations plays an important role in the determination of mitochondrial fusion versus fission upon stress. Despite the fact that direct involvement of OPA1 in FUNDC1-mediated mitophagy upon hypoxia has not been revealed yet, evidence supporting a role of OPA1 in mitochondrial fusion regulation upon hypoxia already exists (Sanderson et al., 2015; Chen M. et al., 2016). Hence, whether mitochondrial fission is a pre-requisite for FUNDC1-mediated mitophagy upon hypoxia or not remains unexplored. Some interesting questions that arise are the following: first, whether MARCH5-dependent degradation of DNM1L acts on the same pathway with FUNDC1- mediated mitophagy or not, and second whether their ubiquitination level is the critical point that regulates mitophagy rates upon hypoxia. Likewise, FUNDC1 phosphorylation on Tyr18 is sufficient to induce mitophagy upon hypoxia even without the presence of mitochondrial fragmentation. This implies that fission events are not required for mitophagy onset upon hypoxia but most probably, enhance the rate of the already ongoing mitophagy events (Kuang et al., 2016). **Figure 2** summarizes key information pertinent to the mechanisms described in this section.

BNIP3/BNIP3L-Mediated Mitophagy

BCL2 and adenovirus E1B 19kDa-interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L)/NIX are two pro-apoptotic proteins which localize to mitochondria and share many common characteristics and functions. BNIP3 contains one transmembrane domain and localizes on the outer mitochondrial membrane. Its C- terminus is exposed inside mitochondria whereas its N-terminus faces the cytoplasm (Ray et al., 2000). Transcriptionally, expression of both genes is highly elevated upon hypoxia in a HIF-1- dependent manner. Specifically, the HIF-1-dependent transcriptional activation of BNIP3 is further enhanced by Ras and E2F-1, while dampened by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and retinoblastoma protein (Rb) which act antagonistically to reduce BNIP3 transcription (An et al., 2006; Tracy and MacLeod, 2007; Tracy et al., 2007; Shaw et al., 2008; Yurkova et al., 2008). Moreover, the Forkhead box O3 (FOXO3) and CREB-binding protein (CBP) also participate in the HIF-1-dependent transcriptional control of BNIP3, while both the Tumor protein p53 (p53) and CBP are regulators of the HIF-1-dependent NIX transcriptional control. Interestingly, the exact factors that participate in the transcriptional regulation of BNIP3 may be cell-specific. Indeed, it was recently shown that FOXO3 negatively regulates NIX under hypoxic conditions in a Cbp/p300-interacting transactivator 2 (CITED2)-dependent manner (Guo et al., 2001; Lee et al., 2017). This finding raises doubts as to whether FOXO3 similarly influences BNIP3, in a context-specific manner.

Recently, it has been suggested that both proteins play important roles in the regulation of hypoxia-induced autophagy. Initially, it was shown that depletion of both BNIP3 and BNIP3L totally abrogated hypoxia-induced autophagy in CCL39 cells. This finding coupled with the fact that autophagy induction promotes cell survival upon hypoxia, rendered the two proteins potential pro-survival factors. In addition, both proteins obtain complementary functions. This is evident by the fact that NIX depletion by itself does not severely affect mitophagy induction upon hypoxia. Despite the fact that BNIP3 depletion has a stronger impact on mitophagy, only depletion of both components can totally abrogate hypoxia-induced autophagy (Bellot et al., 2009). Furthermore, BNIP3 and BNIP3L mechanistically trigger autophagy by regulating

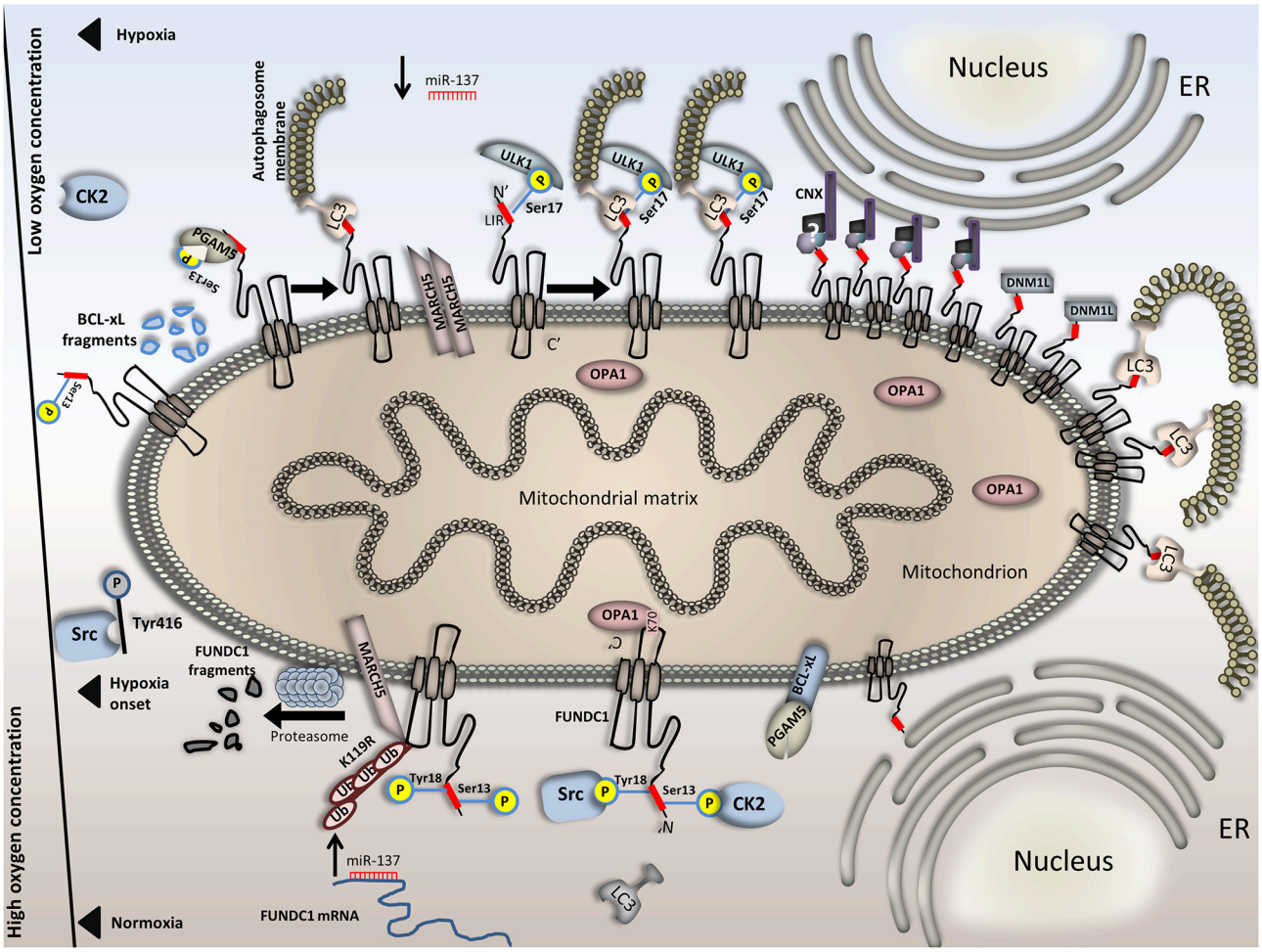


FIGURE 2 | Regulation of FUNDC1-mediated mitophagy in normoxia versus hypoxia. FUNDC1 is inactivated upon normoxia by two phosphorylation events at Tyr18 and Ser13. Phosphorylation at these sites is accomplished by the Src and CK2 kinases, respectively and renders the LIR motif of FUNDC1 inaccessible to LC3. Also, OPA1 binds FUNDC1 in the intermembrane space, promoting mitochondrial fusion. miR-137, which targets the 3′ UTR of FUNDC1 mRNA is upregulated upon normoxia, thus blocking protein synthesis. In parallel, BCL-xL binds PGAM5 and renders it dormant and additionally, the E3 ubiquitin ligase physically interacts with FUNDC1, ubiquitinates it and triggers its proteasomal degradation. This effect is also evident just at the onset of hypoxia. Following, Src kinase is inactivated through phosphorylation on its Tyr416 residue. Src inactivation further blocks Tyr18 phosphorylation on FUNDC1. Moreover, OPA1 dissociates from FUNDC1 and miR-137 is downregulated. FUNDC1 binds PGAM5 phosphatase which cleaves the remaining Ser13 phosphorylation on the first. Interestingly, PGAM5 and CK2 antagonistically bind on FUNDC1. Stoichiometric alterations on FUNDC1 upon hypoxia render its binding affinity for PGAM5 stronger than for CK2. Concomitantly, owing to BCL-xL degradation in response to hypoxia PGAM5 is not trapped on the OMM anymore. Complete dephosphorylation of FUNDC1 triggers LC3 binding and mitophagy onset. Under conditions of prolonged or severe hypoxia, additional mechanisms further increase mitophagy rates. For example, MARCH5 dimerization blocks FUNDC1 degradation and the levels of the second increase. Besides, FUNDC1 phosphorylation at Ser17 by ULK1 increases its affinity for LC3 and last, the CNX-dependent FUNDC1 accumulation in the MAM further boosts mitophagy. This is also facilitated by both DNM1L recruitment and increased affinity of FUNDC1 for LC3 binding. In the bottom half of the Figure, processes that take place under normoxia are illustrated and in the top half, processes under hypoxia. The axis in the left part of the Figure is representative of the available oxygen concentration. Events in the top half (hypoxia) are presented in a specific sequence; when moving from left to right, the severity or duration of the hypoxic event is increased.

the Bcl-2-Beclin complex. Specifically, under normoxia the formation of either BCL-xL-Beclin or Bcl-2-Beclin complexes inhibit autophagy. On the other hand, under hypoxia, these complexes dissociate. Elevation of BNIP3/BNIP3L upon hypoxia triggers the displacement of Beclin from Bcl-2 and BCL-xL. This is achieved because Bcl-2 and BCL-xL preferentially bind on BNIP3/BNIP3L compared to Beclin. So, hypoxia-induced BNIP3/BNIP3L elevation disengages Beclin1. The unbound, free form of Beclin1 is active to induce autophagy while BNIP3 and BNIP3L are now "occupied" by BCL-xL/Bcl-2. Additionally, formation of the latter complexes inhibits cell death upon hypoxia (Bellot et al., 2009). The BH3 domains of these factors are critical for the formation of these complexes both in normoxia and hypoxia.

Additionally, it has been shown that BNIP3 physically interacts with Ras homolog enriched in brain (RHEB) and

triggers a reduction of the RHEB GTP levels, thus inhibiting S6 kinase phosphorylation at Thr389 and ultimately mTOR (Li et al., 2007). This is another mechanism through which BNIP3 triggers general autophagy induction upon hypoxia. Further, it has been proposed that increased expression of BNIP3 and NIX in response to hypoxia causes mitochondrial depolarization and generalized mitochondrial dysfunction (Rikka et al., 2011). This, results in excessive ROS production which causes autophagy induction once again. These actions combined suggest that BNIP3-mediated elevation of general autophagy probably facilitates mitophagy induction as well. To this extent, a question arises relative to ROS homeostasis, BNIP3/NIX regulation, and HIF-1 activation. The notion that prevails up to now is that ROS is the main trigger for HIF-1-stabilization and nuclear localization. HIF-1 nuclear localization and activation triggers the expression of its target genes including BNIP3 and NIX. Albeit, the last example of BNIP3/NIX- mediated autophagy induction supports the idea that BNIP3/NIX elevation precedes ROS augmentation. An interesting question to consider, is whether the aforementioned mechanisms through which BNIP3 regulates autophagy upon hypoxia act in the same or in compensatory pathways.

Interestingly, BNIP3/NIX function as mitophagy receptors apart from their role in general autophagy. The function of both proteins in mitophagy is enhanced upon hypoxia/reoxygenetion. This coupling was initially shown in MEFs where BNIP3 was found to be both necessary and sufficient to trigger mitophagy upon hypoxia and equivalently reduce mitochondrial mass and overall functionality in terms of mitochondrial respiration (Zhang et al., 2008). BNIP3/NIX -induced mitophagy upon hypoxia additionally requires the homodimerization of BNIP3 and the activity of essential autophagy components such as Beclin-1and ATG5 (Hanna et al., 2012). Moreover, both BNIP3 and NIX contain a LIR motif, which is exposed in the cytoplasm, allowing for their physical interaction with LC3/GABARAP (Novak et al., 2010). Despite the fact that an integrated mechanistic insight relative to BNIP3/NIX-induced mitophagy upon hypoxia is still missing, several phosphorylation sites on BNIP3/NIX are decisive for the function of those receptors and for mitophagy induction. First, BNIP3L/NIX phosphorylation at Ser81 seems to be needed for the induction of mitophagy under ischemia-induced conditions although the responsible kinases still remain uncharacterized (Yuan et al., 2017). Second, two phosphorylation events, one at Ser17 and the other at Ser24 of BNIP3 strongly enhance its interaction with the Autophagy-related protein 8 (Atg8) members, LC3B and Golgi-associated ATPase Enhancer of 16 kDa (GATE-16), thus promoting mitophagy. Interestingly, BCL-xL triggers BNIP3-mediated mitophagy in a BH3-dependent manner. Further evidence leads to the conclusion that BNIP3-mediated mitophagy most likely acts as a protective mechanism controlling mitochondrial turnover and counteracting cytochrome c release (Zhu et al., 2013; Liu and Frazier, 2015).

Despite the fact that the pro-survival role of BNIP3 exerted through the control of mitophagy has been extensively tested under hypoxic conditions, still clear evidence regarding the regulation of this receptor upon hypoxic conditions is missing. Furthermore, enzymes that are expected to regulate both the protein levels and the receptor activity upon hypoxic versus normoxic conditions, such as kinases, phosphatases, and E3 ubiquitin ligases have not been identified yet. Interestingly, it has been shown in cardiomyocytes that BNIP3 induction triggers the translocation of Drp1, from the cytoplasm to mitochondria, resulting in mitochondrial fragmentation and subsequently, mitophagy induction (Lee et al., 2011). Importantly, Drp1 localization to mitochondria and mitochondrial fission seem to be a prerequisite for BNIP3-mediated mitophagy in cardiomyocytes. To date, however, strong evidence for direct coupling of Drp1 with BNIP3-mediated mitophagy upon hypoxia is still missing.

Furthermore, despite the fact that the involvement of PINK1/Parkin in hypoxia-induced mitophagy was initially excluded, latest evidence prompted researchers to revisit this theory. Specifically, it was reported that BNIP3 triggers both translocation of Pink1 to mitochondria and elevation of ubiquitination levels in cardiomyocytes (Lee et al., 2011). Moreover, it was recently shown in HEK293 cells that BNIP3 physically interacts with the full-length PINK1 on the OMM, despite the fact that it is not identified yet whether this interaction is direct or not. BNIP3-PINK1 interaction promotes PINK1 stabilization by blocking its proteasomal degradation. Stabilization of PINK1 on the OMM can then trigger Parkin and downstream processes including ubiquitination of OMM proteins which are targeted for degradation. Interestingly, PINK1 deletion did not completely abrogate mitophagy events, implying that BNIP3 can itself induce mitophagy by direct binding on LC3 and/or gamma-aminobutyric acid receptor-associated protein (GABARAP) in a PINK1- independent manner. It is interesting though, that while perturbation of BNIP3 did not have any effect on PINK1/Parkin-mediated mitophagy upon CCCP treatment it did affect hypoxia-induced mitophagy. In response to hypoxia, mitophagy in MEFs is induced through BNIP3-dependent accumulation of PINK1 on mitochondria. On the other hand, BNIP3 depleted cells did not exhibit neither PINK1 accumulation nor mitophagy. So, hypoxic induction of BNIP3 triggers the elevation of PINK1 protein levels and mitophagy (Zhang et al., 2016). This finding contradicts previous research showing that Pink1 deletion did not affect BNIP3-mediated mitophagy upon hypoxia. This discrepancy raises questions relative to whether Pink1 involvement in BNIP3 mitophagy is altered in a cell-type specific manner or whether it depends on the hypoxic conditions applied each time. Another node to the PINK1/Parkin participation in the hypoxic response is added by observations suggesting that Parkin can control HIF-1 and HIF-3 protein levels differentially in normoxia compared to hypoxia. As shown, loss of Parkin increases HIF-1 expression although it decreases HIF-3 in normoxia compared to the control. On the other hand, loss of Parkin upon hypoxia significantly reduces HIF-1 protein levels and also affects its subcellular localization (Maugeri et al., 2016). These data raise the possibility that a feedback loop that coordinates HIF-1, Pink1/Parkin levels and mitophagy exists. However, the possibility that Parkin obtains additional functions cannot be excluded.

At the post-transcriptional level, BNIP3L/NIX is regulated by miR-137, similarly to FUNDC1. miR-137 functions as a negative regulator that when overexpressed, decreases NIX protein levels and mitophagy as shown in HeLa, SKNSH, SY5Y, and HEK293 cells. This effect is very well correlated with hypoxia-induced mitophagy as hypoxia abrogates miR-137 expression (Li et al., 2014). The graphical representation of these mechanisms is shown in **Figure 3**. Evidence up to now suggests that BNIP3-induced mitophagy functions independently from FUNDC-1 mediated mitophagy upon hypoxia (Liu et al., 2012). To this extent, whether the BNIP3- versus BNIP3/PINK1- and FUNDC1-mediated mitophagy are induced upon different hypoxic conditions or in different cell types needs to be tested. For example, in UCB-hMSC cells all *PINK1, BNIP3* and *NIX* are transcriptionally upregulated in response to hypoxia, in contrast to *FUNDC1* which is downregulated (Lee et al., 2017). Also, it is not clear yet whether the PINK1/Parkin activation downstream of BNIP3 is a cellular response to enhanced mitophagy needs. In this respect, it is possible that additional to BNIP3/Nix-mediated mitophagy, activation of Pink1/Parkin-mitophagy serves as a mechanism to boost mitophagy events upon hypoxia. Verification of this hypothesis would highly increase our understanding of the mechanisms that regulate the mitochondrial pool in response to oxygen deficiency.

HYPOXIA-INDUCED DEGRADATION OF OTHER ORGANELLES

The wide variety of metabolic alterations induced by hypoxia are expected to totally reorganize cellular function and affect several if not all cellular compartments in terms of abundance and/or function. Hence, additional targets other than mitochondria are expected to be regulated through selective autophagy upon hypoxia. Toward this direction, evidence exists that selective autophagy of the nucleus (nucleophagy), lipids (lipophagy), ribosomes (ribophagy), ER (ERphagy or reticulophagy), and/or peroxisomes (pexophagy) are activated upon hypoxic stimuli (Carloni et al., 2014; Chen K. et al., 2014; Rashid et al., 2015; Schönenberger et al., 2015; Li L. et al., 2016; Ma et al., 2018). The physiologic relevance and the exact mechanisms governing these selective autophagy types under hypoxia are not well understood yet. For this reason, we will discuss the most important findings on pexophagy and reticulophagy and provide possible future perspectives.

Pexophagy

Peroxisomes are metabolically responsive and highly dynamic organelles in terms of size, number and function. Their key functions are oxygen-dependent and related to lipid synthesis, ROS metabolism and the degradation of both polyunsaturated fatty acids (PUFAs) and very long fatty acids (VLCFAs), among others (Berger et al., 2016). Peroxisomes produce H_2O_2 as a byproduct of their function which is either consumed in downstream reactions or released in tissues (Elsner et al., 2011). To this extent, cell adaptation to oxygen deficiency is expected to seriously readjust peroxisomal number and function. Upon hypoxia, peroxisomes are targeted for selective autophagy, named pexophagy. Through pexophagy induction, cells decrease peroxisomal number and downregulate the high-oxygen demanding processes which take place inside these organelles. This diminishes the cell demands for oxygen and renders them able to preserve their homeostasis even in conditions where oxygen is scarce.

Initial studies in the liver, where peroxisomes are mostly abundant showed that their number drops significantly in a HIF-2a/EPAS1-dependent manner. Despite the fact that this decrease was observed in a von Hippel–Lindau (Vhl) mutant background where HIFs are constitutive active, HIF-1 did not exhibit any involvement in the regulation of peroxisomal number. Since a receptor for HIF-2α -dependent pexophagy under the conditions tested was not identified, it was speculated that the general autophagy receptors Neighbor of BRCA1 gene 1 protein (NBR1) and p62 mediate the effect (Deosaran et al., 2013). Indeed, Nbr1 and p62 are localized on peroxisomes upon HIF-2a stabilization, although Nbr1 was also found there when oxygen is abundant (Walter et al., 2014). Supportive evidence showed that HIF-2α overexpression triggered the concomitant drop of both the peroxisomal number and NBR1 levels. Moreover, both components recognize ubiquitinated proteins on the peroxisomal outer membrane and bind on them. Receptor binding of ubiquitinated substrates subsequently triggers autophagosome formation and engulfment of the organelle. Interestingly, both NBR1 and p62 are degraded in a ROS-dependent manner, although a correlation between ROS, NBR1/p62 and hypoxia has not been established yet (Ishaq et al., 2014).

The field of hypoxia-induced pexophagy is an expanding field in which current understanding is limited. Detailed mechanistic insight would offer better understanding of the hypoxia response mechanisms owing to the fact that peroxisomal function is a determinant of cellular homeostasis. Toward this direction, the identification of specific peroxisomal proteins that function as pexophagy receptors is important. To date, the only specific pexophagy receptors that have been identified are Atg30 and Atg36 in yeast, both of which do not have mammalian orthologs (Farré et al., 2008; Motley et al., 2012). Furthermore, the mechanism by which NBR1 and p62 regulate hypoxia-induced pexophagy is not understood. Moreover, direct evidence for NRB1 and p62 binding on specific ubiquitinated targets does not exist since neither such substrates nor the responsible E3 ubiquitin ligases have been identified. This raises the possibility that these receptors could regulate pexophagy even in a ubiquitin-independent manner.

Our knowledge relative to peroxisomal proteins that participate in pexophagy is still very limited. Only Peroxisomal E3 ubiquitin ligase peroxin 2 (PEX2), which targets PEX5 and 70-kDa peroxisomal membrane protein (PMP70), is a verified peroxisomal component that functions as a pexophagy receptor under starvation conditions. Whether PEX2 mediates hypoxia-induced pexophagy has not been studied yet (Sargent et al., 2016). Interestingly, hypoxia induction downregulates Pex5 in glioblastoma cancer cells but it is not known yet whether this effect is PEX2-dependent (Huang et al., 2012). Moreover, in response to excessive ROS, PEX5 is phosphorylated

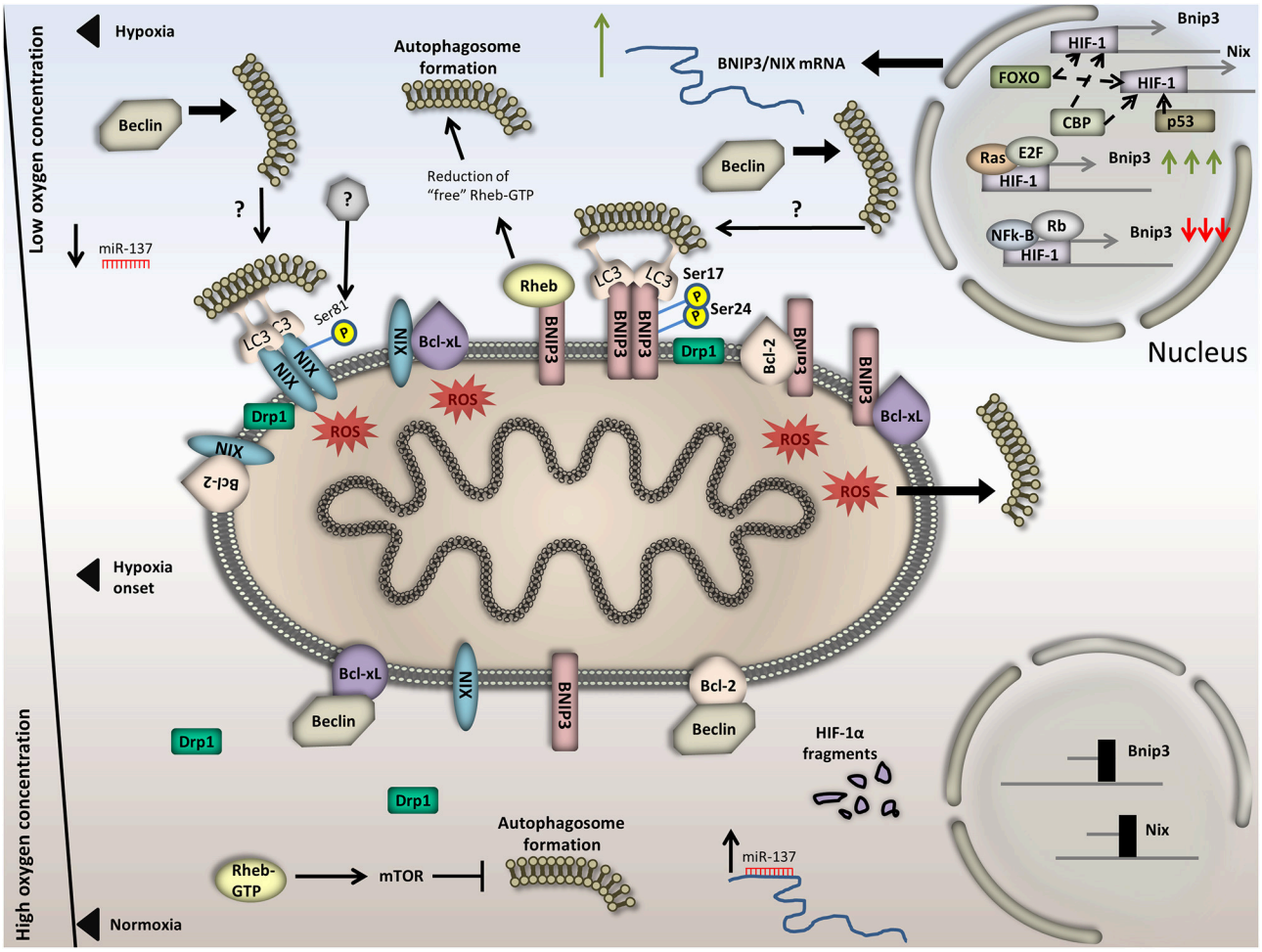


FIGURE 3 | Regulation of BNIP3/NIX-mediated mitophagy in normoxia versus hypoxia. Under normoxia, BNIP3/NIX exhibit basal expression due to HIF-1 degradation in the cytoplasm and hence decreased transcriptional activity. Besides, the OMM localized BCL-xL and Bcl-2 tether Beclin and finally block autophagy induction. In addition, Rheb is free of BNIP3 binding, thus activates mTOR and blocks autophagy induction. Moreover, miR-137, which binds on the 3′ UTR of BNIP3/NIX and suppresses their expression, increases; and last, Drp1 dissipates in the cytoplasm. Upon hypoxia, on the other hand, BNIP3/NIX are highly expressed in a HIF-1-dependent manner. Increased BNIP3/NIX abundance on the OMM triggers Bcl-2/Beclin and BCL-xL /Beclin complex dissociation. Particularly, BNIP3 and NIX are bound on either BCL-xL or Bcl-2, rendering Beclin free to trigger autophagy induction. General autophagy induction could in turn trigger mitophagy. Additionally, BNIP3/NIX accumulation on the OMM triggers mitochondrial dysfunction and membrane depolarization. This leads to excessive ROS production, which can also activate general autophagy. Moreover, BNIP3 binds Rheb, thus diminishes the amount of "free"/cytoplasmic Rheb-GTP and inactivates mTOR, inducing again general autophagy. Concomitantly, BNIP3/NIX phosphorylation and dimerization triggers LC3 binding and finally mitophagy induction. It is also conceivable that Drp1 triggers mitochondrial fragmentation by translocating on the OMM. miR-137 levels drop. In the bottom half of the Figure, processes that take place under normoxia are illustrated and in the top half, processes under hypoxia. The axis in the left part of the Figure is representative of the available oxygen concentration.

at S141 by Ataxia-telangiectasia mutated (ATM) kinase, which translocates from the nucleus to peroxisomes to bind on PEX5. This phosphorylation event subsequently triggers PEX5 monoubiquitination at K209 and activates p62-mediated pexophagy although NBR1 contribution was not tested (Zhang et al., 2015).

ATM signaling also triggers autophagy in a ROS-dependent manner, through both ULK1 activation and mTORC1 inhibition. We speculate that this mechanism could also apply for hypoxic conditions where ROS, ULK1, and mTOR obtain a primary role. Another issue that should be addressed by future studies is the mechanism by which HIF-2α triggers pexophagy. Moreover, taking into account that hypoxic responses depend on ROS and that ROS is also the main stimulus of pexophagy makes the possibility that ROS is the triggering mechanism of pexophagy upon hypoxia very appealing. Notably, the need of ROS for pexophagy induction is further strengthened, as it was shown recently that both genetic and pharmacologic inhibition of catalase, a peroxisomal protein that eliminates ROS and specifically H_2O_2 generated by peroxisomes, triggers pexophagy, and increases ROS levels in HepG2 cells. In fact, initiation of pexophagy upon catalase depletion is ROS- dependent as concomitant treatment with the antioxidant N-acetylcysteine (NAC) abolished pexophagy (Jo et al., 2015; Lee et al., 2018).

The factors mentioned in this section and relative information are summarized in **Table 1**.

ERphagy

Elevation of UPR has been linked to the downstream induction of selective autophagy of the ER, named ERphagy/reticulophagy (Li L. et al., 2016). It is now well established that ER stress is highly induced upon hypoxia, but evidence showing a direct link with ERphagy or reticulophagy is still scarce. Early studies in human cells have revealed the existence of four proteins that specifically function as ERphagy receptors: cell-cycle progression gene 1 (CCPG1), JK-1(FAM134B), SEC62, and Reticulon-3 (RTN3). Interestingly, BNIP3 that was shown previously to participate in mitophagy induction upon hypoxia seems to play a role in ERphagy regulation as well; but the mechanism of action for all the above receptors is still unknown. Additionally, evidence from studies performed in yeast cells, suggests that autophagy of the ER relies on a novel mechanism, independent of the core autophagic machinery (Schuck et al., 2014). Current understanding shows that both RTN3 and FAM134B participate in the starvation-induced ERphagy. Furthermore, SEC62 participates in a specific type of ERphagy that is activated upon acute ER stress in order to alleviate disturbances and re-establish normal homeostasis. Last, CCPG1 is also a stress-induced receptor, but due to the fact that it was only recently identified, still little is known about its mechanism of action (Khaminets et al., 2015; Fumagalli et al., 2016; Grumati et al., 2017; Smith et al., 2018). Notably, FAM134B was found to be among a subset of genes that are upregulated in chronic myeloid leukemia (CML) cells and is correlated with pro-survival phenotypes and poor prognosis. Its upregulation is most probably HIF-1- dependent, like the upregulation of other genes in the same functional subgroup (Ng et al., 2014).

Accumulating evidence showing that Sec62 is highly elevated in the tumor microenvironment point toward a HIF-1-dependent regulation of Sec62 upon hypoxia induction (Linxweiler et al., 2012; Wemmert et al., 2016). Interestingly, Sec62 mediates the translocation of newly synthesized proteins into the ER. This function is achieved through a Sec61-Sec62-Sec63 complex formation on the ER membrane. Especially, the association of Sec62-Sec63 is enhanced by three phosphorylation events on Sec63 at serine residues 574, 576, and 748 by the CK2 kinase (Ampofo et al., 2013). Taking into account that CK2 kinase is responsive to alterations in the oxygen levels, as was observed in the FUNDC1 model upon hypoxia, one would speculate that CK2 could regulate ERphagy in response to hypoxia through a similar mechanism (Mottet et al., 2005). Interestingly, CK2 levels are elevated upon hypoxia and CK2 itself phosphorylates and enhances HIF-1 activity (Mottet et al., 2005; Hubert et al., 2006; Sermeus and Michiels, 2011). On the other hand, the ERphagy receptor RTN3 was found to be downregulated in response to hypoxia in human monocyte-derived macrophages *in vitro* (Fang et al., 2009). In addition, RTN3 protein levels where decreased in fetal heart tissue of sheep exposed to hypoxia, implying the existence of a global mechanism that despite triggering the rest of the ERphagy receptors, downregulates RTN3 (Li et al., 2018). To which extent does RTN3 downregulation affect the overall ERphagy levels upon hypoxia, which is the physiologic relevance of this downregulation and whether the effect is cell-type specific or not needs to be studied in the future. Interestingly BNIP3, which functions as a mitophagy receptor upon hypoxia and is a HIF-1 target gene, has been reported to also function as an ERphagy receptor in HeLa cells (Hanna et al., 2012). Altogether, evidence points toward a physiologic relevance between ERphagy and hypoxia (**Table 2**). We suggest that ERphagy plays a significant role under hypoxic conditions, supported by the emerging roles of ERphagy receptors in pathological conditions such as cancer.

TABLE 1 | Components that are implicated in pexophagy and their association with hypoxia.

Gene name	Function	Additional comments	Direct/Indirect association with hypoxia
NBR1	Receptor	Recognizes ubiquitinated proteins	• Enriched on peroxisomes upon HIF inducing conditions • ROS dependent degradation
p62	Receptor	Recognizes ubiquitinated proteins	• Enriched on peroxisomes upon HIF inducing conditions • ROS dependent degradation
PEX2	E3 ubiquitin ligase Receptor	Binds on PEX5 and PMP70	Not studied
PEX5	Import receptor	• Shuttles between the peroxisomal outer membrane and the cytoplasm • Mono- and poly-ubiquitinated	• Downregulated upon hypoxia • Phosphorylated in a ROS-dependent manner and triggers pexophagy
ATM	Kinase	Phosphotylates Pex5	ROS dependent

HYPOXIA AND CANCER

Cancer cells have the ability to rapidly proliferate and divide giving rise to various types of tumors depending on the tissue of origin. Tumor microenvironment within a solid tumor is characterized by extreme heterogeneities due to the distance a cancer cell obtains from blood vessels. Blood vessels are mostly evident in the periphery of the tumor and function as suppliers of oxygen and other nutritional material to neighboring cells, thereby promoting their proliferation. In contrast, cells located in the more central areas of the solid tumor are often challenged with low oxygen levels. Oxygen scarcity activates HIFs; HIF activation totally alters the metabolic profile of tumor cells by lowering oxidative phosphorylation and promoting glycolysis. Apart from the altered metabolism, activation of additional HIF targets can in parallel promote both vasculogenesis and angiogenesis (Krock et al., 2011). Thus, cells are locally supplied with oxygen and nutrients, boosting their previously stalled proliferation. This is a never-ending phenomenon, as

TABLE 2 | Components that are implicated in ERphagy and their association with hypoxia.

Gene Name	Function	Additional comments	Association with hypoxia
CCPG1	Receptor	Stress-induced, not well studied yet	Not studied
FAM134B	Receptor	Starvation-induced ERphagy	• HIF-1 dependent • Increased in CML
SEC62	Receptor	• ER stress induced ERphagy • Sec62 mediates the translocation of newly synthesized proteins into the ER	Highly increased in tumor micro-environment
RTN3	Receptor	Starvation-induced ERphagy	Downregulated upon hypoxia in human monocyte-derived macrophages and sheep fetal heart tissue
BNIP3	Receptor	It also regulates mitophagy upon hypoxia	HIF-1 target gene
CK2	Kinase	Phosphorylates Sec63	• Oxygen-dependent (altered)functionality • Promotes HIF-1 activity

proliferation would again raise oxygen needs, re-creating a hypoxic microenvironment in a constrained part of the tumor due to the local, abrupt expansion of cells. This re-activates HIFs further promoting tumor expansion, aggressiveness, metastasis, and drug resistance. Taking into account the importance of HIF-1 responses in tumor progression and patient prognosis, it is crucial to gain deep understanding of the mechanisms that are activated downstream of HIFs.

SELECTIVE AUTOPHAGY COMPONENTS IN CANCER

The cell adapts to hypoxic stimuli through the activation of delicate mechanisms which converge on autophagy for recycling of unwanted components or/and organelles, contributing to the preservation of homeostasis. Dysfunction of such mechanisms is coupled with the onset of severe human pathologies such as cancer. In the following section, we will outline recent findings indicating a tight coupling of the aforementioned selective autophagy components to cancer metabolism, emphasizing on studies in mammalian cells.

Mitophagy Components in Cancer

Mitochondrial function is of exceptional importance for cellular and organismal health. Dysregulated mitochondrial homeostasis triggers mitophagy, a process needed to clear damaged mitochondria and prevent their accumulation. Mitophagy impairment leads to accumulation of toxic mitochondrial metabolism byproducts such as ROS that further induce DNA damage and lead to tumorigenesis. Interestingly, components that mediate mitophagy have upcoming roles in several types of cancer. For example, BNIP3 and NIX are highly expressed in breast, macrophage, endothelial and epithelial cancer cells compared to healthy cells from the same patient upon hypoxia induction (Sowter et al., 2001). Furthermore, BNIP3 is highly expressed in lung cancers and follicular lymphomas. On the other hand, BNIP3 is not expressed in other types of cancers such as the pancreatic, colorectal and gastric cancer even under hypoxic conditions. In most pancreatic tumors BNIP3 is methylated. Methylation prevents HIF-1 transcription factor binding, thus inactivating BNIP3. This phenomenon was also observed in many cases of primary colorectal, acute lymphotic, gastric cancer, and multiple myelomas (Li Y. et al., 2017).

BNIP3 loss in pancreatic cancer has been associated with decreased apoptosis in tumor cells, metastatic phenotypes and poor prognosis, rendering BNIP3 a possible anti-tumor gene for this kind of malignancy (Okami et al., 2004; Chourasia et al., 2016; Li Y. et al., 2017). In colorectal cancer, BNIP3 silencing was correlated with increased cell growth and resistance to chemotherapy. In this case, BNIP3 downregulation was associated with aberrant methylation mediated by DNA-methyltransferase 3 beta (DNMT3B) and DNA-methyltransferase 1 (DNMT1) (He et al., 2017). Most possibly, the subcellular localization of BNIP3 is also a measure of functionality. For example, in glioblastoma tumor cells, despite the fact that BNIP3 levels were elevated in the hypoxic areas of the tumors, its localization was not mitochondrial or cytoplasmic as expected, but nuclear. It is not clear whether BNIP3 also has an additional, unknown function in the nucleus, but current evidence suggests that its nuclear localization is a sign of dormancy (Burton et al., 2006). This is in line with observations that BNIP3 is found mainly in the cytoplasm in invasive human breast cancer cells, while in healthy cells BNIP3 is predominantly localized to the nucleus. The physiologic relevance of these observations is not understood yet, although it could reflect the activity status of BNIP3. As previously mentioned, the subcellular localization of BNIP3 protein is altered in invasive human breast cell carcinomas compared to healthy cells and this was oppositely correlated with HIF-1 expression, tumor progression, and good prognosis (Koop et al., 2009). On the other hand, BNIP3 was mainly localized to the nucleus and less in the cytoplasm of laryngeal squamous cell carcinoma (SCC) tumor cells (Jin et al., 2012).

Moreover, BNIP3 is proportionally increased both at the protein and mRNA level by the oncogene Ras. Even in the absence of hypoxic conditions, Ras activation or overexpression could increase BNIP3 levels. This phenomenon was evident in breast, lung, prostate cancer and kidney adenocarcinoma as well as in leukemia (Kalas et al., 2011). On top of that, BNIP3 transcriptional activation by HIF-1, FOXO3A, and E2F is highly induced when Ras is activated (Kalas et al., 2011). Furthermore, microarray analysis performed in patients with renal cell carcinoma (RCC) indicated that increased cytoplasmic levels of BNIP3 correlated with metastasis and poor prognosis implicating that BNIP3 acts as a pro-survival factor and its levels could be used as a prognostic marker for this type of cancer (Macher-Goeppinger et al., 2017). Similar experiments performed in melanoma cell lines under hypoxia revealed a

significant increase in BNIP3; an effect that is correlated with poor prognosis and resistance to pembrolizumab (anti-PD1) immunotherapy (Buart et al., 2017). The effect of BNIP3 does not seem to equally apply in every type of breast tumor. In contrast to other studies, in breast cancer cells, BNIP3 deletion promotes metastasis and is linked with poor prognosis in human triple-negative breast cancer (TNBC). In addition, downregulation of the tumor suppressor retinoblastoma protein highly induces BNIP3 expression under hypoxic conditions (Tracy et al., 2007). On the other hand, the tumor suppressor p53 can directly bind to the BNIP3 promoter and block its expression both under normoxic and hypoxic conditions, thereby inhibiting hypoxia-induced BNIP3-autophagy induction (Feng et al., 2011). Since BNIP3 and NIX are both involved in mitophagy and apoptotic cell death, it is possible that their role may vary depending on the tumor type. For example, in one cancer type they may exert their role through mitophagy, in another through apoptosis and in other cases the balance between mitophagy and apoptosis may determine tumor progression. Future studies are expected to shed light in such speculations.

As far as FUNDC1 is concerned, it was lately shown that cervical cancer cells obtained from early-stage patient tissues had significantly higher levels of the protein compared to adjacent normal cells. Interestingly, this high FUNDC1 expression was negatively correlated with tumor progression and patient prognosis whereas reduction of FUNDC1 levels halted cancer cell proliferation and in parallel induced apoptosis as well as sensitivity to both cisplatin and ionizing irradiation (Hou et al., 2017). Moreover, studies on the PGAM5/FUNDC1/BCL-xL/DRP1 axis described previously in non-small cell lung cancer points toward the direction that targeting mitophagy through FUNDC1 in combination with X-ray irradiation could improve treatment of this type of human cancer (Dong et al., 2017). Additional evidence correlates FUNDC1 and PGAM5 expression with NSCLC and macrophages. Toward this direction, both FUNDC1 and PGAM5 are only expressed in NSCLC epithelial cells and the adjacent macrophages which through yet unknown mechanisms sent signals to neighboring cancer cells, thus determining their fate (Ng Kee Kwong et al., 2017).

Additional factors that regulate hypoxia-induced mitophagy play important roles in cancer cell homeostasis and tumor progression such as the aforementioned kinases Src and CK2. Both kinases retain oncogenic roles and are important players in several types of tumors as reviewed elsewhere, although whether their effect on tumorigenesis is mediated through their role in mitophagy or through other functions has not been well studied (Kim et al., 2009; Trembley et al., 2009, 2010; Zhang and Yu, 2012; Chen et al., 2018). Moreover, CNX, another component implicated in hypoxia-induced mitophagy, is highly increased in cancer cells. This characteristic could render CNX a valuable prognosis marker (Lakkaraju and van der Goot, 2013; Kobayashi et al., 2015; Ryan et al., 2016; Ma et al., 2017). On the other hand, miR-137 acts as a tumor suppressor as evidenced in various cancer cell types (Neault et al., 2016; Chen T. et al., 2017; Ding F. et al., 2018). Finally, the role of the PINK1/Parkin pathway in cancer onset and progression has already been extensively reviewed (Lu et al., 2013; Matsuda et al., 2015; Eid and Kondo, 2017; Palikaras et al., 2017). Interestingly though, it was found that ARIH1 substitutes Parkin in the PINK1/Parkin-mediated mitophagy that takes place specifically in cancer cells. Besides, it is proposed that ARIH1 can be used as a prognostic marker for chemotherapy, as already tested in lung adenocarcinoma patients (Villa et al., 2017).

Pexophagy Components in Cancer

Lately, pexophagy has also been implicated in tumorigenesis and tumor progression. Toward this direction, it is not only the fact that pexophagy rates are highly induced in a HIF-1- and oxygen-dependent manner but also that specific pexophagy components have been implicated in the regulation of tumor homeostasis. For example, Nbr1 and p62 which both function as pexophagy receptors, play a role in cancer homeostasis. Specifically, Nbr1 is expressed in the cytoplasm of low-grade non-musical-invasive bladder cancer cells and is correlated with poor prognosis (Chi et al., 2017). On the other hand, it was recently identified that Nbr1 expression is downregulated in clear cell renal cell carcinoma (ccRCC), phenomenon that is correlated with poor patient prognosis and resistance to sunitinib treatment. As a result, Nbr1 could possibly be used as a prognostic marker for both metastasis and chemoresistance in patients with this type of malignancy (Ruan et al., 2017).

Nbr1 also promotes cell migration and regulates focal adhesion in a breast cancer cell line (Kenific et al., 2016). Furthermore, Nbr1 transcript levels are highly decreased in mammary cancer cell lines compared to their healthy counterparts (Dimitrov et al., 2001). Whether this affects breast cancer progression and prognosis or whether pexophagy is affected and plays a crucial role is expected to be answered in the future. Additional involvement of Nbr1 and p62 to cancer metabolism is indicated through their responsiveness to several compounds with anticancer properties. For example, Gambogic acid (GA), an anti-tumor drug and ROS inducer, cleaves and inactivates both p62 and Nbr1, among others, through ROS-mediated caspase activation (Ishaq et al., 2014). Furthermore, testing for possible anti-tumor effects of copper (I) nicotinate complex (CNC) on squamous cell cancer revealed that the drug could decrease general autophagy levels and elevate Nbr1 expression through yet unknown mechanisms (Abdel-Mohsen et al., 2017).

Except for the aforementioned factors, PEX2 expression is also highly increased in hepatocellular carcinoma (HCC) cells compared to healthy cells. It was shown that increased PEX2 expression was correlated with enhanced tumor growth whereas its depletion leads to increased ROS production, ER stress and autophagy induction. Similar effects were observed for PEX10 and PEX12 (Cai et al., 2018). These findings indicate that liver cancer may behave differentially than other cancer types as in this case ROS and autophagy induction lead to cell death and not to tumor progression. It would be interesting to study whether the PEX2- dependent liver cancer progression is HIF-1-dependent or not.

Furthermore, Pex5 and PMP70 are also implicated in cancer progression. For example, Pex5 mRNA levels were significantly increased in colon carcinoma cells, while they were decreased in C6 glioma cells exposed to hypoxia (Lauer et al., 1999;

Huang et al., 2012). Furthermore, the mRNA levels of PMP70 were unchanged and the protein levels of PMP70 decreased in colon carcinoma cells (Lauer et al., 1999). Following, overexpression of the tumor suppressor H-rev107 triggered the absence of PMP70 from peroxisomes in human embryonic kidney cells 293 (HEK293 cells) (Uyama et al., 2012). Also, PEX3 downregulation reduced the resistance of lymphoma cells to Vorinostat (Vor) by triggering apoptosis (Dahabieh et al., 2017). Furthermore, mutations on the ATM kinase gene are highly oncogenic, predictive of poor prognosis conferring resistance toward therapeutic approaches in various types of cancer such as colorectal, breast, lung and hematopoietic (Squatrito et al., 2010; Feng et al., 2015; Stagni et al., 2015; Weber and Ryan, 2015; Antonelli et al., 2017). Despite the fact that ATM is characterized as a tumor suppressor gene, its activity is not uniform in every type of cancer. Importantly, it was shown that ATM depletion inhibited tumor progression and metastasis in colon cancer cells (Liu et al., 2017).

ERphagy Components in Cancer

ER is one of the most important organelles for cellular homeostasis. Its importance is underscored by the fact that intricate stress response mechanisms have been developed and are activated soon after hypoxia onset. Moreover, most of the proteins implicated in ERphagy are associated with cancer. For example, the recently identified ERphagy receptor CCPG1 has been linked to prostate cancer and in fact was proposed as a predictive biomarker for this type of cancer (Rizzardi et al., 2014). Furthermore, CCPG1 was shown to physically interact with both FIP200 and ATG8 in a lung cancer cell line, thus possibly directly affecting autophagy initiation (Smith et al., 2018). Moreover, CCPG1 was found to be downregulated in colon cancer (Gavert et al., 2013). Finally, downregulation of CCPG1 in retina retinoblastoma cells is correlated with cell proliferation and decreased apoptotic cell death, an effect that is mediated by miR-498 (Yang et al., 2018). Interestingly, miR-498 is downregulated in several types of cancers such as ovarian, non-small cell lung and colon cancers, an effect that is correlated with poor prognosis (Gopalan et al., 2015; Liu et al., 2015; Wang et al., 2015). Whether miR-498 downregulation in the aforementioned types of cancers affects tumor progression through CCPG1 remains to be identified.

Moreover, evidence linking FAM134B, another ERphagy receptor, with cancer recently came to light. Specifically, it was found that decreased FAM134B expression in colorectal adenocarcinomas is coupled with enhanced tumor aggressiveness, poor prognosis, and tumor re-occurrence as well as metastasis. Deeper analysis showed that in this type of cancer cells, FAM134B was inactivated through promoter methylation and interestingly this effect was found to be tumor stage—specific, i.e., late-stage cancer cells exhibited increased FAM134B promoter methylation in comparison to earlier ones (Islam et al., 2017, 2018). Moreover, in colon cancer cells, an alternative FAM134B inhibition pathway through miR-186-5p, has been revealed rendering FAM134B a tumor suppressor, at least for this type of tumor (Kasem et al., 2014; Islam et al., 2017). Additionally, FAM13B is mutated in about half of the colorectal cancer samples tested compared to their healthy counterparts. Different types of mutations were identified ranging from single-nucleotide substitutions to insertions and deletions, among others (Kasem et al., 2014; Islam et al., 2017). Mutations on FAM134B have been identified in other types of cancers as well, such as in oesophageal squamous cell carcinoma (Haque et al., 2016). The localization of FAM134B in colon cancer cells was both cytoplasmic and nuclear with the higher proportion found in the cytoplasm (Islam et al., 2017). Interestingly, FAM134B is a predicted target of an additional miR, namely, miR-4284. This miR is downregulated upon hypoxic conditions, in irradiation-resistant cells and in prostate cancer AMC-22Rv1 cells (McDermott et al., 2017). FAM134B obtains an oncogenic role in chronic myeloid leukemia (CML) cells under hypoxia. Specifically, FAM134B is upregulated in CML promoting cancer cell survival and drug resistance, ultimately associated with poor patient prognosis (Ng et al., 2014). These findings indicate the complex regulation imposed on FAM134B among the different types of cancers. Deeper understanding of the regulatory mechanisms would prove crucial for targeted and successful therapeutic interventions.

Sec62 which also plays a significant role in ERphagy has been correlated with various types of tumors. Sec62 is highly elevated both at the mRNA and protein levels in prostate cancers and is positively correlated with decreased apoptosis in thapsigargin-treated cells, whereas, downregulation of Sec62 makes cells more responsive to this type of therapy (Jung et al., 2006; Greiner et al., 2011). Also, Sec62 is upregulated in other types of tumors, such as the thyroid and non-small cell lung tumor. In all three types of tumors, blockage of Sec62 expression is very well correlated with loss of cell differentiation capacity, tumor invasiveness and metastasis, although cell viability was not significantly affected (Greiner et al., 2011; Körbel et al., 2018). Furthermore, Sec62 was also significantly elevated in more than 80% of thyroid and cervical cancers. In these types of tumors also, ER stress resistance and metastatic capacity were dependent on Sec62 increased protein levels (Linxweiler et al., 2012, 2016). Importantly, increased Sec62 protein levels are evident in post-surgical patients with HBV-related hepatocellular carcinoma recurrence. This finding implies not only that Sec62 could be used as a prognostic marker but also as a new therapeutic target for HCC recurrence (Weng et al., 2012). Additionally, Sec62 overexpression has been detected in head and neck squamous cell carcinomas. In these types of tumors, again, Sec62 overexpression is linked with lymphatic metastasis and poor patient prognosis (Wemmert et al., 2016; Bochen et al., 2017). The exact mechanism of Sec62 tumorigenic activity is not yet understood despite the fact that its role in various types of tumors render it an oncogene.

Finally, RTN3, another ERphagy receptor was found to be downregulated upon hypoxia, as previously mentioned. Despite the fact that information relative to its role in cancer is still limited, it was first shown that RTN3 overexpression triggers tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-, tumor necrosis factor (TNF)-α and Fas-dependent apoptosis. Interestingly, TRAIL selectively induces apoptosis of renal cancer cells without affecting the viability of healthy cells (Lee et al., 2009). These results imply that RTN3 could be

used as a therapeutic target at least in this type of human cancer. Moreover, studies in HeLa cells revealed that RTN3 physically associates with Ras on the endoplasmic reticulum and proposed that RTN3, at least in the model tested, could regulate Ras localization and functionality. Specifically, it is speculated that RTN3 "traps" Ras on the ER, rendering it inactive, by disrupting its redistribution on the plasma membrane (Su et al., 2007). Next, RTN3 was recently identified as a novel prognostic marker for HCC together with UPB1 and SOCS2. RTN3 is positively correlated with HCC and its levels were significantly increased in tumor tissues compared to healthy ones. Additional studies are needed to verify the role of RTN3 in HCC and its mechanism of function (Li B. et al., 2017). Moreover, studies performed in cancer tissues from patients point toward an oncogenic role of RTN3, as it was shown that increased RTN3 levels are observed in astrocytoma whereas no expression was observed in healthy glial cells (Huang et al., 2004). Furthermore, RTN3 was one of the top three upregulated genes in chemotherapy-sensitive epithelial ovarian cancer samples pointing toward an anti-tumor role under these conditions (Zhang and Luo, 2016).These findings highlight the need for tight regulation of selective autophagy components within the tumor microenvironment. The expression changes of selective autophagy components observed in cancer versus healthy cells are summarized in **Table 3**.

CONCLUDING REMARKS

Cells, tissues, and whole organisms may be physiologically exposed to hypoxia, as for instance occurs during embryonic development or when exposed to high altitudes. On the other hand, hypoxia is a common feature of several human pathologies such as ischemia and cancer and importantly stands as the causative link in their onset. Cells respond to hypoxia by adapting their metabolism and function through a number of hypoxia-associated pathways comprising HIFs, mTOR, UPR, and autophagy. HIFs activate several stress response mechanisms, most of which converge on autophagy, to restore homeostasis and ensure cell survival. Importantly, the fact that solid tumors are characterized by hypoxic microenvironment and exhibit HIF activation renders the comprehensive delineation of autophagy pathways necessary. Since autophagy functions as a pro-survival mechanism, its targeted downregulation is a common strategy applied for eliminating cancer cells or making them sensitive to chemotherapy. On the other hand, recent data indicate that this is not always the case. More specifically, it seems that at the stage before the proliferating cells become malignant, i.e., in healthy cells, autophagy induction has a protective, tumor-suppressive role whereas in advanced cancers its role can be both tumorigenic and tumor suppressive. Toward this direction, loss of Beclin1, Atg5, and/or Atg7 has been associated with the onset of several types of tumors. On the other hand, the means by which autophagy can become tumorigenic when activated in the tumor microenvironment is by promoting tumor cell survival and proliferation. At this point, blocking autophagy would be appealing, but concerns toward this direction have arisen. These are based on studies that highlight a possible intervention with anti-tumor inflammatory responses that would in the end convert such handlings from tumor suppressive to tumorigenic (Townsend et al., 2012; Rao et al., 2014).

TABLE 3 | Expression patterns of selective autophagy components linked to hypoxia in various human cancers.

Gene name	Expression pattern per cancer type			
	Increased	**Decreased**	**Mutation**	**Not expressed**
BNIP3	Breast cancer, Macrophage cancer, Endothelial cancer, Epithelial cancer, Lung cancer, Follicular lymphomas, Glioblastoma, Prostate cancer, Kidney adenocarcinoma, Leukemia, Renal cell carcinoma, Melanoma			Pancreatic cancer, Colorectal cancer, Gastric cancer, Acute lymphoma, Myelomas
NIX	Breast cancer, Macrophage cancer, Endothelial cancer, Epithelial cancer			
FUNDC1	Cervical cancer			
Nbr1	Cervical cancer, Bladder cancer, Clear cell renal cell carcinoma	Breast cancer		
ATM			Breast cancer, Lung cancer, Colorectal cancer, Hematopoietic cancer	
PEX2	Hepatocellular carcinoma			
PEX5	Colorectal cancer			
CCPG1	Prostate cancer	Colorectal, Retina retinoblastoma		
FAM134B	Chronic myeloid leukemia	Colorectal cancer	Oesophageal squamous cell carcinoma	
Sec62	Prostate cancer, Cervical cancer, Thyroid cancer, Non-small cell lung cancer, Hepatocellular carcinoma, Head squamous cell carcinoma, Neck squamous cell carcinoma			
RTN3	Hepatocellular carcinoma, astrocytoma			

It is becoming apparent that autophagy inhibition even in the same cell population can differentially impact cancer cell viability. Furthermore, autophagy inhibition at different stages of the tumorigenic process can erratically impact cell viability. All these issues raise the complexity of each tumor entity and render therapeutic strategies in many cases unpredictable. Intervention strategies that globally target the general autophagic machinery or mTOR are proven insufficient and risky, provoking severe side effects for the patient. It is possible that the best strategy for tackling tumor progression would be by regulating specific types of selective autophagy and not general autophagy components that would uniformly affect all types of selective autophagy. Moreover, it becomes apparent that altering a specific type of selective autophagy differentially impacts tumor progression. The same manipulation can either be tumor suppressive or tumorigenic and this is mainly dependent on the tumor stage and cell type. In this respect, we suggest that targeting selective autophagy components instead of general autophagy would be the best approach toward cancer treatment. Such therapies require the development of the appropriate drugs that most possibly would be best combined with chemotherapy or radiotherapy. Ideally, the effectiveness of such therapeutic approaches is expected to be significantly improved if seen in the context of personalized medicine.

AUTHOR CONTRIBUTIONS

ID and IG wrote the manuscript. NT organized and edited the manuscript.

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REVIEW
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The Interplay of Host Autophagy and Eukaryotic Pathogens

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For intracellular pathogens, host cells provide a replicative niche, but are also armed with innate defense mechanisms to combat the intruder. Co-evolution of host and pathogens has produced a complex interplay of host-pathogen interactions during infection, with autophagy emerging as a key player in the recent years. Host autophagy as a degradative process is a significant hindrance to intracellular growth of the pathogens, but also can be subverted by the pathogens to provide support such as nutrients. While the role of host cell autophagy in the pathogenesis mechanisms of several bacterial and viral pathogens have been extensively studied, less is known for eukaryotic pathogens. In this review, we focus on the interplay of host autophagy with the eukaryotic pathogens *Plasmodium spp, Toxoplasma, Leishmania spp* and the fungal pathogens *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans*. The differences between these eukaryotic pathogens in terms of the host cell types they infect, infective strategies and the host responses required to defend against them provide an interesting insight into how they respond to and interact with host cell autophagy. Due to the ability to infect multiple host species and cell types during the course of their usually complex lifestyles, autophagy plays divergent roles even for the same pathogen. The scenario is further compounded since many of the eukaryotic pathogens have their own sets of either complete or partial autophagy machinery. Eukaryotic pathogen-autophagy interplay is thus a complex relationship with many novel insights for the basic understanding of autophagy, and potential for clinical relevance.

Keywords: autophagy, *Plasmodium*, *Toxoplasma*, *Leishmania*, fungi, pathogenesis, host

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INTRODUCTION

Most eukaryotic pathogens are characterized by a wonderfully complicated lifestyle often involving serial infection of multiple host organisms from different orders of life and distinct host cell types within a single host organism. Sequential passage through these very different and diverse host cells is thus a central element of their lifestyle. Consequently, they encounter divergent physiological and cellular environments within a particular host, as well as dramatic shifts in these environments as they alter between these host cell types. In addition, several stages represent major amplification steps where the parasite grows in numbers by several logarithmic fold.

Autophagy is a conserved cell-autonomous catabolic stress response pathway dedicated to the breakdown of cellular material and cell content recycling. Canonical autophagy involves formation of double membrane autophagosomes around the cellular materials to be broken down. The ubiquitin-like machinery, including Atg7 (E1-like), Atg3 (E2-like) and the Atg12-Atg5-Atg16L1

(E3-like) complex brings Atg8 proteins such as LC3 to the autophagosome isolation membrane (Mizushima et al., 2002). Membrane-bound LC3 associates with the cargo via autophagy adaptor proteins on the cargo (Randow, 2011; Gomes and Dikic, 2014). Autophagosome membranes surround the cargo and finally deliver it to lysosomes for destruction (**Figure 1A**).

Apart from its homeostatic role, autophagy is actively involved in the clearance of pathogens. However, the role of autophagy during infection is complex, some pathogens rely on induction of host autophagy to survive within host cells while others are destroyed by it (Gomes and Dikic, 2014). As a result, many pathogens have evolved distinct mechanisms to exploit or subvert these pathways. Consequently, the induction of autophagy during intracellular infection can lead to the capture, breakdown and eventual killing of intracellular pathogens, thereby aiding in their detection by the host cell and subsequent activation of the immune response, e.g., via antigen presentation by professional antigen presenting cells. On the other hand, in the same way that autophagy provides nutrients to host cells during starvation, it has the potential to provide nutrients for intruding pathogens.

A specialized form of autophagy called xenophagy involves recognition of the foreign particle or pathogen by host cell receptors, which initiate autophagosome formation and engulfment of the intruding object by a double membrane autophagosome. Xenophagy, generally, is induced by a pathogen or particle found free within the host cell cytosol, or vacuolar pathogen which expresses a pathogen receptor on vacuole membrane, or pathogen residing inside damaged or perforated vacuole (**Figure 1B**). In all these cases, a double membrane autophagosome engulfs the free pathogen or the vacuole containing the pathogen. Another form of autophagy called LC3-associated phagocytosis (LAP) can also be activated during intracellular infection. This non-canonical form of autophagy involves the recruitment of LC3 and other components of the canonical autophagy pathway to foreign particles that are already contained within a single membraned phagosome or endosome (**Figure 1C**). LAP requires some, but not all of the canonical autophagy machinery. The core PI3KC3 complex involved in nucleation (Beclin 1, Atg14L, VPS34, and VPS15), Atg3, Atg4, Atg7, Atg12, Atg16L are required for LAP while the components involved in initiation including the ULK complex (ULK1/2, Atg13, Atg101, and FIP200), ATG14L, WIPI2, and AMBRA1 are not (Funderburk et al., 2010; Martinez et al., 2015; Heckmann et al., 2017; Schille et al., 2017). LAP also requires the proteins Rubicon and UVRAG which are not required for canonical autophagy (Martinez et al., 2015). The end result of this pathway is the deposition of LC3 on the cytosolic side of the single membrane phagosome membrane, which is thought to lead to faster fusion with lysosomes.

The sub-cellular location of the pathogen and the integrity of the vacuole membrane seems to determine mostly whether a pathogen encounters LAP or xenophagy during host cell infection. While xenophagy occurs against pathogens that have invaded the cytosol of host cells either via invasion from the extracellular space and/or following escape from a phagosome, or reside in phagosomes that are damaged or express pathogen derived receptors on vacuolar membrane, LAP is induced against particles that have actively been taken up by host cells via phagocytosis. A further level of complexity is added by apicomplexan parasites such as *Toxoplasma* and *Malaria* spp. that invade host cells using their own machinery, but reside within membrane-enclosed compartments within host cells that have been co-opted by the parasite from the outer membrane during cellular invasion. These atypical compartments known as parasitophorous vacuoles have membranes derived from the host, but contain parasite derived proteins. Due to this they are not treated by host cells in the same way as a phagosome or autophagosome.

In this review, we will highlight the fascinating aspects of autophagy during intracellular *Toxoplasma* and *Plasmodium* spp growth, development and elimination. We will additionally cover current knowledge of the interplay of host autophagy and several species of the parasite *Leishmania*. We do not review limited literature that suggests host autophagy facilitates *Trypanosoma cruzi* invasion and infection (Romano et al., 2009; Vanrell et al., 2013), and whether autolysosomes form around the parasite is contested (Onizuka et al., 2017). To contrast these eukaryotic parasites, we will discuss another group of eukaryotic pathogens from the fungal kingdom and the induction of LAP against them following phagocytosis by host cells. To our knowledge these organisms are the only eukaryotic pathogens with a substantial amount of literature on their interplay with host autophagy.

AUTOPHAGY AND *Plasmodium*

Parasites of the genus *Plasmodium* cause malaria, a disease that has left indelible imprints on humanity culturally and genetically, while continuing to have devastating impact in terms of mortality and morbidity, leaving lasting social and economic footprints (Carter and Mendis, 2002; Ashley et al., 2018). During their life cycle, the malarial parasite alternates between the mosquito and mammalian hosts.

Host Autophagy During the *Plasmodium* Life Cycle – Mosquito Stages

During the mosquito stages, the malarial parasite undergo dramatic and unique changes. Fertilization of the male and female gametocytes in the gut produces the zygote, which is the only diploid phase of the parasite, followed by ookinete stage, which is the only meiotic stage. The highly motile ookinetes cross the gut lining and develop into oocysts while embedded in the extracellular matrix, resulting in the only extracellular developmental stage. Thousands of sporozoites emerge from the oocysts stage and accumulate in the salivary gland, ready to infect a new mammalian host during the next blood meal (Aly et al., 2009). The role of host response and host cellular processes during these transitions is not well explored, although given the largely extracellular nature of these stages, cell-autonomous mechanisms like autophagy might not have a central role.

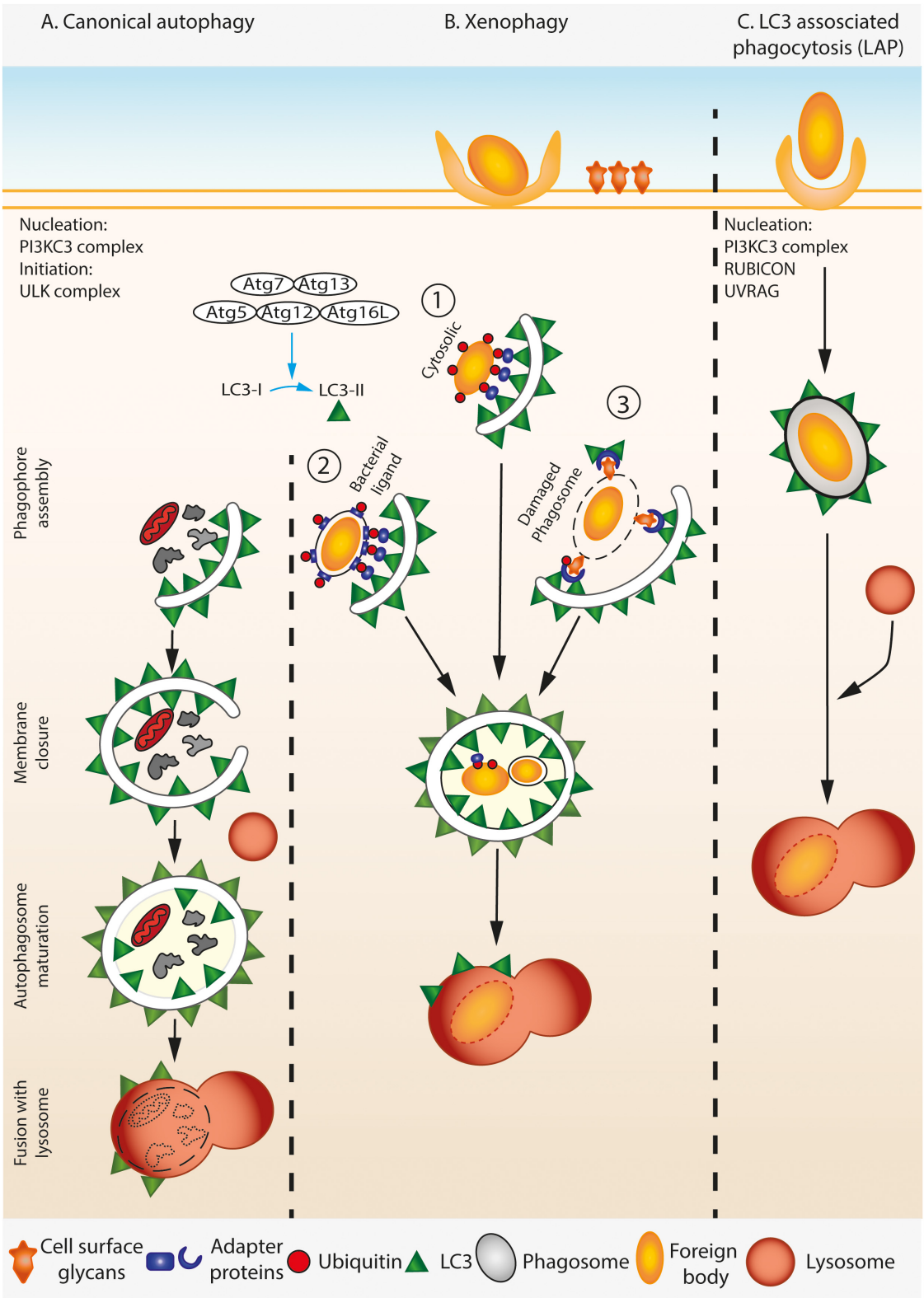


FIGURE 1 | A simplified overview of canonical autophagy, xenophagy, and LAP. **(A)** During canonical autophagy, the nucleation and initiation complexes orchestrate the assembly of phagophore. LC3 is processed and lipidated by covalent conjugation of phosphatidylethanolamine (PE) to its C-terminal Glycine by ATG complex.

(Continued)

FIGURE 1 | Continued
Lipidated LC3 associates with the growing phagophore membrane by inserting the PE. The growing double membrane engulfs the cytosolic contents including damaged organelle (shown here by mitochondria), protein aggregates, etc., trapping them inside when the membranes seal from the growing ends. The resulting mature autophagosome is double membraned and is marked by LC3-II on both its inner and outer membranes. Mature autophagosome fuse with lysosomes, and the cargo is degraded by the acidic environment of the lysosome. **(B)** During xenophagy, foreign particles in the cell such as invading pathogens are specifically identified by the autophagy machinery. Both cytosolic **(1)** and vacuolar pathogens **(2)** that display a pathogen specific ligand are ubiquitylated and bind to distinct adaptor proteins that can recruit LC3, thereby targeting the pathogen to the autophagosome. Alternatively, host cell can infer the presence of pathogen by detecting the usually cell-surface localized glycans on damaged phagosomal membranes in a ubiquitin dependent manner **(3)**, thus marking them as target for the downstream autophagy machinery. **(C)** Lysosomal associated phagocytosis (LAP) involves association of lipidated LC3 on the cytosolic leaflet of the single membrane phagosome that contains the pathogen. Consequently, LAP does not require some components of the autophagy initiation complex, but needs components of the nucleation complex.

Host Autophagy During the *Plasmodium* Life Cycle – Mammalian Stages

When an infected mosquito bites a mammalian host for its blood meal, it injects the infective sporozoites into the skin, from where they home into the liver and infect hepatocytes. Within these cells, the parasites undergo an amplification stage of over 10,000-fold to form merozoites, which egress from the hepatocytes and infect red blood cells. Thus, within mammalian hosts, the parasite encounters two distinct cell types, hepatocytes in the liver and red blood cells in the blood.

Host Autophagy During the *Plasmodium* Life Cycle – Red Blood Stages

The red blood cell is devoid of organelles and autophagy processes do not exist. While *Plasmodium falciparum* and *Plasmodium berghei* invade mature red blood cells, other *Plasmodium spp* (notably *Plasmodium vivax* and *Plasmodium yoelii*) invade immature reticulocytes, sequester in the bone marrow (Thomson-Luque and Scopel, 2015) and remodel the reticulocytes. Reticulocyte remodeling, independent of *Plasmodium* infection, is a critical homeostatic process during hematopoiesis, where autophagy plays a key role (Ney, 2011; Griffiths et al., 2012; Mankelow et al., 2016). Defective autophagy during this step results in strong phenotypes such as severe anemia (Mortensen et al., 2010; Mankelow et al., 2016). Interestingly, it has been proposed that *P. vivax* infection triggers, remodels and indeed accelerates the maturation of immature CD71 positive reticulocytes (Malleret et al., 2015) into CD71 negative red blood cells. Hence it is tempting to speculate that *P. vivax* infection could significantly modulate the host cell autophagy during the infection of immature reticulocytes. However, little information is currently available, largely due to the notorious experimental refractoriness of *P. vivax*.

Host Autophagy During the *Plasmodium* Life Cycle – Liver Stages

Unlike the mosquito and blood stages, the autophagy machinery of hepatocytes plays a central role in the development of the parasite during the liver stages (Agop-Nersesian et al., 2018). Consequently, the interplay of the host autophagy machinery with the malarial parasite during the liver stage development is an active area of investigation. In this review, we will focus on the role of the hepatocyte autophagy machinery during *Plasmodium* liver stage development.

The *Plasmodium* parasite, during their development within the hepatocytes, is shielded from the host cytosolic defense mechanisms by the parasite vacuole membrane (PVM). The PVM is originally derived from the host cell plasma membrane, but is extensively modified by the parasite, which inserts its proteins to this membrane (Meis et al., 1983; Lingelbach and Joiner, 1998; Nyboer et al., 2017). Some of these proteins are therefore likely to directly interact with cytosolic defense mechanisms and subvert them. Although a handful of such proteins have been characterized, molecular functions have been ascribed to only a few of them. Most interestingly, mutants for some of the proteins such as UIS3, UIS4 result in growth arrest of the parasite (Mueller et al., 2005), suggesting their essential function during the liver stage.

Recent years have seen rapid advancement in the knowledge on the interaction of liver stage *Plasmodium* with the host cell autophagy machinery. While the liver stage has been traditionally termed as the "silent stage" of malaria, it is now becoming clear that the host cell indeed senses the parasite and responds accordingly. In fact, many parasites are eliminated by the host cell defense mechanism during *Plasmodium* liver stage development, with autophagy playing a key role (Schmuckli-Maurer et al., 2017). Induction of canonical non-selective autophagy supports parasite development in hepatocytes, as starvation or Rapamycin treatment resulted in an increase in the number of liver stage parasites (Prado et al., 2015; Zhao et al., 2016). Similarly, parasite development is affected by genetic abrogation of the host autophagy machinery (Prado et al., 2015; Wacker et al., 2017), although there could be cell type dependency due to intrinsic differences between hepatoma cells and HeLa cells used in the different experiments (Schmuckli-Maurer et al., 2017). While this has led to a discussion on whether host autophagy is a friend or foe during liver stage infection (Coppens, 2017), an emerging view is that the liver stage *Plasmodium* development could represent a non-canonical form of autophagy, recently termed *Plasmodium* Associated Autophagic-like Response (PAAR; Coppens, 2017; Wacker et al., 2017; Agop-Nersesian et al., 2018).

The molecular mechanisms of the role of host autophagy during liver stage *Plasmodium* infection are being unraveled (**Figure 2**). A hallmark of *Plasmodium* development in the liver stage is the rapid acquisition of LC3, as well as its binding proteins p62, NBR1, NDP52, along with ubiquitin on the PVM (Schmuckli-Maurer et al., 2017). This suggests that either the

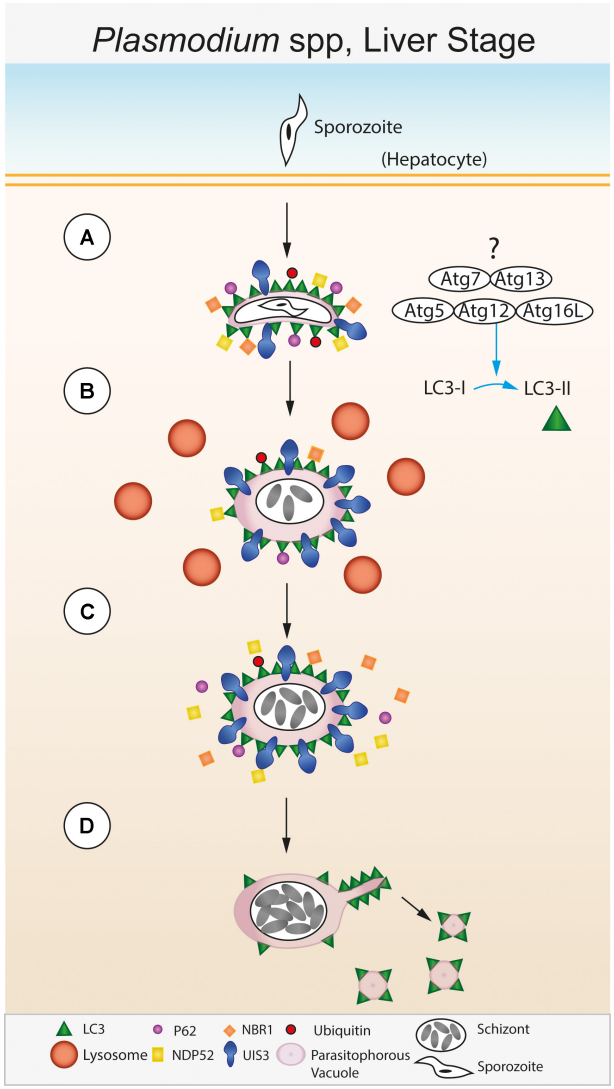


FIGURE 2 | Autophagic control of the liver stage of *Plasmodium* spp. **(A)** The sporozoite stage of the *Plasmodium* parasite invades a hepatocyte within the liver. Following invasion, the parasite resides within a membrane bound parasitophorous vacuole (PV) within the host cell cytosol (Meis et al., 1983; Lingelbach and Joiner, 1998; Nyboer et al., 2017). The PV membrane (PVM) is recognized immediately after infection by the host. Lipidated LC3 is deposited onto the PVM followed by recruitment of host effector proteins including p62, NDP52, NBR1, and ubiquitin (Schmuckli-Maurer et al., 2017). **(B)** The *Plasmodium* PVM resident protein UIS3 sequesters LC3 at the PVM surface and prevents further p62/NDP52/NBR1/ubiquitin binding (Real et al., 2018). Meanwhile, the PVM is surrounded by lysosomes, however, lysosomal fusion and acidification of the PVM compartment does not occur. **(C)** This step is required for parasite development within the host cell and leads to further schizont replication. **(D)** During late stage parasite development, LC3 is shed from the PVM via sequestration by the *Plasmodium* tubo-vesicular network (TVN) and subsequent scission from the PVM (Agop-Nersesian et al., 2017).

parasite is readily recognized by the host or the parasite sequesters and hijacks the host autophagy machinery. There are several striking aspects of the association of LC3 with the PVM that renders it distinct compared to other known forms of autophagy. First, the LC3 decoration on the PVM does not involve the formation of new canonical double membrane autophagosomes, rather LC3 associates with the existing PVM. This is distinct from LAP since sporozoites invade host cells by an active mechanism different from conventional phagocytosis, and the PVM while surrounded by lysosomes does not readily fuse with lysosomes and become acidic, as is the case in LAP. Second, the association of the LC3-binding proteins, including ubiquitin, to the PVM is to a large extent directly mediated by LC3 (Schmuckli-Maurer et al., 2017). This is in contrast to canonical xenophagy, where LC3 recruitment on pathogen vacuole membrane is subsequent to their recognition by receptors. The order of recruitment of LC3 binding proteins to the PVM appears reversed in case of *Plasmodium* liver stage infection, leading to the idea of an "inverted" recruitment of LC3 associated proteins on the PVM (Schmuckli-Maurer et al., 2017). Third, the association of LC3 itself with the PVM is temporary (Prado et al., 2015), with LC3 dissociating from the PVM during the later stages of parasite development. Both recruitment of LC3 onto the PVM at an early time point post-infection (as early as a few minutes) and the disappearance of LC3 from the PVM at later time points (after 40 h) are necessary for proper parasite development (Prado et al., 2015; Agop-Nersesian et al., 2017). Fourth, LC3 recruitment to the PVM is dependent on lipidation of LC3 (Prado et al., 2015), suggesting that the LC3 conjugation machinery involving upstream ATGs such as ATG5 are actively involved in the process. However, initiation complexes of autophagy such as FIP200 are not required (Wacker et al., 2017).

The factors that trigger the LC3 conjugation system upon *Plasmodium* infection and how lipidated LC3 is recruited to the PVM are not clear. However, recent evidence suggests that the parasite protein UIS3 directly binds to and retains LC3 on the PVM (Real et al., 2018). Multiple lines of evidence attest to the role of *Plasmodium* UIS3 in intersecting with the host autophagy machinery by interacting with LC3: first, while *uis3(-)* parasites are arrested in development in wild-type hepatocytes, they develop normally in ATG5$^{-/-}$ MEFs, arguing strongly for a central role for UIS3 in interaction with host autophagy. Second, exogenously expressed UIS3 interacts with LC3 in HeLa cells, which is confirmed by direct *in vitro* interaction of purified recombinant LC3 and UIS3. Third, by modeling the LC3-UIS3 interaction interface, critical residues were identified on UIS3 that were important for binding to LC3. Mutant UIS3 where these residues, singly or in combination, are mutated to alanine, do not show binding to LC3. Interestingly, the residues on UIS3 do not conform to a conventional LIR motif, suggesting a non-canonical interaction (Real et al., 2018). An emerging view is that UIS3, by sequestering LC3 onto the PVM, blocks LC3 binding to its other target proteins, resulting in an inhibitory effect on the host autophagy machinery. Evidence for this comes from the reduced p62 degradation observed in UIS3 transfected HeLa cells (Real et al., 2018). However, since LC3 interacting proteins such as p62, NDP52 also bind to the PVM by binding to LC3, it might be possible that the UIS3 mediated inhibitory effect is either incomplete or occurs after the first wave of LC3 targeting and its associated proteins have already bound to the PVM. The

specificity and non-canonical nature of LC3-UIS3 interaction and the essentiality of UIS3 for parasite development raises the exciting possibility of exploring small molecule disruptors of this protein-protein interaction to target liver stage development. Direct structural information on the UIS3-LC3 interface will be crucial for such studies.

LC3 dissociating from the PVM is necessary for late stage parasite development (Prado et al., 2015; Agop-Nersesian et al., 2017). An interesting "exit" mechanism has been proposed by Heussler and colleagues using elegant live cell imaging experiments, wherein the tubo-vesicular network (TVN) surrounding the PVM siphons off LC3 from the expanding PVM and sheds it into the host cell cytoplasm as vesicles (Agop-Nersesian et al., 2017). This interesting observation raises several exciting questions. What is the fate of the interacting UIS3 during this step? How is the flow of membrane from PVM to TVN regulated while the PVM itself is actively expanding? What are the roles of the host cytoskeletal elements, including the acto-myosin complex, if any, in this process? What mechanisms ensure and regulate sufficient forces and membrane tension for such a sequestration effect? What are the mechanisms involved in scission of the vesicles from TVN, what prevents the "backflow" of LC3 from TVN to PVM? What prevents the re-recruitment of LC3 to PVM? These ongoing studies from multiple laboratories have thus opened up several new and exciting lines of enquiry (**Box 1**).

Most of the results listed above come from studies using murine malarial parasites, notably *P. berghei*. It will be important to address the relevance of these findings during the liver stage infections of human malarial parasites, *P. falciparum* and *P. vivax*. Boonhok et al assessed the effect of IFNγ treatment in hepatocytes during *P. vivax* infections (Boonhok et al., 2016), and identified a LAP-like process that kills *P. vivax* upon stimulation with IFNγ. This process involves autophagy nucleation factors like ATG5, Beclin1, but not the initiation factor ULK1 (Boonhok et al., 2016), consistent with the observations from *P. berghei*. While this study highlights the involvement of IFNγ in *P. vivax* liver stage development, the role of basal autophagy, autophagy components and PAAR like response remain to be elucidated. Given the unique preference of *P. vivax* for dormancy during its liver stages development (Markus, 1980; Krotoski et al., 1982), it is particularly tempting to speculate if there could be differential recruitment of selective host autophagy components between the actively growing schizont and the dormant hypnozoite forms.

Exciting new concepts have emerged in the recent past on the interaction of *Plasmodium* spp with host cell autophagy machinery during the liver stages. Several unusual features define this interaction. They include the necessary, but transient recruitment of LC3 and its binding proteins to the PVM, the "inverted" nature of this recruitment, with LC3 binding to PVM preceding that of its binding proteins, the inhibitory effect of a parasite protein UIS3 on host autophagy machinery via its non-canonical interaction with LC3 and the interesting "exit" mechanism of LC3 from PVM. These observations have opened up new avenues in this rapidly expanding area of research. The relevance of these concepts to human malarial parasites *P. falciparum* and *P. vivax*, and the potential of this interaction for drug discovery make this a particularly exciting if challenging area for future research.

BOX 1 | A collection of unanswered questions concerning each pathogen discussed in this review.

***Plasmodium* Spp. Liver Stage**

- What are the triggers of the autophagy nucleation machinery and LC3 lipidation upon *Plasmodium* infection?
- Why is dissociation of LC3 required for late stage parasite development? Is it coupled to other processes happening at that time, such as merosome formation?
- What role does host autophagy play in human malarial infections? Does it have a role in hypnozoite biology of *P. vivax*?

Toxoplasma gondii

- Why is autophagic control of *Toxoplasma* seemingly different in murine and human cells?
- Do all human cells control *Toxoplasma* via the same autophagic mechanism?
- How do Atg proteins control the recruitment of IRGs and GBPs to the parasite vacuole in murine cells?

***Leishmania* Spp.**

- What is the mechanism of nutrient acquisition by *Leishmania* via autophagy?
- How does autophagy lead to *Leishmania*-specific T cell attenuation?

Fungal Spp. (*Cryptococcus neoformans, Candida albicans* and *Aspergillus fumigatus*)

- What are the defining characteristics of LC3-associated phagocytosis (LAP) aside from LC3 recruitment to the phagosome?
- Does LC3 recruitment to the phagosome during fungal infection always induce LAP?
- Why do some fungal pathogens appear to be more susceptible to LAP than others?
  - Do fungal pathogen induce species-dependent variations of LAP within host cells or have the pathogens evolved to subvert LAP in different ways?
- What is the opsonic receptor responsible for LC3 recruitment to *C. neoformans*?
  - Is Syk activation/reactive oxygen species generations still required to induce LAP following opsonic phagocytosis?

AUTOPHAGY AND *Toxoplasma gondii*

Toxoplasma gondii, like *Plasmodium*, is an apicomplexan parasite that leads a ubiquitous intracellular life. The sexual stage of *Toxoplasma's* life cycle is confined to the feline, while the asexual stage is promiscuously found in all warm-blooded animals. Due to this trait and the fact that *Toxoplasma* establishes a chronic infection in brain and muscle tissue, it can arguably be considered the most successful parasite on the planet with human infection rates of 30% (Torgerson and Mastroiacovo, 2013). *Toxoplasma* infection in immunocompetent people is mostly asymptomatic but can lead to ocular disease when infected with certain parasite strains. Immunocompromised individuals and neonates are also at risk of severe health problems and death (Hill and Dubey,

2002). In North America and Europe, *Toxoplasma* is mostly present as one of three classical strains, types I, II, and III, while an expansion of strain diversity has occurred in South America (Ajzenberg et al., 2004).

CD40-Induced Autophagic Host Control of *Toxoplasma gondii*

Autophagic control of *T. gondii* requires stimulation of the host cells. Almost 20 years ago, it was found that CD40 ligand deficient mice are unable to control *in vivo* replication of *Toxoplasma* in the brain (Reichmann et al., 2000). CD40 activation also controls parasite growth in peripheral tissues during the acute phase of infection (Subauste and Wessendarp, 2006), as well as cerebral and ocular toxoplasmosis (Portillo et al., 2010). However, the predominance of its role, alongside the IFNγ-induced autophagic pathway (see below), in controlling *Toxoplasma* in murine macrophages *ex vivo* has been questioned (Zhao et al., 2007). CD40 ligation was recognized to induce the autophagic clearance of the parasite (Andrade et al., 2006). To date, this mechanism is mostly studied in murine macrophages and probably exists in non-hematopoietic murine cells (Van Grol et al., 2013) and in human macrophages (Andrade et al., 2006).

Presumably following a canonical autophagy route, upon CD40 ligation, LC3 localizes around the *Toxoplasma* parasitophorous vacuole (PV) within 6 h as well as the late endo-lysosomal markers LAMP1 and Rab7 (**Figure 3A**). This suggests that CD40 ligation directs the PV to fuse with endo-lysosomal compartments (Andrade et al., 2006). Importantly, the PVM seems to stays intact throughout this process, however, more detailed investigation is required to confirm this hypothesis. CD40 ligation to combat *Toxoplasma* requires synergy with TNFα (Andrade et al., 2005). CD40 recruits TRAF6 to an intracellular binding site serving two purposes: to enhance autocrine production of TNFα (Mukundan et al., 2005) and to engage TRAF6 signaling downstream of CD40 by synergizing with TNFα to activate autophagy (Subauste et al., 2007) (**Figure 3A**). Resultingly, Beclin1 and ULK1 synergistically signal to promote autophagic clearance of *Toxoplasma* (Liu et al., 2016).

Toxoplasma has to maintain the non-fusogenic nature of the PV to ensure tachyzoite survival. The following proposed mechanism was studied in many cell types including human brain endothelial cells, retinal cells, as well as mouse endothelial cells, microglial cells and macrophages (Muniz-Feliciano et al., 2013). *Toxoplasma* type I and II activate EGFR-Akt signaling in host cells, preventing the targeting of the PVM by the autophagy protein LC3 and thus avoiding Beclin1- and Atg7-dependent autophagic clearance (Muniz-Feliciano et al., 2013) (**Figure 3A**). Phosphorylation of Akt increases with live parasite infection in an IFNγ-independent manner. Two parasite microneme (MIC) proteins containing EGF domains, MIC3, and MIC6, are important contributors to this process (Muniz-Feliciano et al., 2013). Another study recently proposed that in a second mechanism also active in non-CD40 activated cells, *Toxoplasma* invasion activates a focal adhesion kinase (FAK)-Src-EGFR transactivation to STAT3 pathway, which inhibits autophagosome formation and thus *Toxoplasma* killing

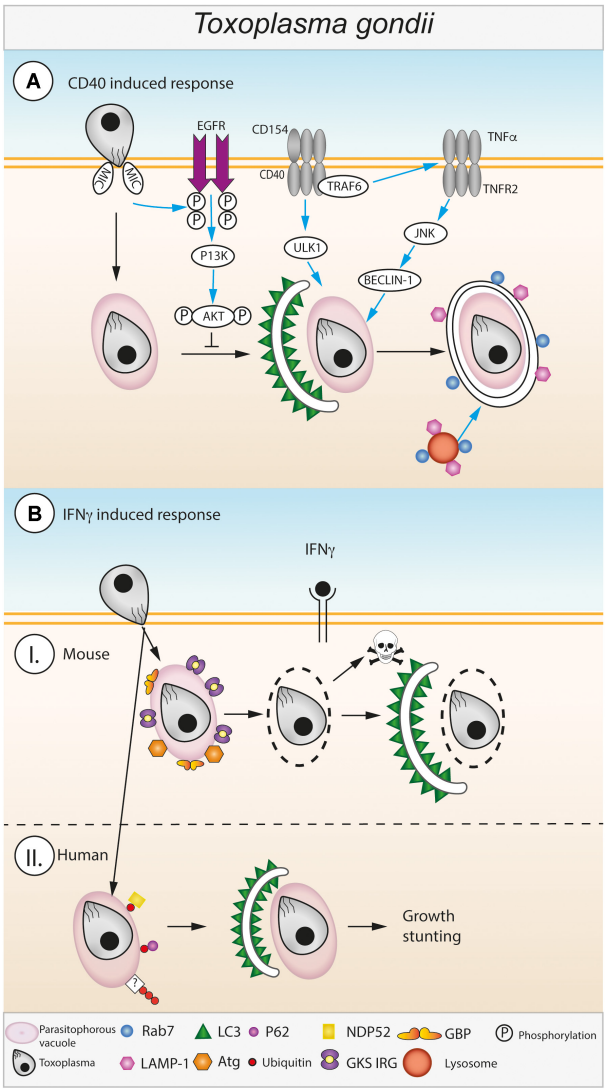


FIGURE 3 | Autophagic control of *Toxoplasma gondii*. **(A)** CD40 induced autophagy during *Toxoplasma* infection. *Toxoplasma* enters into host macrophages via an active invasion process, the parasite resides in the host cytosol within a parasitophorous vacuole (PV). Host autophagy pathways are induced against *Toxoplasma* via interactions between CD40 expressed on the cell surface of infected macrophages and $CD4^+$ T cells expressing CD154 (Andrade et al., 2006). CD40/CD154 ligation leads to recruitment of TRAF6 to CD40 which triggers increased TNFα secretion (Mukundan et al., 2005; Subauste et al., 2007). CD40 mediated ULK1 activation and TNFR2 mediated JNK/Beclin-1 activation leads to the formation of a double membraned autophagosome around the *Toxoplasma* PV (Andrade et al., 2005). The host's autophagic response is actively inhibited by the *Toxoplasma* derived protein MIC which activates host EGFR which in turn activates PI3K leading to activation of the autophagy suppressor protein AKT (Muniz-Feliciano et al., 2013). *Toxoplasma* is destroyed by recruitment of Rab7 and LAMP1 and the subsequent fusion the PV with lysosomes (Andrade et al., 2006). The pathway depicted to the left of the dashed line is found in unstimulated and CD40-stimulated cells. **(B)** IFNγ induced autophagy during *Toxoplasma* infection. **(I)** In mouse cells stimulated with IFNγ the *Toxoplasma* PVM is disrupted by recruitment of GKS-motif containing Immunity Related GTPases (GKS IRG) and Guanylate Binding Proteins (GBPs) to the outer surface of the PVM (Degrandi et al., 2007; Virreira Winter et al., 2011;

(Continued)

FIGURE 3 | Continued
Yamamoto et al., 2012; Selleck et al., 2013). Disruption of the PV leaves the parasite exposed to attack by host autophagy pathways, characteristic autophagosomes (double membrane, LC3 decorated) form leading to destruction and digestion of the parasite (Ling et al., 2006). **(II)** In human cells, the mechanisms responsible for IFNγ mediated destruction of *Toxoplasma* via autophagy are less well-known. Instead of disrupting the PVM, human cells target it with ubiquitination which leads to the subsequent recruitment of ubiquitin binding proteins, e.g., p62 and NDP52. Recruitment of p62 and NDP52 leads to autophagosome formation around the PV via an unknown process which leads to restriction of parasite growth within the cell (Selleck et al., 2015; Clough et al., 2016).

(Portillo et al., 2017). In line with these findings, another study reported that Gefitinib, an EGFR inhibitor, decreased parasite replication in HeLa cells (Yang et al., 2014).

IFNγ-Induced Autophagic Host Control of *Toxoplasma gondii*

A common theme and sometimes prerequisite in autophagic control of intracellular, vacuolated pathogens is the exposure of the pathogen to the cytoplasm. This can either happen spontaneously, such as for *Salmonella typhimurium*, or be driven by host defense proteins, for example for *Chlamydia* and *T. gondii*. Gamma interferon is central to upregulating the expression of host GTPases, the Immunity Related GTPases (IRGs) and Guanylate Binding Proteins (GBPs), both responsible for disrupting pathogen vacuoles by a yet undetermined mechanism (Degrandi et al., 2007; Virreira Winter et al., 2011; Yamamoto et al., 2012; Selleck et al., 2013). *Toxoplasma* then dies in the cytoplasm and is potentially cleared by canonical host cell autophagy, a striking ultrastructural observation now made well over 10 years ago (Ling et al., 2006) (**Figure 3B**). Here, a dependence on the IRG Irgm3 was observed, which localizes to the autophagosomal membranes enveloping the naked parasite (Ling et al., 2006). Another report at that time found LC3 in close vicinity to the PV, suggesting a similar role for autophagy in tachyzoite elimination (Martens et al., 2005).

A hint that the story would not be straightforward arrived with the observation that Atg5 restricted *Toxoplasma* in murine macrophages, but that the PVs were not uniformly acidic in the form of LAMP1 positivity (Zhao et al., 2008). It is now clear that autophagy proteins including the E3-like autophagy complex localize to and recruit host IRGs and mGBPs to the PVM (Zhao et al., 2008; Khaminets et al., 2010; Choi et al., 2014; Park et al., 2016) (**Figure 3B**). For example, Atg5 is essential for the recruitment of Irga6 and Irgb6 to the PV in mouse macrophages, fibroblasts and granulocytes (Zhao et al., 2008; Khaminets et al., 2010). In the absence of Atg5, Irga6, Irgb6 and Irgd aggregated in the host cytoplasm (Zhao et al., 2008; Khaminets et al., 2010). Irgb6 and mGBPs are recruited to the PV in dependence of Atg7 and Atg16L1, yet with Atg9a and Atg14 being dispensable (Ohshima et al., 2014). Similarly, Atg3 is necessary for loading of IRGs and mGBP2 (and possibly other GBPs) onto the PVM and control of *Toxoplasma* infection (Choi et al., 2014; Haldar et al., 2014). Even though the mechanism is unclear, these Atg proteins appear to activate the GTPases, as it was found that a GTP-locked, constitutively active, IRG protein mutant could overcome the targeting defect in Atg3 and Atg5 deficient cells (Haldar et al., 2014). Equally, depletion of all LC3 homologs including GABARAP, GABARAPL1, and GABARAPL2 (GATE-16), led to decreased targeting of the IRGs to the PVM (Park et al., 2016). Relocating the Atg12-Atg5-Atg16L1 complex that marks the LC3 conjugation site onto alternate target membranes led to the host GTPases accumulating at the new target membranes rather than the PVM (Park et al., 2016).

In terms of direct localization of Atg proteins, the Atg12-Atg5-Atg16L1 complex has been postulated to target to the PVM using effector proteins that link phosphoinositides to the Atg complex (Park et al., 2016) (**Figure 3B**). Alternatively, the PVM may be recognized by "missing self," similarly described for GMS IRGs (Haldar et al., 2013; Maric-Biresev et al., 2016; Park et al., 2016). Regardless, currently the factors governing the initial recruitment of Atg proteins to the PVM are unclear. In summary, it is clear, however, that this early involvement of Atgs does not lead to canonical autophagy, since the Atg proteins do not promote the formation of an isolation membrane at the PVM prior to PV breakage (Martens et al., 2005; Ling et al., 2006). Autophagy Atg proteins thus serve a non-canonical autophagy function in *Toxoplasma* control in their capacity to promote recruitment of host GTPases to the PVM. After PVM destruction, the observation of autophagic membranes around the exposed parasite implies their participation in a classical autophagic role or alternatively a LAP-like clearance of the *Toxoplasma* PV.

Interestingly, in humans, no role for the IFN-stimulated IRGs in *Toxoplasma* control has been documented and thus far, the PVM has never been observed as disrupted. This is possibly a consequence of the human genome containing only two IRGs, both non-interferon inducible, IRGC, which is testis specific and IRGM (Bekpen et al., 2005). Humans do possess 7 IFNγ-inducible GBPs. Human GBP1-5 and hGBP1 recruit to *Toxoplasma* in HAP1 and mesenchymal stromal cells, respectively (Ohshima et al., 2014; Qin et al., 2017). However, no recruitment of hGBP1 to the *Toxoplasma* PVM was found in A549 cells (Johnston et al., 2016). Thus, either absence of IRG protein targeting to the *Toxoplasma* PV protects its rupture, or the cell type or circumstance where this may happen has not been found.

Autophagy proteins do play a role in *Toxoplasma* infection of the human epithelial HeLa cell line (Selleck et al., 2015; Clough et al., 2016) (**Figure 3B**). Ablating Atg16L1 and Atg7 resulted in increased parasite replication. This again was described as non-canonical autophagy, as it did not lead to lysosomal fusion, with no evidence for LAMP1 staining. Instead, parasites were growth-restricted by an unknown mechanism involving recruited LC3B and membranes to the type II and III PV (Selleck et al., 2015) (**Figure 3B**). Other studies demonstrated that the key autophagy mediators Atg5 and Atg16L1are not required for parasite restriction in human foreskin fibroblast (HFF) and HAP1 cells, respectively (Niedelman et al., 2013; Ohshima et al., 2014). Again, this may be a cell-type specific difference in human *Toxoplasma* restriction. A common theme between human epithelial and endothelial cell types seems to be

ubiquitin recognition of type II and III PVs (Selleck et al., 2015; Clough et al., 2016). Ubiquitin recognition is the prerequisite to parasite destruction, a process that involves the autophagy adaptor proteins p62 and NDP52, but again, no obvious PVM disruption (Selleck et al., 2015; Clough et al., 2016). Interestingly, minimal recognition by galectin 8 was found in an IFNγ and type II parasite specific manner, potentially indicating a slight permeability of the PVM (Clough et al., 2016).

Much progress has been made to elucidate how autophagy can restrict *Toxoplasma* in murine cells, with some understanding how this pathway operates in human cells. It has become clear that there are differences in pathways depending on organism infected, cell type under study and *Toxoplasma* strain. It will be critical to unravel these differences, as well as understand their importance during human infection (**Box 1**). For example, CD40 ligation has been suggested to restore IFNγ and IL12 production *ex vivo* in patients with hyper IgM syndrome, possibly linking some of the discussed pathways (Subauste et al., 1999). Conversely, studies have also pointed out that *Toxoplasma* can benefit from autophagic degradation as a means to provide nutrients (Wang et al., 2009; Pernas et al., 2018). Further studies will be needed to address how the autophagy-*Toxoplasma* interplay is balanced.

AUTOPHAGY AND *Leishmania*

Leishmania spp. are protozoan parasites that cause a variety of diseases in humans ranging from cutaneous lesions to visceral leishmaniasis. *Leishmania* is ranked second in mortality to malaria among parasitic infections and is primarily found in tropical and subtropical countries (GBD 2015 DALYs and HALE Collaborators, 2016). *Leishmania* invades macrophages in the dermis (Liu and Uzonna, 2012). The promastigote stage at that point evolves into the amastigote stage in the phagolysosome. Thus, *Leishmania* has developed ways to block phagolysosomal maturation in order to survive (Kaye and Scott, 2011). Amastigotes multiply and disseminate to the reticulo-endothelial system through the lymphatic system, then infiltrating macrophages in the bone marrow. Autophagy could thus benefit the parasite by providing nutrients or play a role in pathogen defense.

Several *Leishmania* species have been found to induce autophagy. This is thought to be a means for the parasite to acquire critical nutrients. *Leishmania infantum* disease severity seems to be associated with upregulation of the autophagy genes Atg7 and LC3, as well as the LAP-like accumulation of LC3 around the parasite vacuoles (Esch et al., 2015). Increased *Leishmania amazonensis* parasite burden could be found in Balb/c mice after the induction of autophagy (Pinheiro et al., 2009) and the parasite has itself been found to induce autophagy in macrophages, concurrent with an increased infection index after inhibiting autophagy with 3-methyladenine (Cyrino et al., 2012). Patient data from a *Leishmania donovani*-infected individual showed induction of autophagy by LC3 conversion from the patient's bone marrow samples (Mitroulis et al., 2009). Direct acquisition of macromolecules has been demonstrated for *Leishmania mexicana* via an autophagy-sensitive pathway (Schaible et al., 1999).

Induction of autophagy by *Leishmania* can be a means to attenuate T cell responses against the parasite. Single bilayers positive of LC3 seem to surround apoptotic *Leishmania major* with the consequence of dampening the parasite-directed CD4 T cell response (Crauwels et al., 2015). Reducing T cell exhaustion by blocking PD1-L signaling inhibited autophagy and reduced *Leishmania donovani* burden (Habib et al., 2018).

Only one report has seemingly observed autophagy as a mechanism for *Leishmania* destruction. *L. major* was found to increase the presence of autophagosomes, vacuoles and myelin-like structures, concurrent with the clearance of amastigotes (Frank et al., 2015). Clearly more mechanistic work is needed to elucidate the exact model of interaction between *Leishmania* species and the host autophagy machinery (**Box 1**).

AUTOPHAGY AND FUNGAL PATHOGENS

Invasive fungal infections cause around 1.5 million deaths per year, the majority of deaths are due to just three fungal species – *Cryptococcus neoformans, Candida albicans,* and *Aspergillus fumigatus* (Brown et al., 2012). All three species pose a significant risk to individuals who have become immunocompromised, e.g., via HIV AIDS, hematological malignancies, major physical trauma or immune suppression therapy for solid organ transplant. *C. neoformans* and *A. fumigatus* are environmental fungi that can cause respiratory infection following inhalation of infectious spores, *A. fumigatus* remains within the lungs where it causes severe inflammation and tissue damage (van de Veerdonk et al., 2017), whereas *C. neoformans* disseminates to the central nervous system where it can cause fungal meningitis (Ma and May, 2009; Evans and May, 2014; Gibson and Johnston, 2015). *C. albicans* is a commensal organism that can opportunistically outgrow its niche in the intestinal tract, oral cavity or vaginal cavity if an individual is immunocompromised. Fatal candidiasis occurs when *C. albicans* invades epithelial barriers and enters the bloodstream resulting in sepsis (Mayer et al., 2013). Initially, *C. neoformans* resists the intracellular killing within the macrophage and is able to proliferate within mature phagosomes. Infected macrophages require a $CD4^+$ Th1 helper cell mediated adaptive immune response to control intracellular infection (Kawakami et al., 1995; Voelz et al., 2009). Like *C. neoformans, C. albicans* is able to survive within the macrophage phagosome, however, *C. albicans* is able to form hyphae, which disrupt host cell membranes leading to the escape of the fungus (Mayer et al., 2013). *A. fumigatus* spores or conidia are inhaled into the alveolar space, where alveolar macrophages initially phagocytose and kill conidia (van de Veerdonk et al., 2017).

LC3-Associated Phagocytosis (LAP)

Cryptococcus neoformans, C. albicans, and *A. fumigatus* are targeted by host autophagy proteins during infection and remain within single membrane phagosomes throughout. As previously discussed LAP requires some, but not all of

the canonical autophagy machinery. LAP is triggered by host cell pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) unique to the pathogen. PI(3)P is deposited on the phagosome membrane by the PI3KC3/Rubicon/UVRAG complex, this recruits NADPH oxidase and NOX2 to the phagosome resulting in production of reactive oxygen species (ROS) which attracts the LC3 conjugation complexes Atg7-Atg3 and Atg5-Atg12-Atg16L, LC3 as well as Atg3 and Atg4. The end result of LAP is the lipidation of LC3-I into LC3-II, which is attached to the phagosome membrane to form a structure called the LAPosome. LAPosomes are able to fuse with lysosomes leading to phagosome maturation and destruction of the pathogen (**Figure 4**). Following destruction, the pathogen is digested, it is then possible for components of the pathogen to be passed to endosomal PRRs such as TLR2 and TLR7 or processed for antigens that can be presented on MHC-II complexes for antigen presentation.

Recognition of Fungal Pathogens During LAP

LC3-associated phagocytosis mediated deposition of LC3 onto phagosome membranes can be induced by toll-like receptor (TLR) activation, but in the context of fungal infection the C type lectin receptor Dectin-1 can also mediate LAP. Dectin-1 is a cell surface PRR expressed mainly on myeloid cells that recognizes β-1,3-glucan – a polysaccharide found in the fungal cell wall (Brown and Gordon, 2001; Brown et al., 2002; Brown, 2006). Genetic mutations in Dectin-1 are known to increase susceptibility to *C. albicans* and *A. fumigatus* (Marakalala et al., 2011). Ligand binding to Dectin-1 leads to phosphorylation of an ITAM located on the cytoplasmic tail of the receptor. This subsequently recruits and activates spleen tyrosine kinase (SyK) which activates NADPH oxidase leading to production of ROS in the phagosome (Gantner et al., 2003) (**Figure 4**). Dectin-1 activation is required for LC3 recruitment to phagosomes containing *C. albicans* and *A. fumigatus* within infected macrophages (Ma et al., 2012; Kyrmizi et al., 2013). Dectin-1 mediated LC3 recruitment is Syk dependent and relies on ROS generation by NADPH oxidase (Ma et al., 2012; Kyrmizi et al., 2013).

The PPR that leads to LC3 recruitment to *C. neoformans* phagosomes is still not known. Nicola et al. report that only antibody-opsonized *C. neoformans* recruit LC3 (Nicola et al., 2012), however, LC3 recruitment to phagosomes containing unopsonized *C. neoformans* cells has been reported by Qin et al. (2011). This suggests that Dectin-1 activation may not be fully responsible for mediating LC3 recruitment to phagosomes containing *C. neoformans*. Unopsonised *C. neoformans* cells are very poorly phagocytosed by host macrophages (Evans et al., 2015; Bojarczuk et al., 2016; Lim et al., 2018), due to the polysaccharide capsule produced by *C. neoformans* during infection that can hide β-1,3-glucan from Dectin-1. It is possible that a host recognition receptor other than Dectin-1 is responsible for LAP induction against *C. neoformans*, in this respect Fc-receptor activation has been shown to induce LC3 recruitment to phagosomes (Huang et al., 2009). Interestingly, recent research by Lim et al. shows that although unopsonised *Cryptococcus* cells are poorly phagocytosed by macrophages, the phagocytosis that does occur is Syk-dependent and can be blocked with the Syk inhibitor piceatannol. Furthermore, Syk activation was found to localize to an area around phagocytic cup formation during phagocytosis and the uptake of non-opsonized *Cryptococcus* cells could be blocked by pharmacological or genetic ablation of Dectin-1 (Lim et al., 2018). This suggests that Dectin-1 activation is seen during macrophage recognition of *C. neoformans,* but further work must be performed in order to explore whether this leads to LC3 recruitment.

Recruitment of LC3 to Phagosomes Containing Fungi

One of the defining features of LAP is the deposition of LC3 on the phagosome membrane. LC3 recruitment to phagosomes containing *C. neoformans* infection has been observed as early as 1 h post-infection and persists for at least 24 h post infection. Recruitment levels differ between studies but range from ∼40 to 80% at 12 h post-infection (Qin et al., 2011; Nicola et al., 2012), furthermore, as discussed above, Nicola et al. show that for *C. neoformans* the route of uptake can determine LC3 recruitment. Phagosomes containing both unopsonized (Qin et al., 2011; Nicola et al., 2012) and opsonized (Pandey et al., 2017) *C. neoformans* cells recruit LC3. It has been found that phagocytosis of *C. neoformans* cells by macrophages leads to the activation of the host autophagy initiation complex (AIC) as well as upstream regulatory components LKB1 and AMPKα, which regulate autophagy induction through their kinase activity. Depletion of AIC components (ULK1, Atg13, and FIP200) and AMPKα reduces LC3 recruitment to *C. neoformans* containing phagosomes (Pandey et al., 2017). On phagosomes containing *C. albicans,* LC3 recruitment is observed for both live and heat-killed cells, heat-killed *Candida* elicit higher LC3 recruitment compared to live at 30 min post infection, however, at 60 min this phenotype is reversed (Tam et al., 2014). This could suggest that *C. albicans* actively inhibits LAP, but it is also possible that the heat killing leads to increased availability of LAP activating PRRs by changing the cell wall composition. Recruitment of LC3 to phagosomes containing *C. albicans* is Dectin-1/ROS dependent and leads to increased intracellular killing of *C. albicans* by macrophages (Tam et al., 2014). For *A. fumigatus,* Kyrmizi et al. report that monocyte phagosomes containing the *Aspergillus* conidia only recruit LC3 after the conidia begin to germinate or "swell" within the phagosome. The swelling process leads to changes in the cell wall composition of conidia including increased β-1,3-glucan display. As with *C. albicans,* LC3 recruitment to *A. fumigatus* conidia was ROS dependent. Furthermore, monocytes from patients with Chronic Granulomatous Disease (CGD), who have inactivating mutations in NADPH oxidase, fail to recruit LC3 to swollen conidia (Kyrmizi et al., 2013).

The Contribution of LAP to Host Defense

Although LC3 recruitment to the phagosome has been observed for all three fungi it is still unclear what downstream effects LAP has on fungal infection. A number of studies have investigated genetic knockdown of autophagy related proteins such as Atg5, Atg9a, Atg7, Atg12, and LC3 (Qin et al., 2011; Nicola et al., 2012; Smeekens et al., 2014; Kanayama et al., 2015). For *C. neoformans*, Qin et al. report that Atg5 and Atg9a

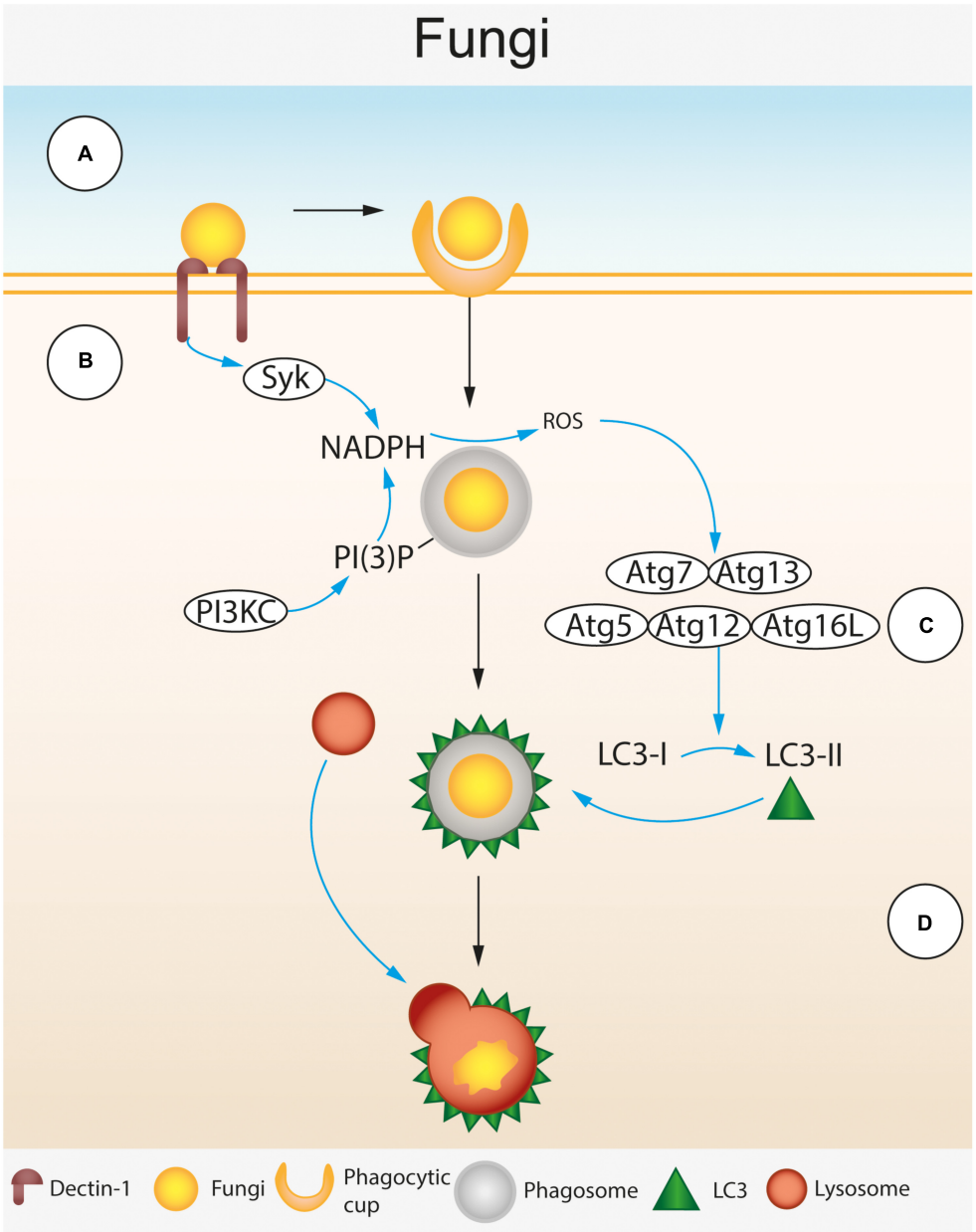


FIGURE 4 | Autophagic control of Fungal spp. LC3-associated phagocytosis (LAP) during fungal infection of macrophages. **(A)** β 1,3 glucan residues in the fungal cell wall are recognized by the cell surface receptor Dectin-1 expressed on the surface of the macrophage (Brown and Gordon, 2001; Brown et al., 2002; Brown, 2006). Dectin-1 recognition leads to phagocytosis of fungal cells. Following phagocytosis, the phagocytosed fungus is enclosed by a single membraned phagosome within the cytosol. **(B)** Dectin-1 activation triggers spleen tyrosine kinase (SyK) activation (Gantner et al., 2003). Activated Syk and phosphatidylinositol 3-phosphate (PI(3)P) deposited on the surface of the phagosome by the phosphoinositide 3-kinase complex (PI3KC) recruit NADPH oxidase leading to the production of reactive oxygen species (ROS) within the phagosome (Gantner et al., 2003). **(C)** ROS production attracts the LC3 lipidation complexes (Atg7-Atg13 and Atg5-Atg12-Atg16L) that convert LC3-I to LC3-II and deposit it on the phagosome surface (Ma et al., 2012; Kyrmizi et al., 2013). **(D)** LC3 deposited on the phagosome membrane leads to lysosomal fusion, acidification of the phagosome and destruction of the fungus.

recruit to infected phagosomes, however, knockdown of these proteins reduced the growth of *C. neoformans* within infected macrophages (Qin et al., 2011), similar findings in respect to Atg5 knockdown are reported by Nicola et al (Nicola et al., 2012). Further evidence that induction of host autophagy promotes *C. neoformans* growth is provided by Pandey et al. who find that knockdown of AIC components leads to reduced growth of the fungus within macrophages (Pandey et al., 2017). Studies in *C. albicans* have revealed conflicting data. Nicola et al. have shown that Atg5-deficient mice are more susceptible to *Candida* infection than wildtype mice and that Atg5 knockdown in J774 murine macrophages decreases LC3 recruitment to

phagosomes (Nicola et al., 2012). Additionally, Kanayama et al. have shown that mice with myeloid specific deficiencies in Atg7 are also more susceptible to *Candida* infection (Kanayama et al., 2015). In contrast to this study, Smeekens et al. report that myeloid specific Atg7 knockout does not affect *Candida* susceptibility in mice. Furthermore, a clinical study by Rosentul et al. that analyzed a cohort of patients with SNPs in the *ATG16L* gene found no correlation between SNPs *ATG16L* and susceptibility to oropharyngeal candidiasis (Rosentul et al., 2014). For *A. fumigatus,* Kyrmizi show that Atg5 knockdown in human THP1 macrophages reduces their ability to kill *A. fumigatus*. This phenotype correlated with reduced acidification of phagosomes containing *A. fumigatus* in $Atg5^{-/-}$ cells (Kyrmizi et al., 2013).

It is clear that LAP is induced against intracellular fungal pathogens, however, there are still many unanswered questions (**Box 1**). At a fundamental level, a better understanding is required about what constitutes LAP. LC3 recruitment to the phagosome is currently one of the only hallmarks to define LAP. As discussed above, studies investigating genetic ablation of autophagy-related genes remain inconclusive as to whether LC3 recruitment to the phagosome leads to improved host defense. The link between LAP and host defense appears to be strongest for *A. fumigatus* (Kyrmizi et al., 2013), while data for *C. albicans* is currently inconclusive (Nicola et al., 2012; Rosentul et al., 2014; Smeekens et al., 2014; Kanayama and Shinohara, 2016) and phagosomes containing *C. neoformans* recruit LC3 but autophagy appears to be required for fungal growth in the phagosome (Qin et al., 2011; Nicola et al., 2012). Interpreting these studies to produce a gestalt picture of LAP's importance in the defense against fungal pathogens is difficult not only because of the diversity of these fungi, but also because of the variety of strains and models used in each study. One standout issue is that the genes targeted by these studies are also involved in canonical autophagy and therefore their knockdown may affect other processes within the host. It is necessary at the moment to target these genes because very few LAP specific proteins are known other than Rubicon. However, resolving these two pathways should become easier as more components become known. Additionally, very little is also known about what happens to the LAPosome downstream of LC3 recruitment other than its eventual fusion with the lysosome. It is conceivable that *C. neoformans, C, albicans* and *A. fumigatus* could provoke very different host autophagic responses downstream of LAPosome formation which could explain why the outcome for each pathogen is so different. Hopefully as a better understanding of the LAP pathway is gained these questions will be addressed and LC3 recruitment to the phagosome may be seen as more of a staging post to a variety of different pathogen and host dependent outcomes rather than a single fixed pathway.

CONCLUSION AND OUTLOOK

This review summarizes current knowledge and emerging concepts in the interaction of host cell autophagy with several key eukaryotic pathogens, a field that is only recently emerging, in contrast to bacterial pathogens where autophagy has been established as a crucial mediator in both host defense and bacterial exploitation strategies. While clearly much work needs to be done in the contexts of the individual pathogens addressed, one emergent idea points to the unconventional nature of these interactions, with LAP, or LAP-like processes utilizing selective subsets of core autophagy components playing an important role. The diverse nature of responses and outcomes to LAP-like processes from individual pathogens suggests distinct variations of a core theme, the molecular details of which are likely to emerge in the near future. Importantly, the non-canonical nature of these interactions makes them attractive as drug targets against these pathogens.

AUTHOR CONTRIBUTIONS

RE, VS, and E-MF developed the ideas for the manuscript, and wrote and read the manuscript.

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REVIEW
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The Ins and Outs of Autophagy and Metabolism in Hematopoietic and Leukemic Stem Cells: Food for Thought

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Discovered over fifty years ago, autophagy is a double-edged blade. On one hand, it regulates cellular energy sources by "cannibalization" of its own cellular components, feeding on proteins and other unused cytoplasmic factors. On the other, it is a recycling process that removes dangerous waste from the cytoplasm keeping the cell clean and healthy. Failure of the autophagic machinery is translated in dysfunction of the immune response, in aging, and in the progression of pathologies such as Parkinson disease, diabetes, and cancer. Further investigation identified autophagy with a protective role in specific types of cancer, whereas in other cases it can promote tumorigenesis. Evidence shows that treatment with chemotherapeutics can upregulate autophagy in order to maintain a stable intracellular environment promoting drug resistance and cell survival. Leukemia, a blood derived cancer, represents one of the malignancies in which autophagy is responsible for drug treatment failure. Inhibition of autophagy is becoming a strategic target for leukemic stem cell (LSC) eradication. Interestingly, the latest findings demonstrate that LSCs show higher levels of mitochondrial metabolism compared to normal stem cells. With this review, we aim to explore the links between autophagy and metabolism in the hematopoietic system, with special focus on primitive LSCs.

Keywords: HSCs, LSCs, autophagy, mitophagy, metabolism, quiescence

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MAINTENANCE IN THE NICHE

Hypoxia, a Key Role in the Regulation of HSCs Quiescence

Decades of research has allowed scientists to characterize and describe the unique role of hematopoietic stem cells (HSCs) in the lifelong homeostasis of mature blood cells. The mammalian hematopoietic system is maintained by self-renewal of quiescent long-term (LT)-HSCs, which subsequently can differentiate into short-term (ST)-HSCs or multipotent progenitors (MPPs). The latter two will commit to either myeloid or lymphoid lineages exclusively. Because of their vital and long-term function, HSCs are provided with unique survival mechanisms. In this context, their localization in a complex endosteal niche, that is characterized by low levels of oxygen, is central (Szade et al., 2017). Changes in the bone marrow niche, such as increased production of growth factors and cytokines, as well as transplantation procedures and injuries, can stimulate HSCs to proliferate and differentiate. Once recovery is restored, HSCs return to a dormant state. Despite the

fact that hypoxia tolerance in HSCs is poorly understood, it has been proposed to be responsible for the quiescence and slow cell cycling of HSCs (Cipolleschi et al., 1993; Parmar et al., 2007). Takubo et al. (2010) elucidated this mechanism by demonstrating that hypoxia-inducible factor-1α (HIF-1α), a transcriptional factor that plays a central role in cellular response to oxygen levels, is stabilized by hypoxia in LT-HSCs. In HIF-1α deficient mice, loss of LT-HSCs numbers is directly proportional to loss of quiescence. This now raises the question: in what ways hypoxia can assume a protective role and assure LT-HSCs maintenance? HIF-1α and hypoxia have in fact, been linked with the distinct metabolic phenotype of HSCs.

HIF-1α and the Regulation of HSCs Metabolism

Metabolomic approaches indicate that LT-HSCs, when compared to MPPs and more differentiated cells, specifically upregulate glycolysis and represses influx of glycolytic metabolites into mitochondria, via regulation of pyruvate dehydrogenase kinase (PDK) activity by HIF-1α (Takubo et al., 2013; **Figure 1**). Furthermore, glycolytic adenosine triphosphate (ATP) production, commissioned by the HIF-1α/PDK regulatory system, is necessary to maintain HSCs during cell cycle quiescence. A recent discovery demonstrates that mitochondrial protein mitofusin 2 (MFN2), which has roles in mitochondrial fusion and in tying mitochondria to the endoplasmic reticulum, is essential for the maintenance of HSCs with wide lymphoid potential (Luchsinger et al., 2016). A different study identified that the mitochondrial unfolded protein response (UPRmt) is activated upon transition from quiescence to proliferation in HSCs (Mohrin et al., 2018). Remodeling the activity of sirtuin 7 (SIRT7), a component of the UPRmt, is translated into reduction of quiescence, higher mitochondrial unfolded protein stress, and insufficient self-renewal ability of HSCs. Furthermore, SIRT7 expression is lower in more mature HSCs whose regenerative capacity is improved following upregulation of SIRT7 (Mohrin et al., 2015). Kim et al. (1998), using a cationic fluorescent dye that selectively accumulates in the mitochondria of eukaryotic cells, demonstrated that HSCs have relatively less mitochondria when compared to proliferating progenitors. A subsequent study showed that differentiation of primary human HSCs (quantified by CD34 loss) is connected with increased mitochondrial content (Piccoli et al., 2005). In agreement with this, Simsek et al. (2010) showed that LT-HSCs are characterized by low mitochondrial potential and utilize cytoplasmic glycolysis for ATP production. Contrarily, cells that need to cycle and expand do not rely on anaerobic glycolysis. This may be because pyruvate produced during glycolysis will generate only 2 ATPs per molecule of glucose following anaerobic respiration (with lactate being the by-product), while it will produce 32 ATPs per molecule of glucose upon entering in the mitochondria to be used for oxidative phosphorylation (OXPHOS). However, de Almeida et al. (2017) using dye-independent methods, such as mitochondria DNA quantification and enumeration of mitochondria nucleoids, have recently suggested that while HSCs have high mitochondrial content they have compromised respiratory and turnover capacity, concluding that mitochondria perform an essential and yet unknown function in HSCs, which may not be directly linked with ATP production.

Stem Cell Proliferation and Maintenance, a Key Role for Fatty Acid Oxidation

LT-HSCs glycolytic phenotype can be seen as a protective mechanism to reduce reactive oxygen species (ROS) generation, which would cause oxidative stress and induce differentiation (Folmes et al., 2012). Based on this, a recent study demonstrates that while mitochondrial complex III subunit Rieske iron sulphur protein (RISP) in fetal HSCs is not essential for mitochondrial membrane potential maintenance, it is crucial for stem cell genes and multilineage potential retainment (Ansó et al., 2017). Based on the critical role of mitochondria in driving cell differentiation, RISP null fetal HSCs were unable to generate an adequate number of MPPs indicating compromised HSCs differentiation. In addition, products of the tricarboxylic acid (TCA) cycle, such as citrate, could be exported to the cytosol to contribute to lipid metabolism that is required for cell growth, proliferation and differentiation (Lum et al., 2007).

HSCs fate is dictated by their decision to undergo symmetric or asymmetric cell division when HSCs leave quiescence. Asymmetric division generates two daughter cells of which one will show same features of the initiator cell, such as self-renewal and quiescence, and the other will differentiate and enter the circulatory system. Otherwise, symmetric division will generate two daughter cells that will only be able to undergo cell cycling and differentiation. Fatty acid oxidation (FAO), which occurs in the mitochondria, also plays an important role in HSCs maintenance. FAO metabolism prevents HSCs exhaustion when HSCs proliferation and division are required, resulting in asymmetric division and thus assuring self-renewal (Ito et al., 2012). A role for peroxisome proliferator-activated receptor delta (PPARδ), which is a member of a nuclear receptor superfamily of transcription factors that controls nutrient sensing and FAO, has been reported in HSCs. PPARδ deletion or pharmacological inhibition of FAO stimulates the symmetric commitment of HSCs leading to stem cell depletion, while PPARδ activation, via use of an agonist, increased asymmetric cell division. In agreement with this, a subsequent study showed that weakening the mitochondrial phosphatase protein tyrosine phosphatase 1 (PTPMT1), drives the conversion from glycolysis and FAO to mitochondrial aerobic metabolism, resulting in unsuccessful hematopoiesis (Yu et al., 2013). This is linked with accumulation of HSCs unable to differentiate due to increased entry of quiescent stem cells into the cell cycle and a following pause at the G1 phase.

HSCs MAINTENANCE: GLYCOLYSIS VERSUS OXPHOS

As introduced above, HSCs maintenance is affected by a balance between HSCs metabolic status and ROS levels. In fact, dormant

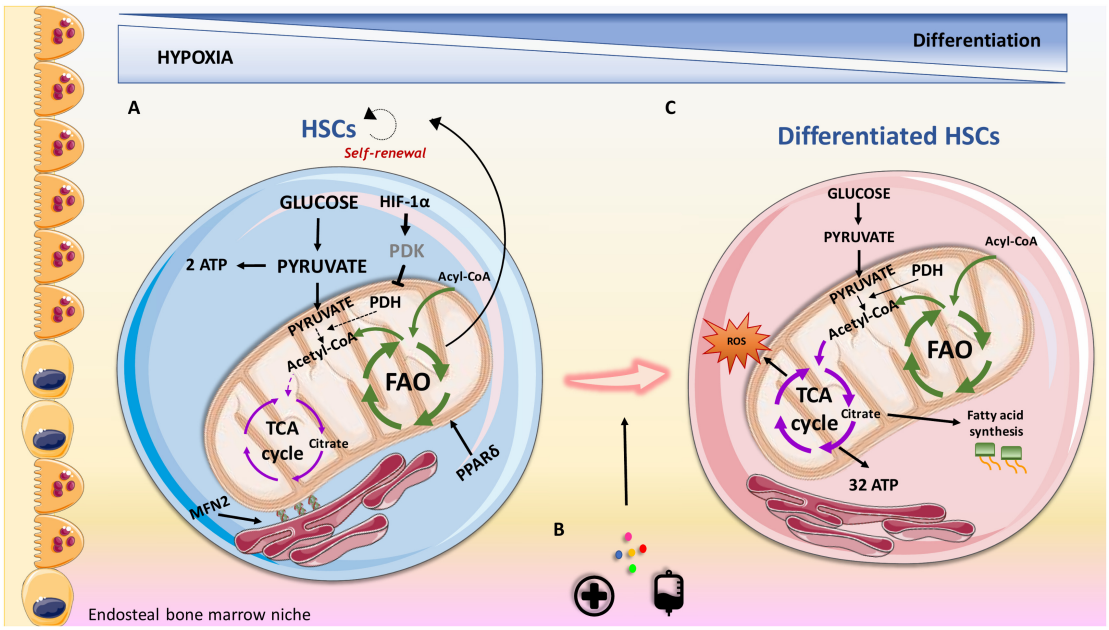


FIGURE 1 | Overview of metabolic features contributing to HSCs maintenance and differentiation in the endosteal niche. **(A)** HSCs are characterized by the expression of the surface marker CD34 and utilize anaerobic glycolysis and fatty acid oxidation (FAO) as the sources of energy. Nutrient sensor peroxisome proliferator-activated receptor delta (PPARδ) promotes FAO, contributing to their self-renewal capacity. HIF-1α represents a fundamental feature in regulating oxidative metabolism via regulation of pyruvate dehydrogenase kinase (PDK). **(B)** Transplantation procedures, cytokines and injuries can promote HSCs to proliferate and differentiate. **(C)** Differentiated HSCs lose their stemness surface marker CD34 and adapt their energy source to address energy demand for proliferation. $CD34^{-}$ HSCs, rely on increased oxidative metabolism that provides a higher production of ATP compared to anaerobic glycolysis. Oxidative metabolism can result in increased production of reactive oxygen species (ROS), which can contribute to differentiation. PDH, pyruvate dehydrogenase; MFN2, mitofusin 2.

HSCs seem to rely on glycolysis to avoid a decline in HSCs maintenance and HSCs with defective glycolysis will switch to a mitochondrial metabolic profile with increased production of ROS (Wang et al., 2014). ROS have been largely found to contribute to bone marrow failure as one of the main sources for DNA damage and genome instability (Richardson et al., 2015), thus ROS can play a role as sensor dictating HSCs fate. Mitochondria, which are the main source of energy and indirectly of ROS, are considered as minor player in the maintenance of HSCs maintenance. However, HSCs highly rely on mitochondria when a metabolic switch is required (i.e., HSCs need to increase their proliferation rate). FOXO3, a transcriptional factor that shows multiple functions associated with longevity, is a regulator of HSCs metabolism. Loss of FOXO3 alters mitochondria function, inducing deleterious accumulation of ROS (Rimmele et al., 2015; Bigarella et al., 2017). Specifically, deletion of FOXO3 in HSCs compromise DNA repair pathway leading to DNA damage, which compromises HSCs function. Mutation in tuberous sclerosis complex 1 (TSC1), a negative regulator of mTOR and a key regulator for cellular metabolism, induces levels of ROS and loss of quiescence in HSCs (Chen et al., 2008). Additionally, mTOR activity contributes to erythroid differentiation favoring mitochondria activity and is also increased with aging (Luo et al., 2014; Liu et al., 2017). Moreover, mTOR is one of the main regulators of autophagy, a process that itself has a critical role in HSCs biology.

AUTOPHAGY

Autophagy, from the Greek *auto*-self and *-phagy* eating, is an evolutionally conserved process first described in yeast in 1963 by Christian de Duve (de Reuck, 1963). It is a lysosomal catabolic process that has several functions. First of all, it has a role as a cell cleaner by reducing the chance of cell misfunction due to accumulation of damaged cellular components and organelles. It is also involved in microbe's demolition and sustains metabolism during stressful situations, such as starvation, providing building blocks for energy production and cellular homeostasis. The assembly of the catabolic machinery of autophagy takes place in the cytoplasm, in double membrane vesicles known as autophagosomes. Numerous autophagy-related (*ATG*) genes are involved in their biogenesis and function that can be organized in three main stages (Mizushima et al., 2011). The very first step consists in the autophagy initiation and formation of the phagophore. Signals of cellular nutrient status are sensed by the unc-51 like autophagy activating kinase 1 (ULK1) initiation complex which then activates autophagy and recruits a second complex, known as VPS34 complex, resulting in the formation of a flat unique membrane known as phagophore (Ganley et al., 2009; Russell et al., 2013). Phagophores will then elongate and expand leading to autophagosomal maturation. The last step is represented by their fusion with lysosomes where proteases will be in charge of their content demolition (Kim et al., 2002; Yang and Klionsky, 2009). Although, keeping LT-HSCs

in their hypoxic niche seems to satisfy maintenance of their "dormant" state, it may not be the only factor that contributes to their metabolism adaptation. Lately, autophagy has been shown to be essential in preserving the organization and the welfare of this small cell compartment (Warr et al., 2013b; Nguyen-McCarty and Klein, 2017). The maintenance of cell health and prevention of stem cell aging is also vital for hematopoiesis, and the role of autophagy in degrading damaged cellular components and organelles may be essential in this context. Furthermore, autophagy flux negatively correlates with cell decline in several cell subtypes including HSCs (Revuelta and Matheu, 2017).

Autophagy, Key Player in HSCs Maintenance

The fact that HSCs have a slow turnover increases the chance to reduce or dilute damaged cellular components and autophagy might be indispensable for the necessary increase in catabolic rate. Several studies propose that autophagy can sustain glycolytic flux, protecting HSCs from metabolic stress and expansion stimulus in the bone marrow, thereby reducing the chance of HSCs exhaustion. FOXO3a, a transcriptional factor that shows multiple functions associated with longevity, regulates levels of autophagy in HSCs in case of metabolic stress (Warr et al., 2013a). Specifically, FOXO3a deficient mice showed a pronounced reduction in autophagy capacity in protecting HSCs. FIP200, a component of the ULK1 initiation complex, has also been associated with maintenance of HSCs (Liu et al., 2010). This study found that mice depleted with FIP200 resulted in increased HSC proliferation, in which mitochondrial mass was higher when compared with HSC with no depletion. ATG7, whose role is only associated with autophagy, regulates mitochondrial homeostasis of HSCs, as well as ROS production and cell differentiation (Mortensen et al., 2011). Deleting ATG7 in the hematopoietic compartment results in the loss of normal HSCs functions and severe myelo-proliferation, causing mice death. The authors also show that the hematopoietic stem and progenitor cell (HSPC) compartment exhibit accumulation of mitochondria and ROS, in addition to an increased proliferation rate and DNA impairment.

Autophagy and HSCs Cell Cycle

Orientating HSCs to quiescence and a slow cell cycle ensure preservation and health of long-lived stem cells. Fewer replication cycles with minimum telomere shortening ensure that HSCs age more slowly (Liu and Rando, 2011). Despite the fact that cyclin D family members, including cyclins D1–D3, are expressed at different levels in HSPCs, Cao et al. (2015) showed that only cyclin D3 responds to nutrient stress and identified autophagy as a driving force for cell cycle entry. Therefore, they suggest that the lower levels of autophagy activity observed in aged mice may be due to lower cyclin D3 levels, and thus a postponed HSCs entry into the cell cycle. This ultimately results in a defect in self-renewal (**Figure 2**). The most common way to test HSCs ability to undergo self-renewal is represented by serial transplantation of murine HSCs from an original donor to a recipient, whose own HSCs were completely ablated before transplantation. Recent studies indicate that autophagy is an essential process for self-renewal. Ho et al. (2017), demonstrated that mice with hematopoietic-specific deletion of the essential autophagy factor ATG12 had increased numbers of cells in the peripheral blood and spleen. The authors then performed serial transplantation of ATG12 deficient HSCs into recipient mice. The mice receiving the ATG12 deficient HSCs showed dramatically impaired engraftment and reduced number of regenerated HSCs. It has recently been reported that ATG7 can bind p53 and modulate TP53/p53 checkpoint in cell cycle exit in response to metabolic stress (Lee et al., 2012). Here authors showed that starved murine fibroblasts lacking ATG7 fail to undergo cell cycle arrest.

Mitophagy Controls Oxidative Stress in HSCs

It is likely that to maintain latency in HSCs, it is fundamental to have low mitochondrial activity. A phenomenon known as mitophagy is the only known process for mitochondrial clearance, and this function has been demonstrated to control levels of oxidative stress. One of the key regulators of mitophagy is PTEN-induced putative kinase 1 (Pink1) that interacts with the outer mitochondrial membrane and with Parkin, a E3 ubiquitin ligase, guiding mitochondria to autophagosomal degradation (Michiorri et al., 2010; Baudot et al., 2015). In their study Jin et al. (2018) reported that ATAD3A is a major regulator of mitophagy. During mitophagy in the hematopoietic system, ATAD3A functions as a bridge between the translocase of the outer membrane complex and the translocase of the inner membrane complex to facilitate the import of Pink1 into mitochondria. Deletion of ATAD3A results in enhanced mitophagy with mitochondria depletion and blockage in differentiation, restoring HSCs pool. Another study showed that PPAR–FAO pathway mediates clearance of damaged mitochondria, an important process in the self-renewing population expansion of $Tie2^{+}$ HSCs in mice (Ito et al., 2016). Tie2, a receptor tyrosine kinase, expression on HSCs is a marker of quiescence (Iwama et al., 1993; Arai et al., 2004). Additionally, ATG5 and ATG7 have been shown to regulate mitophagy and oxidative stress (Zhang et al., 2009). A robust increase in mitochondrial mass is detected in mice with conditional depletion of ATG5 or ATG7. This is translated in increased production of ROS and higher DNA damage in ATG7 deficient cells than that of their wild-type counterparts. Authors therefore highlight mitophagy as a critical mechanism for normal HSPC function. Furthermore, it has been reported that mice deficient in ULK1 have compromised mitochondrial clearance during the stages of erythrocyte maturation (Kundu et al., 2008). As previously mentioned, ROS have a major role in HSCs decline. Since mitochondria are the main source for ROS, mitophagy might also represent a crucial regulator for HSCs aging. Reducing oxidative stress via mitochondria degradation might therefore prevent HSCs exhaustion and immature aging, although further investigation to address this hypothesis is needed.

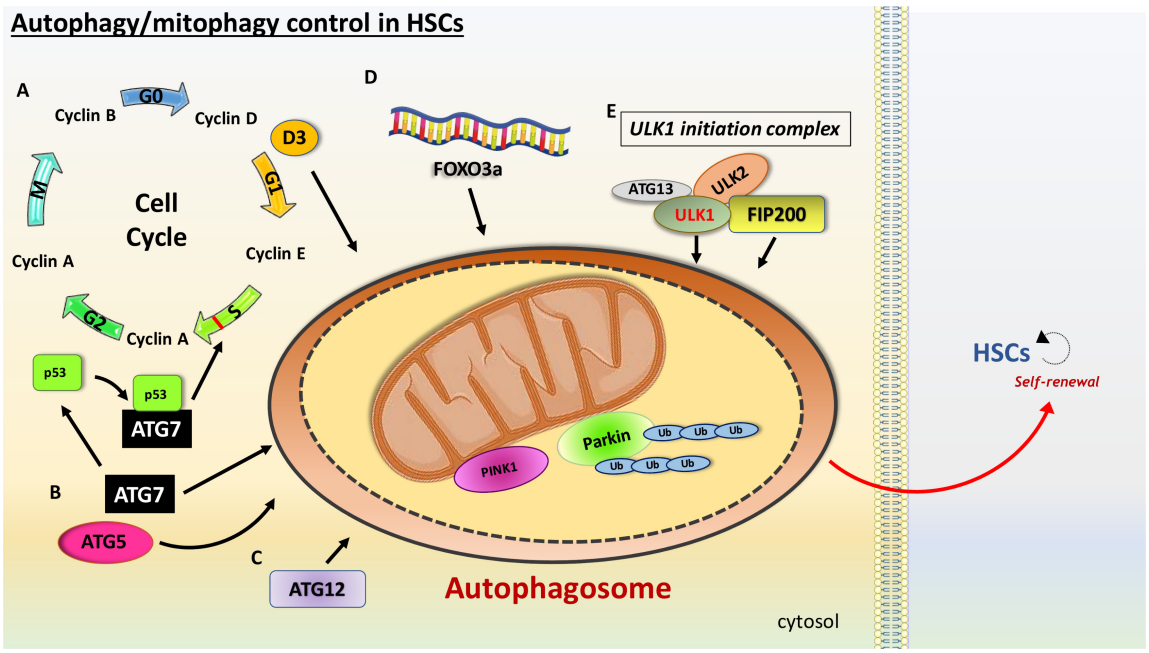


FIGURE 2 | Schematic view of the main features involved in the autophagy promotion in HSCs. HSCs are characterized with high levels of autophagy. **(A)** Slow cell cycle is essential to ensure maintenance of HSCs. Cyclins are a family of proteins that control the progression of cells through the cell cycle. Cyclin D3, a member of the cyclin D family involved in the G1 phase of the cell cycle, is a nutrient stress sensor, promoting autophagy as a fuel source for cell cycle entry. ATG7 and ATG5 have been linked with mitophagy induction in HSCs. **(B)** ATG7 binds p53/TP53, an S phase checkpoint control that senses nutrient stress, modulating cell cycle exit. **(C)** ATG12 has also been linked with HSCs maintenance. **(D)** FOXO3a reduces HSCs exhaustions via regulation of autophagy. **(E)** Members of the ULK1 initiation complex, such as ULK1 and FIP200, have been linked with mitophagy in HSCs.

LSCs AND THE ORIGIN OF MYELOID AND LYMPHOID LEUKEMIA

The ground-breaking discovery of the HSCs niche was made by Schofield (1978). Since then, significant advances have been made in describing what orchestrates HSCs maintenance. Mutagenesis or epigenetic changes in HSCs, together with fluctuations in the bone marrow microenvironment, are important events to cause blood malignancies as leukemia. Based on functional and immunophenotypic investigation of various subtype of cells, the existence of cancer stem cells (CSCs) was firstly described in the hematopoietic system and it is proposed that leukemia is a stem cell disorder, initiated by as little as a single leukemic stem cell (LSC). These cells can originate either from rare transformed HSCs or from more abundant and more differentiated progenitor cells. The origin of LSCs can vary with the stage of the disease, whether the leukemia is chronic or acute, its immunophenotype, myeloid or lymphoid, and the nature of the transforming event. HSCs are equipped with intrinsic self-renewal activity that persists for the whole life of an individual. In this context, HSCs have much higher chance to accumulate mutations than less primitive cells, which are not as long lived. LSCs initiation and maintenance is based on enhanced self-renewal activity (Argiropoulos and Humphries, 2007; Wang et al., 2010). In the case of leukemic cells, they could potentially originate from more restricted progenitors by acquiring mutations that allow them to self-renew, or from HSCs that accumulate genetic and epigenetic changes that down-regulate cell death and increase their self-renewal capability. To fully understand the vast heterogeneity of the disease we will briefly introduce each type of leukemia.

Origin and Aberrations in AML

Acute myeloid leukemia (AML) is the most common leukemia in adults and the origin of AML has been thoroughly investigated. The *t*(8,21) and the *t*(15,17) are the most frequent chromosomal abnormalities associated with AML (Downing et al., 2000). Nucleophosmin (*NPM1*), CCAAT/enhancer-binding protein alpha (*CEBPA*), FMS like tyrosine kinase 3-internal tandem duplication (*FLT3-ITD*) and proto-oncogene receptor tyrosine kinase (*KIT*) are the most common mutations in AML patients, dictating the development of the leukemia and rearranging them into different prognostic groups (Welch et al., 2012; Yohe, 2015; **Figure 3**). Mutations such as isocitrate dehydrogenase (*IDH*) that occurs in early stages of the disease, lead to pre-leukemia development of AML (founder mutation) (Thomas and Majeti, 2017). Later in the disease progression, acquisition of driver mutations such as *FLT3-ITD* can cause the full-blown disease phenotype and further tertiary mutations can contribute to disease heterogeneity. In 1994 it was shown that leukemic cells possessing the $CD34^{+}CD38^{-}$ cell-surface markers were able to initiate leukemia in severe combined immunodeficiency

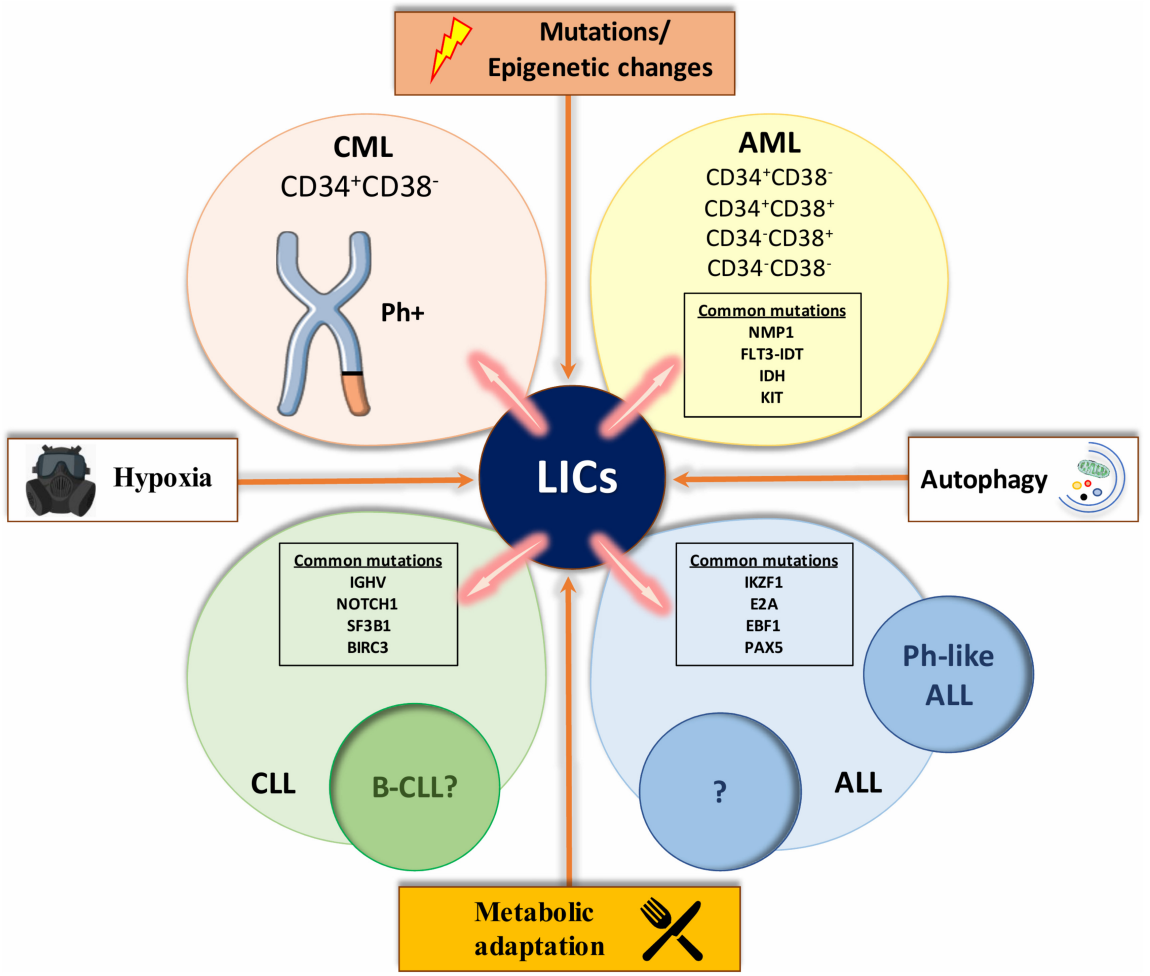


FIGURE 3 | A compilation of factors involved in leukemic transformation. The figure represents a compilation of the various influences involved in the leukemic initiation process that characterizes each type of leukemia. Mutations and epigenetics changes, such as a distinct metabolic profile that drives leukemic stem cells (LSCs) expansion, autophagy which contributes to fuel LSCs energy demand and hypoxic environment, seem to be some of the main inducers of changes in HSCs and initiate leukemia. With the help of extended research in the field, we might be able to study and or perturb these influences for a better understanding of each type of leukemia and ultimately better-tailored therapeutics. List of abbreviations; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; B-CLL, B cell CLL like phenotype; ALL, acute lymphoblastic leukemia; Ph-like ALL, Philadelphia chromosome-like ALL; Ph^+, Philadelphia positive; *NPM1*, Nucleophosmin; *FLT3-ITD*, like tyrosine kinase 3-internal tandem duplication; *KIT*, proto-oncogene receptor tyrosine kinase; IKZF1, IKAROS family zinc finger 1; E2A, transcription factor 3; EBF1, early B-cell factor 1; PAX5, paired box 5; *IGHV*, non-mutated immunoglobulin heavy chain variable genes; *NOTCH1*, Notch homolog 1, translocation-associated; *SF3B1*, splicing factor 3B subunit 1; *BIRC3*, baculoviral IAP Repeat Containing 3. CD34 and CD38 are markers of hematopoietic stem and progenitor cells.

(SCID) mice, while $CD34^+$ or certain $CD34^+CD38^+$ expressing cells were unable to do so. Moreover, limiting dilution assays showed that leukemic-initiating cells (LICs) were a small fraction of the entire disease, representing roughly 1 in 250,000 leukemic cells (Lapidot et al., 1994). Bonnet and Dick, the pioneers of developing and refining transplantation techniques of human cells into recipient mice, demonstrated that only $CD34^+CD38^-$ fractions of cell types isolated from AML patients could engraft in recipient mice (Kamel-Reid et al., 1989; Lapidot et al., 1994). This observation has been further supported by the finding of Blair et al. (1997) indicating that LICs from human AML samples were also $Thy\text{-}1^-$. However, Taussig et al. (2010) indicate that LICs from AML patients with mutated NPM1 reside in the $CD34^-$ fraction.

Origin and Aberrations in CML

Almost 100% of chronic myeloid leukemia (CML) patients are positive for the Philadelphia (Ph) chromosome, a shortened chromosome 22 that arises from a reciprocal translocation *t*(9q34,22q11) (Rowley, 1973; Raskind and Fialkow, 1987). The Ph chromosome is the hallmark of the disease, in which fusion of *BCR* and *ABL* genes encode for an constitutively active protein kinase (Daley et al., 1990; Sawyers, 1999). Since BCR-ABL fusion can occur in myeloid, B lymphoid, erythroid and sporadically T lymphoid cells in the majority of CML patients,

the consensus is that the original translocation takes place in LT-HSCs (Fialkow et al., 1977). The presence of BCR-ABL in endothelial cells originating from CML patient, raises the question: does the aberration take place even in more primitive cells than LT-HSC (Gunsilius et al., 2000)? An elegant experiment conducted by Fialkow et al. (1967, 1981) using patterns of inactivation in X-linked genes, showed that erythrocytes and myeloid cells in female CML patients with heterozygous X-linked glucose-6-phosphate dehydrogenase (G6PDH) had the same single isoenzyme type for G6PDH in contrast to normal cells, which were heterogeneous. These results suggested that both erythrocytes and granulocytes share a common stem cell, demonstrating that CML is a clonal disease with a stem cell origin. A recent study showed that while BCR-ABL expressing progenitor cells were eliminated following imatinib treatment in patients with a major molecular response (MMR), BCR-ABL expressing HSCs were still detectable (Abe et al., 2009). In chronic phase, the leukemic clone seems to be maintained by a small number of BCR-ABL positive $CD34^{+}CD38^{-}$ cells, a population enriched for HSCs (Fialkow et al., 1977). These LSCs differentiate normally and proliferate slowly like normal HSCs. However, as these cells progress into intermediate phases of lineage restriction, their progeny proliferate losing their primitive marker CD34. By analyzing different subpopulation of primitive CML cells it has been shown that an unusual autocrine IL-3-granulocyte colony-stimulating factor mechanism can provide a strong rational for the unusual performance of BCR-ABL expressing stem and progenitor cells (Chang et al., 1989; Holyoake et al., 2002). This mechanism only moderately offsets the *in vivo* signals, which maintain normal HSCs quiescence but, when active in BCR-ABL expressing LSCs, drives their differentiation at the expense of their self- renewal. In less primitive CML progenitors, the same mechanism has a more potent mitogenic effect that is then quenched when the cells progress into the final phases of differentiation.

Origin and Aberrations in ALL

Acute lymphoblastic leukemia (ALL) defines a group of blood malignancies that frequently have chromosomal or intra-chromosomal changes. These rearrangements can impact on immunoglobulin or T-cell receptor genes that drive commitment to the lymphoid lineage. What has been elusive is proving the existence of a rare stem cell-like population that are capable of maintaining ALL. There have been conflicting results from studies investigating whether there is a CSC-like subpopulation in ALL. The heterogeneity of ALL itself may be responsible for some of these inconsistencies. Possible explanations for the pronounced variation in response to therapy include the presence of primitive LICs for each subtype of ALL and the different biology of the cell of origin. While chromosomal abnormalities are a key hallmark of ALL, on their own they are insufficient to generate the disease. Characteristic translocations include *t*(1,19), *t*(12,21), and *t*(9,22) (Mullighan et al., 2009). Cytogenetic abnormalities and transcription profiling approaches divide ALL into several subcategories, in which prognosis and frequency differ significantly in different age groups (Mullighan, 2014). One of these subcategories is Ph chromosome-like ALL (Ph-like ALL), which gives rise to the *BCR-ABL* oncogene and this is one of the most adverse abnormalities seen in ALL patients. Ph-like ALL is the group with Ph-negative B-lineage ALL but has a transcription profile similar to those of patients that have Ph-positive ALL (Den Boer et al., 2009). Genetic abnormalities associated with ALL cases are not homogeneous, and the most common mutations associated with the Ph-like subtype are the IKAROS family zinc finger 1 (IKZF1), paired box 5 (PAX5), early B-cell factor 1 (EBF1), and transcription factor 3 (E2A) (Mullighan et al., 2007). Likewise, kinase-activating mutations are seen in 90% of the Ph-like ALL. The most frequent of these include rearrangements involving abelson murine leukemia viral oncogene homolog 1 (*ABL1*), Janus kinase 2 (*JAK2*), and FLT3 (Roberts et al., 2012).

Origin and Aberrations in CLL

Chronic lymphocytic leukemia (CLL) is defined by a very heterogeneous clinical course. Genomic aberrations are present in more than 80% of cases including 11q deletion (11q-), trisomy 12 (11-), 17p deletion (17p-), and 13q deletion (13q-) and each of these is associated with a specific clinical outcome. Inactivation or mutation of the tumor suppressor 53 (TP53) results in a more aggressive CLL phenotype in patients with 17p- (Döhner et al., 1995). Non-mutated immunoglobulin heavy chain variable genes (*IGHV*) is linked with high-risk clinical characteristics and shorter survival (Damle et al., 1999; Hamblin et al., 1999). Using next generation sequencing techniques has revealed a more detailed panel of aberrations such as Notch homolog 1, translocation-associated (*NOTCH1*), baculoviral IAP Repeat Containing 3 (*BIRC3*) and splicing factor 3B subunit 1 (*SF3B1*) (Puente et al., 2011; Quesada et al., 2012). *NOTCH1* and *SF3B1* represent the most frequently mutated genes in CLL, being present in the majority of patients (Wang et al., 2011). The results of xenogeneic transplantation studies have shown that HSCs isolated from patients with CLL firstly differentiate into B cell progenitors and only later stimulus and rearrangements can address them within a B-CLL-like phenotype (Kikushige et al., 2011). While it's not certain that these cells constitute a type of CSC for CLL, it does seem that further genetic/or epigenetic transformation are needed for such B cells to turn into malignant cells. Furthermore, investigations into telomere length and telomerase expression indicate that CLL cells with no mutations in the immunoglobulin heavy variable (*IGHV*) proliferate rapidly and undergo extensive cell division, which are characteristic abilities of LSCs (Damle et al., 2004). In brief, these features can lead them to leukemic transformation.

LSCs DEPENDENCY ON HYPOXIA

We have previously described how LSCs can arise from HSPCs that reside in the hypoxic bone marrow niche. However, the role of hypoxia in the maintenance of LSCs is still controversial, perhaps due to point of stemness at which the hypoxia is introduced in conflicting studies, and the length and level of hypoxia (Deynoux et al., 2016; Huang et al., 2018). Nevertheless, further studies are required in order to elucidate its complex effect on LSCs maintenance and survival. Hypoxia via HIFs

may drive disease maintenance and development through other mechanisms such as energy metabolism, cell cycle, quiescence, and immune function. These physiological processes can be up- or down-regulated in cancer. In the case of AML, the existence of an oxygen gradient in the bone marrow allows maintenance of primary AML cells (Griessinger et al., 2014). Rouault-Pierre et al. (2013) reported that down-regulation of HIF-2α or HIF-1α to lesser extent, induces apoptosis and prevents leukemic engraftment upon transplantation of human AML cells into mice. These results suggest that HIF-2α or HIF-1α is necessary for the maintenance of LSCs and may potentially be therapeutically targeted for AML. On the other hand, the Velasco-Hernandez et al. (2014) study reported that HIF-1α deletion does not affect mouse AML maintenance, which highlights the contradictions in the role of HIFs in AML disease. However, these differences in effect might be dependent on the particular genetic alteration that initiates the malignancy, revealing once more the enormous heterogeneity of this disease. Furthermore, Vukovic et al. (2015) developed a conditional genetic model to investigate the effect of deletion of both HIF-1α and HIF-2α during leukemogenesis. The authors showed that while HIF-2α had no influence on proliferation of AML cells in a murine model, it's important in blocking the progression of LSCs into a malignancy. HIF-2α deletion accelerates LSCs differentiation but does not affect LSCs maintenance of AML (Vukovic et al., 2015). In CML, Zhang et al. (2012) reported that deletion of HIF-1α blocks CML progression through weakening cell cycle progression and induction of apoptosis in LSCs. BCR-ABL oncogene in CML-LICs, in fact, stabilizes HIF-1α to promote cell proliferation. Whether HIF-1α has a role in the survival mechanisms of LSCs in CLL is still unknown. In CLL, HIF-1α is stabilized even under normoxia through down regulation of von Hippel Lindau (VHL) protein, whose expression is compromised by HIF-1α microRNAs (Ghosh et al., 2009). This mechanism allows the formation of a complex (HIF-1α/p300/p-STAT3) responsible for the expression of the vascular endothelial grow factor (VEGF) (Ghosh et al., 2009). The authors indicate that up-regulation of factors such as VEGF by HIF-1α plays an important role in the microenvironment's control of leukemic cell survival. In T-ALL, HIF-1α stabilization induces Wnt signaling through enhanced transcription of β-catenin (Giambra et al., 2015). Loss of HIF-1α decreases the LSCs frequency without affecting the growth and viability of leukemic bulk cells.

LSCs METABOLISM

Hematopoietic stem cells have a distinct energy demand to sustain maintenance. However, demand for energy and nutrients increase drastically upon cell division and differentiation. Cells in order to proliferate, on top of increasing biomass and duplicate their genome, upregulate the metabolic rates of nucleotides, proteins and lipids. Consequently, cancer cells must adapt their metabolism, particularly by increasing nutrient uptake, to maintain their uninhibited proliferation (Dang, 2012). In fact, the metabolism of cancer cells is not an indirect by-product of proliferation but also a direct reprogramming orchestrated by oncogenic signaling (Ward and Thompson, 2012; Pavlova and Thompson, 2016). Investigating the metabolic phenotype of LSCs might clarify their survival mechanism and their persistence and progression though the development of the disease. Understanding how they are metabolic distinct from HSCs could help for a better characterization of each type of leukemia.

Glucose Metabolism in LSCs

Glycolytic flux is the main feature in the metabolism of HSCs. As a rule, HSCs are energetically dormant with active glycolysis, until they differentiate, moving to mitochondrial respiration to survive. Song et al. (2014, 2016) showed that bone marrow cells isolated from AML patients with no remission have higher expression of HIF-1α, as well as glucose transporter 1 (GLUT1) and higher expression of two of the main controlling stages of the glycolytic flux, hexokinase 2 (HK2) and lactate dehydrogenase (LDH) compared to patients with complete or partial remission and healthy donors. Bhanot et al. (2015) using metabolomics approaches demonstrated that UDP-P-Glucose, which is a glycogenic precursor of glucose, is increased in AML regardless of low levels of glycogen. Likewise, changes in glucose metabolism have been linked with clinical outcome and therapeutic resistance. Herst et al. (2011) showed that high glycolytic primary blast AML are resistant to treatment. They indicate that myeloblast glycolytic rate could be an effective and easily employed method to determine the pre-treatment prognosis of AML. This conclusion is also supported by the results of a separate study conducted in AML patients by Chen et al. (2014). Unfortunately, recent studies are not enough to profile the glycolytic phenotype of LSCs and further studies are required. In support of the above results, a study conducted in mice demonstrated that deletion of lactate dehydrogenase A (LDHA) and of pyruvate kinase and muscle 2 (PKM2), two enzymes that regulates last steps of glycolysis, reduced the chance to induce leukemia (Wang et al., 2014). Finally, CML leukocytes have higher aerobic glycolytic rates when compared to normal and CLL leukocytes (Beck and Valentine, 1953).

Glutamine Metabolism in LSCs

An alternative source of energy is glutaminolysis, in other words the metabolism of glutamine, the most abundant amino acid in circulating blood. How tumor cells regulate the balance between glycolysis and oxidative metabolism to meet their energy need is not fully understood. While it's been long known that the Warburg shift is a notable hallmark of proliferating cancer cells, they have an intact TCA cycle that becomes progressively more reliant on glutamine metabolism when compared to normal cells. As well as use for ATP generation, the cancer cells can use TCA cycle intermediates as precursors for biosynthetic pathways and glutamine anaplerosis can help sustain this. Furthermore, glutamine is also required for protection against antioxidants by increasing glutathione (GSH) levels that in turn neutralize ROS. In a recent study, Gallipoli et al. (2018), exploiting CRISPR-Cas9 screen, identified that glutaminase (GLS), the first enzyme in glutamine metabolism, is synthetically lethal when combined with FLT3-tyrosine kinase inhibitor (TKI) treatment. Here the

authors combined complementary metabolomics with a CRISPR screen to show that glutamine metabolism, through its ability to support both the TCA cycle and GSH synthesis, becomes a metabolic dependency of FLT3-IDT AML, specifically unmasked by FLT3-TKI treatment. Knoechel and Aster (2015) recently showed that signal from the PI3K-AKT pathway shifts NOTCH-dependent T-ALL cells from glutamine metabolism to aerobic glycolysis. Using murine models and xenograft transplantation model of primary human T-ALL, the authors showed that T-ALL cells with activating mutations of NOTCH1 use glutamine as the main substrate of anaplerotic reactions that feed the TCA cycle.

Fatty Acid Metabolism in Leukemia

As discussed in the previous paragraph, LSCs may have a high demand for glutamine to feed oxidative metabolism. Adipose tissue represents one of the major sources of glutamine in cells. The high amount of energy required from LSCs can be satisfied by using fatty acid as a fuel source. Additionally, adipocytes have a well establish role in the LSCs energy demand. Adipocytes can store energy as triglycerides, which during lipolysis can be catabolized into glycerol and free fatty acids (FFA). Thus, adipocytes may deliver FFA to cancer cells, to help meet their demands for energy and lipid synthesis. A recent study demonstrated that adipocytes provide FFA as fuel source to leukemia cells (Ye et al., 2016). Using a mouse model of blast-crisis CML, Ye et al. interestingly found that LSCs have a niche in gonadal adipose tissue (GAT). The authors used limiting-dilution transplantation assays to show that these GAT-resident LSCs gave rise to leukemia with a frequency similar to that of bone-marrow-derived LSCs. Also, the GAT-associated LSCs have high expression of the fatty acid transporter CD36. Gene expression analysis showed LSCs have a pro-inflammatory phenotype that increases lipolysis that fuels the LSCs' high levels of FAO when compared to their more differentiated progeny or normal HSCs. These features are responsible for LSC quiescence and resistance to chemotherapy. A previous study examining primary human samples of AML identified in the $CD34^+$ LSCs, a subpopulation expressing CD36, and this $CD36^+$ phenotype was been linked with poor prognosis (Perea et al., 2005). In these cases, the $CD36^+$ LSCs also displayed an increase in uptake of FFAs and their subsequent oxidation, suggesting that CD36 can regulate LSCs metabolism in at least a subset of human myeloid leukemia. Tucci et al. (2014) reported that ALL cells stimulate adipocyte lipolysis and use the resulting FFAs to supplement *de novo* lipogenesis and proliferation. In a separate study is shown that CLL cells, in contrast with normal B-lymphocytes, are able to catabolize lipids in order to use FFAs for oxidative respiration (Rozovski et al., 2015). FFAs can also bind the nuclear receptor peroxisome proliferator activated receptor α (PPARα). The interaction between FFAs and PPARα generates a complex that, similar to a transcription factor, activates the transcription of enzymes necessary for OXPHOS (Kersten, 2014). Adipose tissue can also be a protective compartment for LSCs during stressful condition such as drug treatment. Ehsanipour et al. (2013) report that adipocytes protect leukemia cells from L-asparaginase treatment by secreting glutamine. This is particularly relevant when considering that L-asparaginase is used in ALL treatment due to leukemic lymphoblasts being highly sensitive to the depletion of exogenous asparagine and glutamine (Oettgen et al., 1967; Kitoh et al., 1990).

Mitochondrial Metabolism and LSCs

LSCs are resilient cells and able to exploit multiple metabolic pathways in order to survive. In fact, LSCs can, in addition to glucose, utilize fatty acids and amino acids such as glutamine in order to provide precursors of the TCA cycle to sustain mitochondrial metabolism in LSCs. Most CSCs that are dependent on OXPHOS generally upregulate this energy source. For this reason, CSCs can be sensitive to mitochondrial inhibition. IDH mutated AML-LSCs acquire increased enzymatic function generating R-2-hydroxyglutarate (R2HG) from α-ketoglutarate (α-KG), as opposed to non-mutated IDH, which catalyzes the conversion of isocitrate to α-KG. The resulting accumulation of the onco-metabolite R2HG inhibits the α-KG dependent ten-eleven translocation (TET) protein family leading to DNA demethylation which contributes to tumorigenesis (Dang et al., 2009). Furthermore, cytarabine resistant AML cells enriched in quiescent LSCs, have increased levels of mitochondrial mass, hold functional mitochondria, which is translated into increased OXPHOS levels with subsequent peak in ROS. Interestingly, even though cytarabine wasn't effective, residual cells displayed an increased expression of OXPHOS genes together with an augmentation in FAO and upregulation of CD36 that can be predictive for treatment response in patients with AML (Farge et al., 2017). In a different study it was shown that even though AML cells have higher levels of mitochondrial mass compared to normal, they still have lower respiratory chain complex activity and lower spare respiration (Sriskanthadevan et al., 2015). Interestingly, Marlein et al. (2017) recently demonstrated that NADPH oxidase 2 (NOX2) generates superoxide that stimulates bone marrow stromal cells to transfer mitochondria to AML blast cells through AML-derived tunneling nanotubes. CLL patients also display an increased metabolic oxidative profile, which is linked to alterations in their lymphoid compartment (Jitschin et al., 2014). In this study, CLL cells were found to adapt to intrinsic oxidative stress by increasing levels of the stress-responsive heme-oxygenase 1 (HO-1). The results of this study implicate that HO-1, beyond its function as an antioxidant, has additional roles in promoting mitochondrial biogenesis and reducing the high levels of ROS present in CLL cells. A study conducted in quiescent CLL-LSCs exposed to three different stromal cell lines demonstrate that OXPHOS was significantly higher when compared to CLL cells cultured alone. Here the authors co-cultured 28 CLL patient-derived cells with bone marrow derived natural killer cells, M2-10B4 fibroblasts or HS-5 stromal cells, This study, highlights the importance of considering cell-cell interactions (Vangapandu et al., 2017). Cai et al. (2016) reported that exposure of T-ALL cells to mesenchymal stem cells (MSCs) lowered their mitochondrial ROS levels and promoted a Warburg-like shift that is characterized by an increase in glucose uptake and production of lactate with associated reduction in ATP production and mitochondrial membrane potential. In addition, T-ALL cells co-cultured with MSCs have altered mitochondrial morphology

due to the extracellular signals and mediate phosphorylation of the pro-fission factor, dynamin-related protein 1 (Drp1) at residue S616. Supporting this was the observation that expression of S616-phosphorylated Drp1 recapitulates mitochondrial ROS levels, the mitochondrial dynamics, metabolic switching and chemo-resistance observed in T-ALL cells co-cultured with MSCs. A study conducted in primary lymphocytes and $CD34^+$ progenitors from ALL patients indicates that since the inhibitor of mitochondrial translation, tigecycline is able to sensitize them to increased apoptosis, ALL might have higher levels of oxidative metabolism (Fu et al., 2017). Interestingly, T-ALL with higher expression of Golgi-localization of oxysterol binding protein-related protein 4L (ORP4L) are characterized by higher levels of OXPHOS compared to normal T-lymphocytes (Zhong et al., 2016). Since higher ORP4L is associated with higher OXPHOS rate and it is upregulated in 80% of CML cases, CML cells are thought to have higher levels of oxidative metabolism compared to their normal counterparts (Henriques Silva et al., 2003). This was further investigated by Kuntz et al. (2017). Performing metabolic analyses on both stem cell-enriched $CD34^+CD38^-$ and $CD34^+$ and differentiated $CD34^-$ cells derived from patients with CML, the authors demonstrated that most primitive LSCs have higher mitochondrial activity than more differentiated LSCs and normal $CD34^+CD38^-$ cells. Importantly, they show that primitive CML cells are reliant on higher rates of oxidative metabolism for their survival.

AUTOPHAGY IN LSCs

In the last few decades the hypothesis that LSCs, as many other CSCs, have a high-energy demand has been supported by the results of a large number of studies. High rates of metabolism also correspond to high levels of cellular stress and this can damage cellular components. In this scenario, LSCs take advantage of a survival "sustainer" process, autophagy by using it as building block provider to address their metabolic requirements. As well as this, autophagy contributes in keeping cells healthy by reducing oxidative stress. Watson et al. (2015) indicated that human and mouse HSPCs exhibited lower mitochondrial stress and increased mitochondrial clearance due to increased autophagy when compared to more differentiated cells. Interestingly, they showed that ATG genes are in chromosomal regions that in AML are frequently deleted. Mice with ATG7 and ATG5 deficiency in the HSPCs compartment developed an early leukemic phenotype that compromised animals, leading to death. Importantly, deleting autophagy in both alleles in mixed lineage leukemia-eleven nineteen (MLL–ENL) model of AML enhanced glycolysis and proliferation *in vitro* and caused a more hostile leukemia *in vivo*. Since loss of ATG genes have also been identified in other malignancies, different studies have indicated that reduction in autophagy flux represents a "plus" for tumorigenesis. However, a recent study conducted in human $CD34^+$ AML cells demonstrated that low risk AML have enhanced autophagy while intermediate risk AML is associated with limited autophagy flux (Folkerts et al., 2017). To further characterize autophagy, $CD34^+$ AML cells were distributed into ROS-low and ROS-high sub-fractions. AML $CD34^+$ ROS-low cells exhibited higher basal autophagy and decreased survival following treatment with hydroxychloroquine (HCQ), an inhibitor of lysosomal fusion during autophagy, when compared with ROS-high cells. Furthermore, knockdown of ATG5 reduced maintenance of AML $CD34^+$ cells in NSG mice. Karvela et al. (2016) provided further understanding of how autophagy inhibition affects energy metabolism of CML cells. Loss of ATG7 impaired glycolysis and induced a distinctive mitochondrial metabolism profile, and the subsequent induction of ROS encouraged differentiation of $CD34^+$ CML cells in the erythroid lineage. Further investigations are still required to understand the metabolic profile of LSCs, which will also provide additional tools to elucidate the role of autophagy in sustaining leukemic metabolism.

Mitophagy and LSCs

As described previously, mitochondrial metabolism is one of the main sources of energy for LSCs. To assure that oxidative metabolism can meet LSCs energetic demand, their mitochondria need to be conformed to meet this. We have previously indicated mitophagy as the mechanism that removes injured mitochondria and, in this context a high demand for mitophagy will probably assure the replenishment of functioning mitochondria. However, this still remains to be investigated in detail. One of the few studies conducted in leukemia regarding mitophagy is a recent study in AML (Pei et al., 2018). The authors indicate that primitive AML-LSCs have higher expression of the mitochondrial dynamics regulator F1S1 compared to non-LSCs. Using valinomycin to stress mitochondria, they show that AML-LSCs overexpressing FIS1 have higher levels of mitophagy than non-LSCs. Loss of FIS1 in AML-LSCs impairs mitophagy, leading to myeloid differentiation, block in cell cycle and restricted LSC self-renewal ability.

Autophagy in the Initiation of Leukemia

Understanding what are the main features in the initiation of leukemia is still of high importance. Remodeling of autophagy function has been largely investigated in the transformation of HSCs to LSCs. Auberger and Puissant (2017) have recently proposed a detailed analysis of autophagy's primary participation in leukemogenesis (Auberger and Puissant, 2017). In the case of AML, several studies have demonstrated that autophagy genes are down regulated in AML cells (Brigger et al., 2013, 2014; Walter et al., 2009). A recent study by Visconte et al. (2017), conducting sequencing of the entire exome in patients with different type of myeloproliferative disorder including AML, found that expression of most relevant autophagy genes is compromised in 14% of patients. In accordance with this, two different studies have elucidated the effect of disrupted autophagy in AML initiation and progression. Mortensen et al. (2011) demonstrated that loss of the ATG7 gene in the mice hematopoietic niche enhanced the development of a myeloproliferative disorder. Based on that, Watson et al. (2015) demonstrated that deletion of ATG5 impairs autophagy, which results in developing a myeloproliferative disease in

the animals. Specifically, using a mixed lineage leukemia-eleven nineteen leukemia (MLL-ENL) AML mouse model the authors demonstrated that loss of ATG5 contributes to decreased glycolytic flux enhancing disease progression. Interestingly, they indicate that heterozygotes loss of ATG5 increased the progression of the disease while deletion in both alleles resulted premature death of mice prior to developing malignancy. Differently from AML, autophagy genes are upregulated in CML and loss of autophagy in $CD34^+$ CML cells results in compromised initiation of leukemia (Rothe et al., 2014; Karvela et al., 2016). A high-energy demand is also required for leukemia expansion. Ianniciello et al. (2017) reported that primary $CD34^+$ LSCs leaving hypoxic environment require metabolic adaptation to repopulate as well as for disease expansion. In the lymphoid counterpart, mRNA analysis showed that patients with CLL show increased expression of BECLIN1 and ATG5 genes than healthy controls (Kong et al., 2018). Based on these studies, autophagy seems to dictate LSC fate depending on the stage of the transformation, the type of leukemia and the presence of multiple mutations that can affect the progress in the disease.

Autophagy in LSCs Drug' Resistance

Several studies have shown that LSCs escape from drug treatment by upregulating autophagy. TKIs, the front-line treatment of CML, inhibit the oncogenic BCR-ABL tyrosine kinase activity and this induces autophagic flux (Bellodi et al., 2009; Helgason et al., 2013; Baquero et al., 2018). Arsenic trioxide is an alternative way to remove BCR-ABL, which requires induction of the cathepsin B, a lysosomal protease (Puissant et al., 2010; Goussetis et al., 2012). Interestingly, CML LSCs balance autophagy between a survival and apoptotic function. Autophagy is induced for BCR-ABL degradation upon TKIs treatment and at the same time promotes leukemic cell recovery following cessation of treatment (Crowley et al., 2013). Additionally, Helgason et al. (2011) highlighted autophagy inhibition with TKIs treatment as a strategic approach to eradicate LSCs. While current treatment for AML is based on combining anthracyclines with cytarabine, use of m-TOR inhibitors has been investigated. The results of these studies indicated that treatment with inhibitors of m-TOR increases protective autophagy flux in AML cells (Altman et al., 2014; Chen et al., 2017). A different approach investigated in AML is to combine conventional chemotherapy with statins, which act by restricting the last step of cholesterol synthesis (Hartwell et al., 2013). However, statins induce autophagy, which reduces the effect of treatment in targeting LSCs. A different approach tested for AML uses a recombinant arginase to deplete arginine levels in AML patients (Tanios et al., 2013). This is based on the observation that AML blast cells are dependent on income of arginine for their survival. While the effect of targeting arginine was promising in AML blasts, cytoprotective autophagy was increased. The same scenario has been indicated when the disease affects the lymphoid compartment. In T-ALL, approaches using inhibitors of NOTCH1 are used to weaken glutamine metabolism. However, NOTCH inhibitors act as a double edge sword: the effect on glutaminolysis is null since NOTCH inhibitors induce autophagy, which contributes to T-ALL metabolism (Herranz et al., 2015). Glucocorticoids are also used in the treatment for T-ALL (Jiang et al., 2015). Unfortunately, finding a cure for this disease seems to constantly run in to the same issues. Glucocorticoid effect on repressing m-TOR is translated in increased autophagy, which contributes to resistance to treatment. In the case of B-ALL, bortezomib, a proteasomal inhibitor has been indicated to increase cytotoxicity (Murata et al., 2009). However, to compensate for the loss of proteasomal activity and re-establish protein homeostasis following bortezomib treatment, autophagy is upregulated as a rescue mechanism (Milan et al., 2015; Wang et al., 2015).

CONCLUSION

Autophagy is indeed an important physiological process. However, what seems the answer in certain circumstances can be the problem in others. While HSCs use autophagy to protect themselves, it can also be involved in their malignant transformation. Importantly, LSCs upregulate autophagy potentially to provide building blocks/energy in stressful conditions, such as drug treatment. The relevance of combining current treatment with autophagy inhibition in LSCs have come to of a phase II clinical trial. The name of the study is CHOICES (CHlOroquine and Imatinib Combination to Eliminate Stem cells) and combines first line treatment for CML patients with HCQ. However, HCQ is a non-specific autophagy inhibitor and high doses are required to target autophagy in patients that might not be achievable, thus there is a need to develop more specific autophagy inhibitors to target autophagy in patients. ULK1 and VPS34 inhibitors have been established and *in vitro* results are promising (Dowdle et al., 2014; Egan et al., 2015; Petherick et al., 2015; Baquero et al., 2018). However, further analysis and *in vivo* studies using suitable robust pre-clinical models are still necessary to validate their ability to target autophagy in cancer patients and specifically in the context of leukemia.

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AI wrote the manuscript. All authors contributed editing and proofreading of the article.

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REVIEW
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Crosstalk Between Mammalian Autophagy and the Ubiquitin-Proteasome System

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Autophagy and the ubiquitin–proteasome system (UPS) are the two major intracellular quality control and recycling mechanisms that are responsible for cellular homeostasis in eukaryotes. Ubiquitylation is utilized as a degradation signal by both systems, yet, different mechanisms are in play. The UPS is responsible for the degradation of short-lived proteins and soluble misfolded proteins whereas autophagy eliminates long-lived proteins, insoluble protein aggregates and even whole organelles (e.g., mitochondria, peroxisomes) and intracellular parasites (e.g., bacteria). Both the UPS and selective autophagy recognize their targets through their ubiquitin tags. In addition to an indirect connection between the two systems through ubiquitylated proteins, recent data indicate the presence of connections and reciprocal regulation mechanisms between these degradation pathways. In this review, we summarize these direct and indirect interactions and crosstalks between autophagy and the UPS, and their implications for cellular stress responses and homeostasis.

Keywords: autophagy, UPS, proteasome, ubiquitylation, protein quality control, mitophagy, proteostasis, organelle homeostasis

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INTRODUCTION

The ubiquitin–proteasome system (UPS) and macroautophagy (hereafter referred as autophagy) are two major intracellular protein degradation pathways. Degradation of short-lived proteins through the UPS is initiated by sequential addition of ubiquitin chains to target proteins (Hershko, 1983, 2005; Finley, 2009). Polyubiquitylated proteins are then recognized by the subunits of multicatalytic protease complexes called proteasomes (Hershko and Ciechanover, 1998; Schwartz and Ciechanover, 2009).

Proteasomes are extremely efficient organelles that degrade short-lived proteins and soluble unfolded/misfolded proteins and polypeptides. On the other hand, long-lived proteins, insoluble protein aggregates (usually originating from misfolded proteins, disease-related mutant proteins) and dysfunctional organelles, such as degenerated mitochondria and peroxisomes, are eliminated by the autophagy-lysosome system (Groll and Huber, 2003, 2004; Klionsky, 2007). Autophagy is characterized by the formation of double-membrane structures termed as autophagosomes, which later on fuse with lysosomes, forming autolysosomes degrading autophagosome contents.

The UPS and autophagy are interconnected, and inhibition of one system was shown to affect the other. There is accumulating evidence in the literature about connections between the UPS

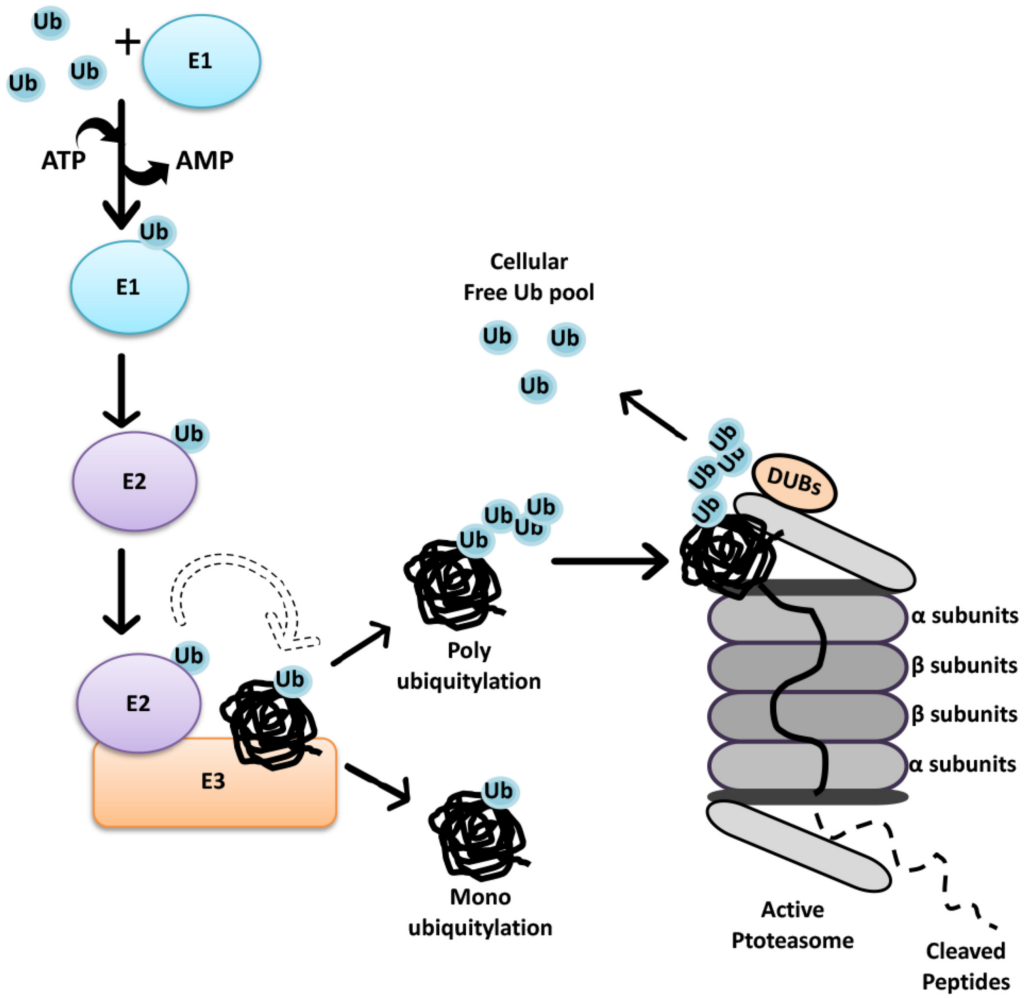


FIGURE 1 | The Ubiquitin-Proteasome System. Initially through C-terminal glycine, ubiquitin is attached to a cysteine residue of an activating enzyme, E1, in an ATP-dependent manner. The active ubiquitin is then associated with a cysteine residue of an ubiquitin conjugating enzyme, E2. Finally, specificity of ubiquitin transfer is ensured by E3 ubiquitin ligase family of proteins that bind to selected protein subsets (Hershko and Ciechanover, 1998). In the case of RING finger E3 ligases, the transfer of ubiquitin is direct from E2-ubiquitin to the substrate, even if the presence of E3 is required for substrate selection. At present, 2 genes are known to encode E1 isoforms, at least 40 genes encode E2's, and over 600 E3 ubiquitin ligases were defined in the human genome (Pickart and Eddins, 2004; Clague et al., 2015). Each E1 isoform reveals a distinct preference for different E2 enzymes, while association of E2 and E3 depend on cellular context, generating extensive combinatorial complexity.

and autophagy. In this review article, we will first briefly summarize the two systems, and then discuss in detail various examples of coordination and crosstalk between them. For more detailed discussion on individual systems, the readers are referred to recently published excellent review articles (Collins and Goldberg, 2017; Kwon and Ciechanover, 2017; Mizushima, 2018; Yu et al., 2018). This review article mainly focuses on the mammalian system and advances in this field. For crosstalk in other systems, such as plants, readers should check other recent and relevant reviews [for example see, (Minina et al., 2017)].

The Ubiquitin-Proteasome System

Ubiquitylation-dependent degradation is involved in the regulation of several cellular processes, including protein quality control, transcription, cell cycle progression, DNA repair, cell stress response and apoptosis. For example during cell cycle regulation, timely progression of each phase of the cycle rely on sequential transcription and degradation of cell cycle proteins, such as cyclins (Glotzer et al., 1991; Benanti, 2012). During apoptosis, ubiquitylation leading the degradation of survivin depends on ubiquitin ligase XIAP (Arora et al., 2007; Altieri, 2010; Delgado et al., 2014).

Ubiquitylation involves the addition of the small protein ubiquitin to specific lysine residues on the target proteins. Covalent attachment of ubiquitin to protein targets occurs through a three-step mechanism involving E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligase) enzymes as summarized in **Figure 1** (Hershko and Ciechanover, 1998). At least seven lysine (K) residues in the ubiquitin protein are involved in the polyubiquitin chain

formation (K6, K11, K27, K29, K33, K48, or K63). Initially, K48-linked ubiquitin chain formation was introduced as the degradation signal for proteasomal degradation. In contrast, K11 or K63 chains or single ubiquitin moieties (monoubiquitylation) were initially connected to non-proteolytic functions (Welchman et al., 2005; Behrends and Harper, 2011). However, recent reports indicate that K63-linked ubiquitin chains as well as various other chains prime substrates for autophagic elimination (Tan et al., 2008b).

The 26S proteasome is an ATP-dependent protease complex, consisting of a core complex, the 20S proteasome and a regulatory complex, the 19S proteasome cap. The 20S proteasome forms a barrel-shape structure with two end rings formed by α subunits regulating the entry of unfolded proteins, and two middle rings are composed of β subunits harboring proteolytic activity (Heinemeyer et al., 2004). Substrates must be unfolded and then guided by α subunits prior to catalytic cleavage. At the end, polypeptides are chopped into 3–25 amino acid long fragments, and further cleavage to single amino acids is carried out by peptidases (Tomkinson and Lindås, 2005) (**Figure 1**). By this way, recycling of proteins result in the generation of amino acids that are ultimately reused by cells in the synthesis of new proteins.

The 26S proteasome contains an additional 19S cap structure that further regulates the internalization of ubiquitylated substrates (Lander et al., 2012). The central part of the 19S cap consists of six AAA ATPases (Rpt1–Rpt6) forming the Rpt ring that is responsible for substrate binding and unfolding as well as substrate transfer through the channel (Collins and Goldberg, 2017). Non-ATPase proteins such as Rpn10 and Rpn13 in the 19S cap, possess ubiquitin-binding domains and therefore function as receptors for ubiquitin-labeled substrates (Finley, 2009).

Recent studies showed that ubiquitylation is a reversible phenomenon. Deubiquitinating enzymes (DUBs) are proteases that remove ubiquitin or ubiquitin-like molecules from substrates and disassemble polyubiquitin chains. DUBs regulate UPS-mediated degradation in different cellular contexts (Reyes-Turcu et al., 2009; He et al., 2016; Pinto-Fernandez and Kessler, 2016). Moreover, they play an important role in the control of available free ubiquitin pool in cells, allowing recycling and reuse of ubiquitin. Some DUBs are also responsible for processing newly synthesized ubiquitin precursors (Komander et al., 2009; Lee et al., 2011; Grou et al., 2015; Collins and Goldberg, 2017).

Autophagy

There are three major types of autophagy: Macroautophagy, microautophagy and chaperon-mediated autophagy (CMA). In this review, we chose to focus on macroautophagy (herein autophagy). CMA and microautophagy were discussed in elsewhere (Kaushik and Cuervo, 2018; Oku and Sakai, 2018).

Autophagy is characterized by the engulfment of cargo molecules by double-membrane vesicles, called autophagosomes (Klionsky, 2007; Mizushima, 2010, 2018; Lamb et al., 2013). Following closure, autophagosomes are transported by the microtubule system, leading to their fusion with late endosomes and lysosomes, forming autolysosomes. In this new compartment, sequestered cargos are degraded by the action of lysosomal hydrolases. Building blocks that are generated by hydrolysis of macromolecules (e.g., amino acids from protein degradation) are then transferred back to cytosol for reuse (**Figure 2**). Active at a basal level, autophagy is upregulated following a number of stimuli and stress conditions. Amino acid deprivation, serum starvation and growth factor deprivation, hypoxia, exposure to various chemicals and toxins might be counted among stress conditions activating autophagy.

Most autophagy inducing signals converge at the level of mTOR protein complexes (mTORC1 and mTORC2) that coordinate anabolic and catabolic processes (Sabatini, 2017; Saxton and Sabatini, 2017) (**Figure 2**). Cellular energy sensor AMPK directly regulates mTOR and therefore contributes to the regulation of the autophagic activity. Moreover, the ERK/RSK pathway, PI3K/AKT pathway, amino acid sensor RAG system as well as hypoxia are among autophagy-related pathways converging at the level of mTOR. Under normal conditions, mTORC1 limits the autophagic activity through inactivation of the ULK1/2 autophagy complex. mTORC1-dependent phosphorylation of ULK1 and Atg13 (Hosokawa et al., 2009) result in the inactivation of ULK1/2 complex and down regulation of autophagy. Under stress, mTORC1 is inhibited and ULK1/2 complex dephosphorylated. ULK1/2 then phosphorylates itself, Atg13 and FIP200 and activate autophagy.

A class III phosphatidylinositol 3-kinase (PI3K) complex, including the lipid kinase VPS34 and the regulatory protein Beclin1, controls the membrane nucleation stage and initial phagophore formation. Phosphatidylinositol 3-phosphate (PtdIns3P) that is generated by PI3K activity serves as a landing pad for autophagy-related proteins containing PI3P-binding domains (e.g., FYVE-domains). Among them WIPI1-4 and DFCP1 were involved in the formation of a membrane structure called omegasome or cradle, a structure that creates a platform for the elongation of autophagosome precursor isolation membranes (Mauthe et al., 2011; Mercer et al., 2018).

Elongation of the isolation membrane depends on two ubiquitin-like conjugation systems. In the first system, autophagy-related gene 12 (ATG12) is covalently conjugated to the ATG5 protein through the action of ATG7 (E1-like) and ATG10 (E2-like) proteins. Then, recruitment of the ATG16L1 protein to ATG12-5 dimer results in the formation of a larger complex. Then forming ATG12-5-16L1 oligomers serve as E3 ligases that conjugate lipid molecules (such as phosphatidylethanolamine) to ATG8 orthologs MAP1LC3, GATE16, GABARAP (Mizushima et al., 2011; Shpilka et al., 2011; Tsuboyama et al., 2016). Lipid-conjugated ATG8 proteins are required for the elongation, expansion and closure of autophagosome membranes (Nakatogawa et al., 2007).

In order to acquire lytic capacity, autophagosomes fuse with late endosomes or lysosomes. In mammalian cells, fusion requires lysosomal integral membrane protein LAMP-2, several SNARE proteins (e.g., STX17 and WAMP8) and RAB proteins (e.g., RAB5 and RAB7) (Tanaka et al., 2000; Jager, 2004). Following fusion of the outer membrane of autophagosomes, materials contained in the inner membrane are degraded by the action of lysosomal hydrolases (Tanida et al., 2004). Building blocks (e.g.,

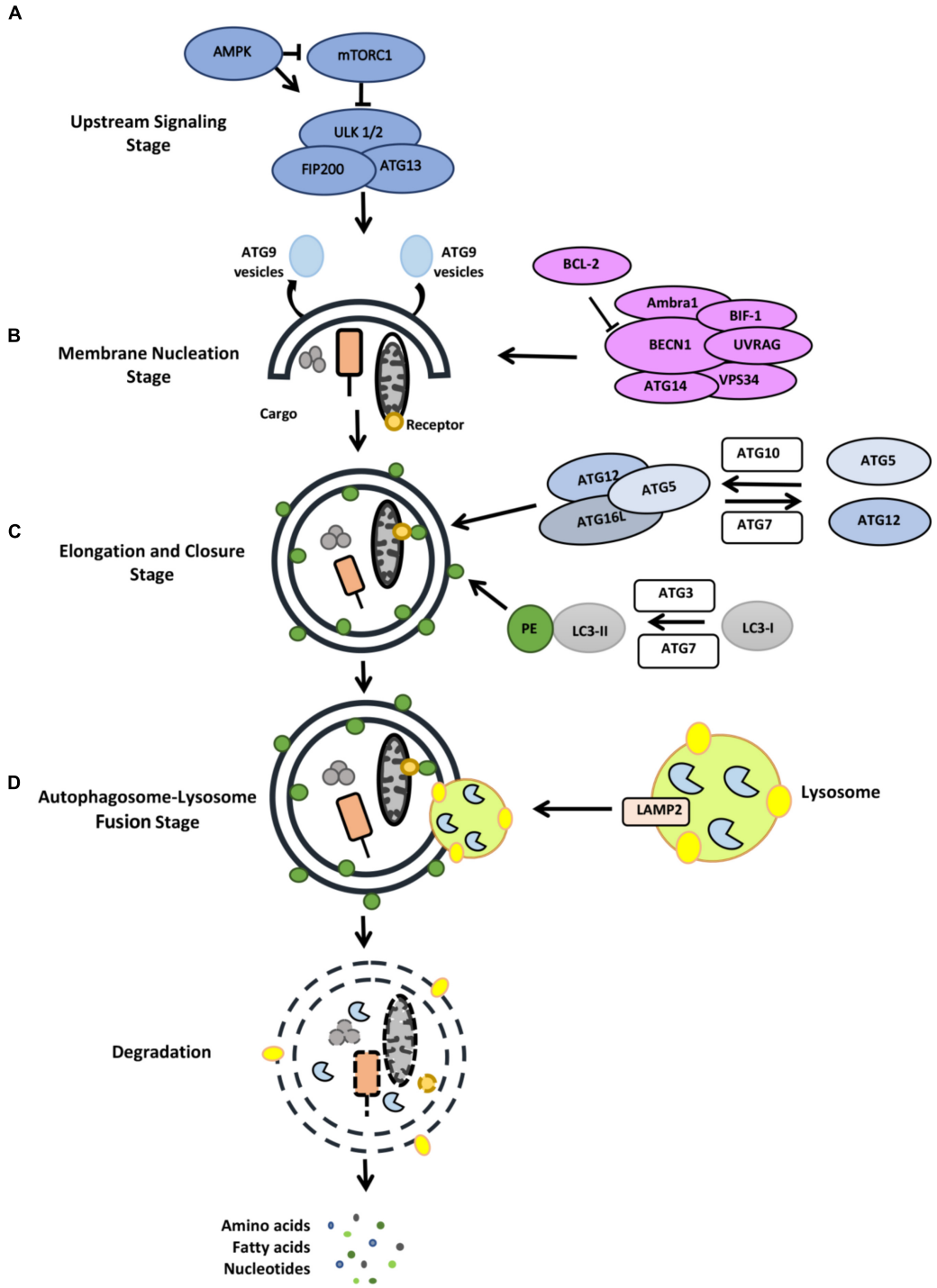


FIGURE 2 | Stages of the autophagy pathway (for detail, see the text). **(A)** Upstream signaling, **(B)** membrane nucleation stage, **(C)** elongation and closure stage, **(D)** autophagosome-lysosome fusion stage.

amino acids, fatty acids etc.) are then transported back to cytosol for reuse in the metabolic processes of the cells.

Autophagic vesicles engulf targets such as portions of cytoplasm and various cytoplasmic components in a non-selective manner. On the other hand, several selective forms of autophagy have been described (Kraft et al., 2010; Anding and Baehrecke, 2017). In most cases, ubiquitylation of the cargo constitutes a key step in the chain of events leading to its autophagic removal (Kirkin et al., 2009; Rogov et al., 2014). Selective targets include mitochondria (Okamoto

et al., 2009), peroxisomes (Till et al., 2012), lysosomes (Hung et al., 2013), endoplasmic reticulum (ER) (Khaminets et al., 2015), ribosomes (An and Harper, 2018), cytoplasmic protein aggregates (Lamark and Johansen, 2012), pathogenic intracellular invaders (Wileman, 2013) and even certain free proteins and RNAs (Huang et al., 2015) were shown to be targets of selective autophagy. By this way, cells control number of the organelles, eliminate dysfunctional components and get rid of potentially harmful aggregates and invaders.

Selectivity is ensured by target-specific autophagy receptors that form a bridge between the ubiquitylated cargo and LC3 component of autophagic membranes. Selective autophagy relies on the recognition and binding capacity of autophagy receptors to various types of cargo, including mitochondria (OPTN, NDP52, Tax1BP1, NIX, FUNDC1) (Novak et al., 2010; Sarraf et al., 2013; Wong and Holzbaur, 2014; Lazarou et al., 2015; Chen et al., 2016), peroxisomes (NBR1) (Deosaran et al., 2013), lysosomes (galectin-3) (Hasegawa et al., 2015), ER (FAM134B, SEC62, RTN3, and CCPG1) (Khaminets et al., 2015; Fumagalli et al., 2016; Grumati et al., 2017; Smith et al., 2018) and intracellular ubiquitylated aggregates (p62, NBR1, OPTN, TOLLIP receptors) (Pankiv et al., 2007; Kirkin et al., 2009; Korac et al., 2013; Lu et al., 2014), bacterial invaders (p62, OPTN, NDP52 receptors) (Thurston, 2009; Zheng et al., 2009; Wild et al., 2011). LC3-interacting region (LIR) is the common motif which allows autophagy receptors to bind lipidated LC3. On the other hand, ubiquitin-associated domain (UBA domain) on autophagy receptors are responsible for the recognition of ubiquitin decorated cargos (Khaminets et al., 2016). Cargos that are wrapped and packed in autophagosomes are then ready for delivery and degradation in lysosomes.

THE UPS-AUTOPHAGY CONNECTION

The UPS and autophagy are the two major and evolutionarily conserved degradation and recycling systems in eukaryotes. Although their activities are not interdependent, recent studies show that connections and crosstalks exist between the two systems. Mitophagy constitutes a prominent example connecting these two degradative systems, yet several other examples exist. In this section, we will summarize biological events involving autophagy and the UPS, and discuss molecular details of the crosstalk mechanisms.

Compensation Between the Two Degradative Pathways

Initial observations about functional connections between the UPS and autophagy systems revealed that inhibition of one led to a compensatory upregulation of the other system. In order to maintain homeostasis, cellular materials that accumulate following inhibition of one degradative system needs to be cleared, at least in part, by the other system (**Figure 3**). Here, we will give examples of scenarios where these compensation mechanisms are operational.

Inhibition of the UPS using various compounds (e.g., MG132, bortezomib, lactacystine etc.) (Wu et al., 2008; Selimovic et al., 2013; Fan et al., 2018) or by genetic approaches (Demishtein et al., 2017) resulted in the upregulation of the autophagic activity in cells (**Figure 3**). For example, inhibition of proteasomal activity by the proteasome inhibitor and chemotherapy agent bortezomib led to an increase in the expression of autophagy genes ATG5 and ATG7, and induced autophagy. In fact, autophagy gene upregulation depended on an ER stress-dependent pathway that involved eukaryotic translation initiation factor-2 alpha (eIF2α) phosphorylation (Zhu et al., 2010). In another study, proteasome inhibition was associated with an increase in p62 and GABARAPL1 levels by Nrf1-dependent and -independent pathways prior to autophagy activation (Sha et al., 2018). In other contexts, MG132-mediated proteasome inhibition resulted in a decrease in cell proliferation, cell cycle arrest at G2/M phase and stimulation of autophagy through upregulation of Beclin1 and LC3 (Ge et al., 2009).

Autophagy induction following proteasome inhibition correlated with AMPK activation as well. A number of studies provided evidence that proteasomal inhibition is sensed by both AMPK and mTORC1, two major regulators of autophagy.

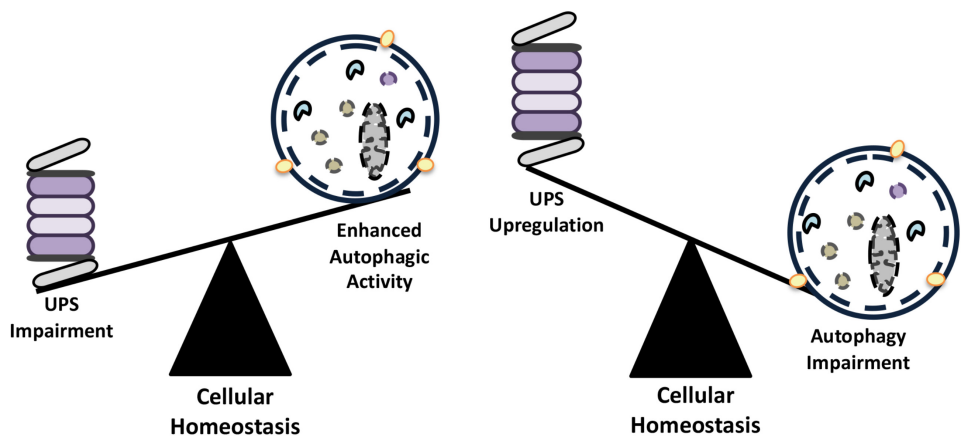


FIGURE 3 | The compensatory balance between the activities of autophagy and the UPS in order to maintain cellular homeostasis.

For instance, in macrophages, epitelial and endothelial cells, proteasome inhibition using chemicals resulted in the activation of AMPK (Xu et al., 2012; Jiang et al., 2015). In some other cancer cell types, CaMKKβ and glycogen synthase kinase-3β (GSK-3β) were identified as upstream regulators of AMPK activation, proteasome inhibition was linked to a decrease in GSK-3β activity and to the activation of AMPK and autophagy (Sun et al., 2016). On the other hand, Torin-1- or rapamycin-mediated inhibition of mTOR stimulated long-lived protein degradation through activation of both UPS and autophagy (Zhao et al., 2015; Zhao and Goldberg, 2016). In retinal pigment epithelial cells, inhibition of proteasome by lactacystin and epoxomicin was shown to block the AKT-mTOR pathway and induce autophagy (Tang et al., 2014). SiRNA-mediated knockdown of Psmb7 gene coding for the proteasome β2 subunit, resulted in enhanced autophagic activity, and it was linked the mTOR activation status of cultured cardiomyocytes (Kyrychenko et al., 2013).

Similarly, impairment of autophagy correlated with the activation of the UPS. In colon cancer cells, chemical inhibition of autophagy and small RNA mediated knock down of ATG genes resulted in the upregulation of proteasomal subunit levels, including the catalytic proteasome β5 subunit, PSMB5 and led to increased UPS activity (Wang et al., 2013). In another study, 3-MA-mediated autophagy inhibition in cultured neonatal rat ventricular myocytes (NRVMs) increased chymotrypsin-like activity of proteasomes (Tannous et al., 2008).

Since proteasomes were identified as autophagic degradation targets (proteaphagy), enhanced proteasome peptidase activity following autophagy inhibition might be associated with the accumulation of proteasomes (Cuervo et al., 1995; Marshall et al., 2015). Yet in several cases, autophagy inhibition correlated with the accumulation of ubiquitylated proteins. For instance in independent studies with ATG5 or ATG7 knockout mice, accumulation of ubiquitylated conjugates were observed, especially in the brain and the liver of the animals (Komatsu et al., 2005, 2006; Hara et al., 2006; Riley et al., 2010). Similar results were observed in other animal models such as *Drosophila* (Nezis et al., 2008). In line with these data, inhibition of autophagy through siRNA-mediated knockdown of ATG7 and ATG12 in HeLa cells resulted in the impairment of UPS, accumulation of ubiquitylated proteins as well as other important UPS substrates, including p53 and β-catenine (Korolchuk et al., 2009a). In above-cited papers, autophagy impairment followed by the autophagy receptor p62 accumulation in cells, and played a key role in the observed UPS defects.

Ubiquitylation was proposed to be a common component that directs substrates to the proper degradation system and even contribute to the UPS-autophagy crosstalk (Korolchuk et al., 2010; Dikic, 2017). According to this view, proteins that are predominantly linked to K48-based ubiquitin chains are generally directed for degradation through UPS. Conversely, aggregates that are linked to K63-based ubiquitin chains are directed for autophagic degradation. P62 binding capacity was introduced as the critical step in the choice between the UPS and autophagy. Although, p62 is able to attach both K48- and K63-linked ubiquitin chains through its UBA domain, binding affinity of the protein for K63-linked chains seems to be higher (Long et al., 2008; Tan et al., 2008a; Wooten et al., 2008). Due to this dual ubiquitin binding ability, p62 might show UPS inhibitory effects in some contexts. A competition between p62 and p97/VCP (a ubiquitin binding ER-associated degradation protein) determined the fate of ubiquitylated proteins in cells (Korolchuk et al., 2009a,b). Over expression of p97/VCP protein prevented binding of p62 to ubiquitylated substrates, and directed them for degradation by the UPS. On the other hand, accumulation of p62 following autophagy inhibition led to the sequestration of proteins that were otherwise p97/VCP targets.

In summary, in the case of a defect in one of the two degradation systems, the other system is upregulated in order to eliminate ubiquitylated protein substrates. Yet, compensation does not always work and its success largely depends on cell types, cellular and environmental conditions and target protein load.

Interplay Between the UPS-Autophagy in the Selective Clearance of Cytosolic Proteins

Function of proteins depend on their proper folding and 3D structures. Various insults, including heat shock, organellar stress, oxidative stress etc., might lead to the accumulation of unfolded or misfolded proteins. Moreover several disease-related mutations were associated with folding problems. Failure to refold result in dysfunctional or malfunctional, hence toxic protein accumulations, activation of stress and even cell death pathways. In order to control toxic protein accumulations, an active process of protein aggregate formation comes into play. Additionally some proteins, including mutant proteins are already prone to form aggregates. Selective clearance of most cytosolic proteins require ubiquitylation. Depending on their solubility, ubiquitylated proteins and protein aggregates are then cleared by the UPS or autophagy.

Soluble fractions of proteins with a folding problem are recognized by the chaperone machinery and directed to the UPS for degradation. Hsp70 and Hsp90 chaperone interactor CHIP was identified as one of the E3 ligases that are responsible for K48-linked ubiquitin chain addition to unfolded/misfolded proteins. BAG family proteins, especially BAG1, interact with the Hsp70 complex and induce proteasomal degradation of client proteins.

On the other hand, clearance of insoluble aggregate-prone proteins require formation of aggresomes. Ubiquitylation by a number of different E3 ligases, including CHIP, Parkin, HRD1 and TRIM50 prime aggregate-prone proteins (Olzmann et al., 2007; Mishra et al., 2009; Zhang and Qian, 2011; Mao et al., 2017). HDAC6 is another protein that plays a key role in the process of aggresome formation. HDAC6 was shown to provide the link between K63-based ubiquitylated aggregates and microtubule motor protein dynein (Matthias et al., 2008; Olzmann et al., 2007). Then, dynein-mediated mechanism direct the aggregates toward microtubule organizing centers (MTOCs), resulting in their piling of as aggresomes (Johnston et al., 1998; Kopito, 2000) (**Figure 4**). Following aggresome formation, direct interaction of adaptor proteins p62 and NBR1 with ubiquitylated aggregates result in their delivery to autophagosomes (Ichimura et al., 2008; Lamark and Johansen, 2012). Another autophagy-related protein, ALFY, was also identified as a player in the selective autophagy

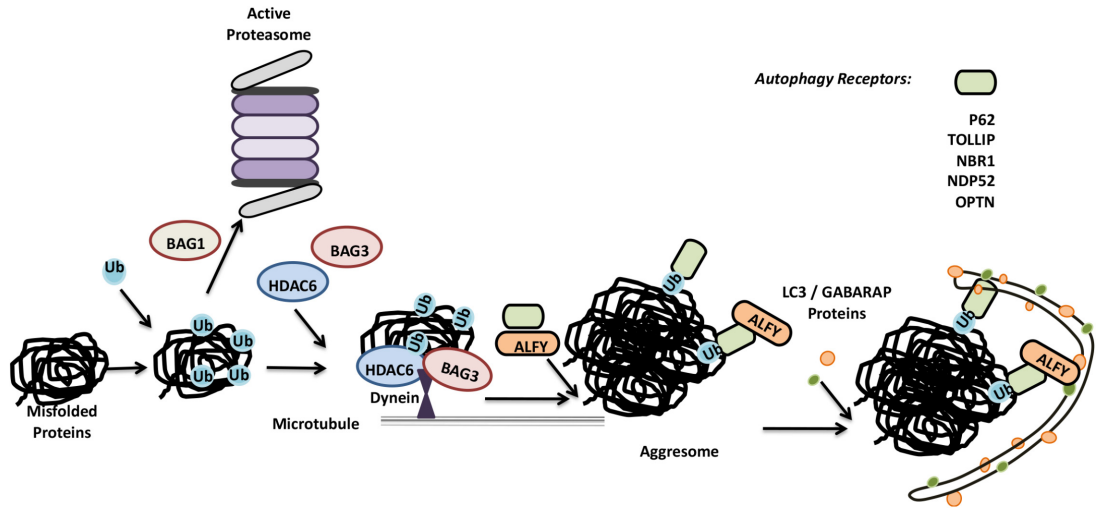


FIGURE 4 | Misfolded proteins can be eliminated by both the UPS and autophagy system. Misfolded proteins are ubiquitylated and based on the differences in ubiquitin linkages and ubiquitin binding proteins, they are directed for proteasomal degradation or further accumulated in aggresomes. Aggresomes are selectively cleared by autophagy.

and degradation of aggresomes (Clausen et al., 2010; Filimonenko et al., 2010).

An alternative pathway for aggresome formation require Hsp70 partner proteins BAG3 and CHIP (Zhang and Qian, 2011). Similar to HDAC6, BAG3 binds to dynein, and this directs Hsp70 substrates to aggresomes. However, BAG3-dependent aggresome formation was not dependent on the ubiquitylation of substrates as in the case of HDAC6, and CHIP E3 ligase activity was dispensible (Gamerdinger et al., 2011; Zhang and Qian, 2011). Yet, E3 ligases such as CHIP were required for BAG3-dependent aggresome clearance by autophagy (Klimek et al., 2017).

Proteolytic Degradation of the UPS or Autophagy Components as a Mutual Control Mechanism

Until so far, we focused on the UPS and autophagy as complementary but independent mechanisms. However, there are cases where components of one system were reported to be a proteolytic target of the other system. For example, a number of autophagy proteins were regulated through degradation by the UPS. On the other hand, even the whole proteasomes were shown be selective targets of autophagic degradation. Here, we will give examples of how mutual regulation through proteolysis contributes to the crosstalk and the interplay between the two systems.

Control of the UPS by the Autophagic Activity

Early studies indicated that proteasomes could be degraded in lysosomes (Cuervo et al., 1995). Later on, plant studies revealed that lysosomal degradation of 26S proteasomes occurred by a specific form of selective autophagy, proteaphagy (Marshall et al., 2015). RPN10 protein was introduced as an ATG8 interacting plant proteaphagy receptor. Unlike the plant protein, yeast and mammalian RPN10 failed to interact with ATG8/LC3. Instead, Cue5 protein in the yeast and its human ortholog TOLLIP, were introduced as selective receptors regulating proteasome clearance by autophagy (Lu et al., 2014). Moreover, p62 was also described as another proteaphagy receptor (Cohen-kaplan et al., 2016). For example, in mammals, amino acid starvation significantly upregulated ubiquitylation of 19S proteasome cap components RPN1, RPN10, RPN13, and led to their p62-mediated recruitment to autophagosomes (Cohen-kaplan et al., 2016) (**Figure 5**). Interestingly during carbon or nitrogen starvation, plant and yeast proteasomes were shown to localize in proteasomal storage granules (PSGs), protecting them from autophagic degradation during stress (Peters et al., 2016; Marshall and Vierstra, 2018). Whether similar mechanisms exist in the mammals is currently an open question. These observations underline the importance of selective degradation of proteasome by autophagy in the control of proteasome numbers as well as overall UPS and lytic activity in cells.

Control of Autophagy Components by the UPS

Modulation of the half-life of some proteins in the autophagy pathway by the UPS serves as a means to control cellular autophagic activity. For instance, LC3 protein was shown to be processed in a stepwise manner by the 20S proteasome, a process that was inhibited by p62 binding (Gao et al., 2010). On the other hand, E3 ligase NEDD4-mediated K11-linked ubiquitylation of Beclin1 prevented its binding to the lipid kinase VPS34, and led to its degradation (Platta et al., 2012). Another E3 ligase, RNF216 ubiquitylated Beclin1 adding K48-linked ubiquitin chains on the protein (Xu et al., 2014). Beclin1 ubiquitylation resulted in autophagy blockage in both cases. Conversely, reversal of Beclin1 ubiquitylation by the DUB protein USP19 stabilized the protein under starvation conditions and promoted autophagy (Jin et al., 2016). USP10 and USP13 as well as USP9X were characterized as other DUBs that regulated

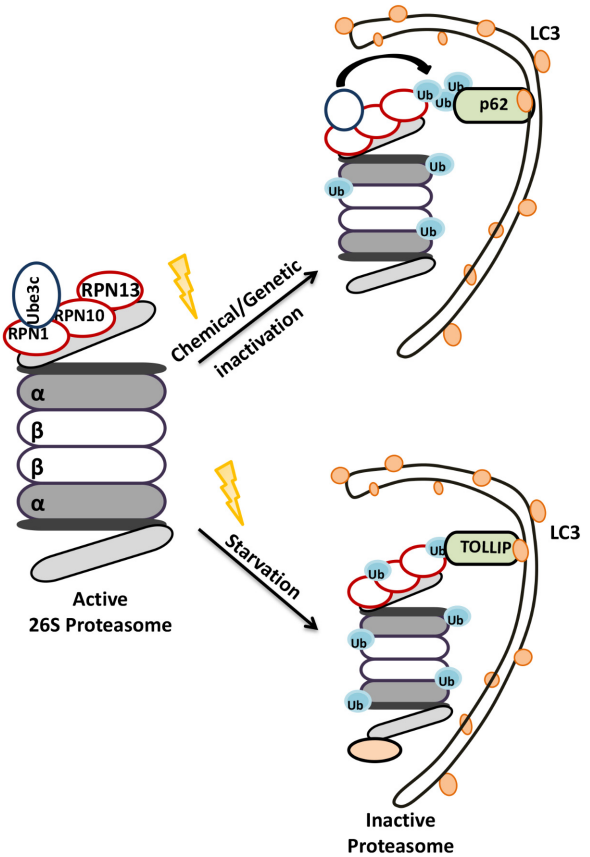


FIGURE 5 | Schematic representation of the selective degradation of proteasomes by autophagy. Upon starvation and functional defects proteasomes become ubiquitylated and degraded by autophagic machinery.

autophagy through control of Beclin1 stability (Liu et al., 2011; Jin et al., 2016).

Beclin1 is not the only autophagy protein that is targeted by the UPS in a controlled manner. G-protein-coupled receptor (GPCR) ligands and agonists were reported to regulate cellular Atg14L levels, and therefore autophagy, through ZBTB16-mediated ubiquitylation of the protein (Zhang T. et al., 2015). Serum starvation increased GSK3β-mediated phosphorylation of ZBTB16, leading to its degradation. Under these conditions, stabilization of Atg14L restored of autophagy. AMBRA1 is another UPS-controlled autophagy protein. Cullin-4 was identified as an E3 ligase that was responsible for the ubiquitylation of AMBRA1, dooming it for degradation under nutrient-rich conditions where autophagy should be inhibited (Antonioli et al., 2014). The PI3K complex subunit p85b is another example. Ubiquitylation of this autophagy signaling component by the E3 ligase SKP1 led to a decrease in its cellular levels and stimulated autophagic activity (Kuchay et al., 2013).

Ubiquitylation of some autophagy proteins did not result in their immediate proteasomal degradation, yet the post-translational modification provided an extra layer of control for the autophagy pathway. For instance, autophagy receptor OPTN was ubiquitylated as a target of the E3 ligase HACE1, and K48-linked ubiquitylation regulated the interaction of the protein with p62 (Liu Z. et al., 2014). TRAF6, a central E3 ligase of the NF-κB pathway, participated controlled ULK1 activity through K63-linked ubiquitylation. Under nutrient-rich conditions, mTOR phosphorylated AMBRA1 leading to its inactivation. When nutrients were limiting, mTOR inhibition resulted in AMBRA1 dephosphorylation and increased the interaction of the protein with TRAF6. This event facilitated ULK1 ubiquitylation by TRAF6 (Nazio et al., 2013). Ubiquitylation of ULK1 resulted in the stabilization of the protein, controlled its dimerization and regulated its kinase activity. Another ubiquitin-dependent regulation mechanism involved AMBRA1-Cullin-5 interaction in the regulation of mTOR complex component DEPTOR (Antonioli et al., 2014). Above-mentioned AMBRA1-Cullin-4 complex dissociated under autophagy-inducing conditions, allowing AMBRA1 to bind another E3 ligase, Cullin-5. This newly formed complex was shown to stabilize DEPTOR and induce mTOR inactivation, providing a negative feed-back loop in the control of autophagy (Antonioli et al., 2014).

In another study, TLR4 signaling triggered autophagy through Beclin1 ubiquitylation and stabilization. TLR4-associated TRAF6 protein was identified as the E3 ligase responsible for K63-linked ubiquitylation of Beclin1 at its BH3 domain. This modification blocked inhibitory BCL-2 binding to the protein, and free Beclin1 could activate autophagy (Shi and Kehrl, 2010). On the other hand, the deubiquitinating enzyme A20 reversed TRAF6-mediated ubiquitylation of Beclin1, resulting in autophagy inhibition (Shi and Kehrl, 2010). Another K63-linked ubiquitylation event on Beclin1 was promoted by AMBRA1 protein. In the same context, the WASH protein interacted with Beclin1, blocked AMBRA1-mediated Beclin1 ubiquitylation, and suppressed autophagy (Xia et al., 2013).

LC3 and p62 were also subjected to regulatory ubiquitylation. NEDD4 was identified as the E3 ligase in these reactions. NEDD4 was reported to interact with LC3 (Sun et al., 2017) and p62 (Lin et al., 2017), and LC3 binding to NEDD4 stimulated its ubiquitin ligase activity on the p62 protein (Sun et al., 2017). Moreover, NEDD4 deficient cells exhibited aberrant p62 containing inclusions, indicating the defect in aggresome clearance (Lin et al., 2017). Hence, NEDD4 is important for the regulation of p62 function and autophagy.

Xenophagy: Removal of Intracellular Invaders

Another essential function of autophagy is the clearance of intracellular pathogens. This special form of autophagy, called xenophagy, is a result of a cooperation between the ubiquitylation machinery and the autophagy pathway. Pathogens such as *Streptococcus pyogenes, Mycobacterium tuberculosis, Listeria monocytogenes,* and *Shigella flexneri* were identified as autophagy targets (Gutierrez et al., 2004; Kirkegaard et al., 2004; Ogawa et al., 2005). As a form of selective autophagy, xenophagy involves cargo labeling with ubiquitin, followed by the recognition by autophagy receptors (**Figure 6**). K48- and K63-linked and linear M1-linked ubiquitin chains were shown to mediate recognition

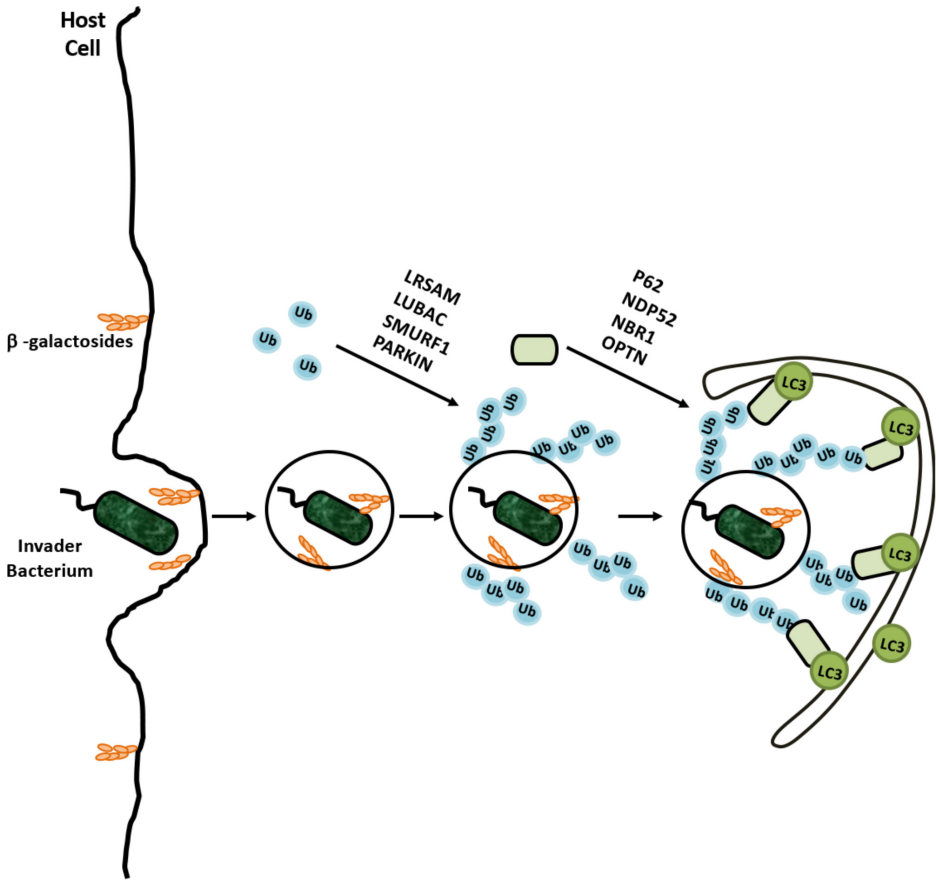


FIGURE 6 | Selective degradation of invaders by xenophagy is example of coregulation of the UPS and autophagy. Cellular degradation of invading bacterium was ubiquilated by various E3 ligases and recognized by adaptor proteins for recruitment autophagic membranes around bacterium.

of different pathogens by the xenophagy machinery (Collins et al., 2009; Randow and Youle, 2014).

Ubiquitylation frequently occurs on various cell penetrating parasites as well as on disrupted endosomes, providing an "eat me" signal for xenophagy. For example, *Salmonella enterica* serovar Typhimurium was heavily ubiquitylated in mammalian cells, and activation of xenophagy restricted intracellular bacteria numbers (Birmingham et al., 2006). Recent studies showed that, bacterial outer membrane-associated and integral membrane proteins were targets of ubiquitylation (Fiskin et al., 2016). A number of E3 ligases were involved in xenophagy, including Parkin, RNF166, ARIH1, HOIP, and LRSAM1 (Huett et al., 2012; Manzanillo et al., 2013; Heath et al., 2016; Franco et al., 2017; Lobato-Márquez and Mostowy, 2017).

For example, both K48- and K63-linked ubiquitylation were observed on Mycobacterium, and Parkin was identified as the E3 ligase catalyzing the K63-linked ubiquitylation (Collins et al., 2009; Manzanillo et al., 2013). Moreover endosome-free areas on the intracellular *Salmonella* Typhimurium contained a directly attached ubiquitin coat, and addition of linear M1-linked ubiquitin chains by the E3 ligase HOIP of the LUBAC on these ubiquitins contributed to the autophagy of the intracellular parasite (Noad et al., 2017). Xenophagy receptors that were described to date include p62, OPTN, NDP52, and NBR1 (Thurston, 2009; Zheng et al., 2009; Wild et al., 2011). These receptors were reported to bind pathogen- and/or endosome-associated ubiquitin, and directing the selective targets to autophagic membranes (Wild et al., 2011; Richter et al., 2016).

The interplay between ubiquitylation and autophagy achieves the important task of keeping host cells pathogen-free and providing an intracellular innate immune defense mechanism against invaders. In some reports, ubiquitylated bacteria were found to be surrounded by proteasomes as well (Perrin et al., 2004) and proteasomal activity might also be required for efficient killing of intracellular parasites (Iovino et al., 2014). Whether in the elimination of invading organisms, the crosstalk between the UPS and autophagy systems goes beyond ubiquitylation, needs further consideration. As discussed below, cellular mechanisms controlling commensal-turned ancient intracellular microorganisms, namely mitochondria, indeed rely on the function of both the UPS and autophagy.

Mitophagy: Mitochondrial Turnover

Mitochondria are vital organelles that form an intracellular dynamic network in the cytosol of eukaryotic cells. Through

fusion and fission, they are constantly made and destroyed. Under steady state conditions, mitochondria might be eliminated by basal in a non-selective manner. On the other hand, elimination of damaged, dysfunctional or superfluous mitochondria requires a selective form of autophagy called mitophagy (Lemasters, 2005). Programmed elimination of mitochondria during development and differentiation (e.g., reticulocyte maturation to erythrocyte, in oocytes after fertilization, during lens formation in the eye) also relies on mitophagy (Schweers et al., 2007; Song et al., 2016; Esteban-Martínez et al., 2017). Recent studies showed that mitophagy is a biological phenomenon that involves both the UPS and autophagy. In this section, we will discuss mechanisms of mitophagy, and analyze connections between the UPS and autophagy in this context.

PINK1/Parkin-Dependent Mitophagy

Depending on the E3 ligase that ubiquitylates proteins on mitochondria, mitophagy can be divided into two major forms: Parkin-dependent and Parkin-independent mitophagy. The E3 ligase Parkin was first characterized as the product of the gene PARK2, mutations of which were linked to early-onset of Parkinson's Disease. Strikingly, Parkin recruitment to mitochondria was found to be necessary for mitophagy (Narendra et al., 2008). Further studies showed that Parkin, together with another familiar Parkinson's Disease-associated gene, PINK1 (PARK7), was responsible for priming mitochondria for autophagic degradation (**Figure 7**).

Under normal conditions, after being synthesized as precursor in the cytoplasm, PINK1 was imported to mitochondria by its N-terminal mitochondria targeting sequence (MTS). Then, PINK1 was post-translationally modified within mitochondria by resident proteases: MPP and PARL (Jin et al., 2010; Deas et al., 2011). Cleavage by PARL resulted in destabilization of the protein and its degradation by cytoplasmic proteasomes (Yamano and Youle, 2013). Under mitochondrial stress however, PINK1 cleavage did not occur and the protein accumulated on the outer mitochondrial membrane (OMM) (Lazarou et al., 2012; Hasson et al., 2013). Recruitment of cytoplasmic E3 ligase Parkin onto mitochondria required stabilization and the kinase activity of the PINK1 protein (Lazarou et al., 2012). Parkin itself was a substrate of PINK1 (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012). Phosphorylation of Parkin by PINK1 resulted in a conformational change overcoming an

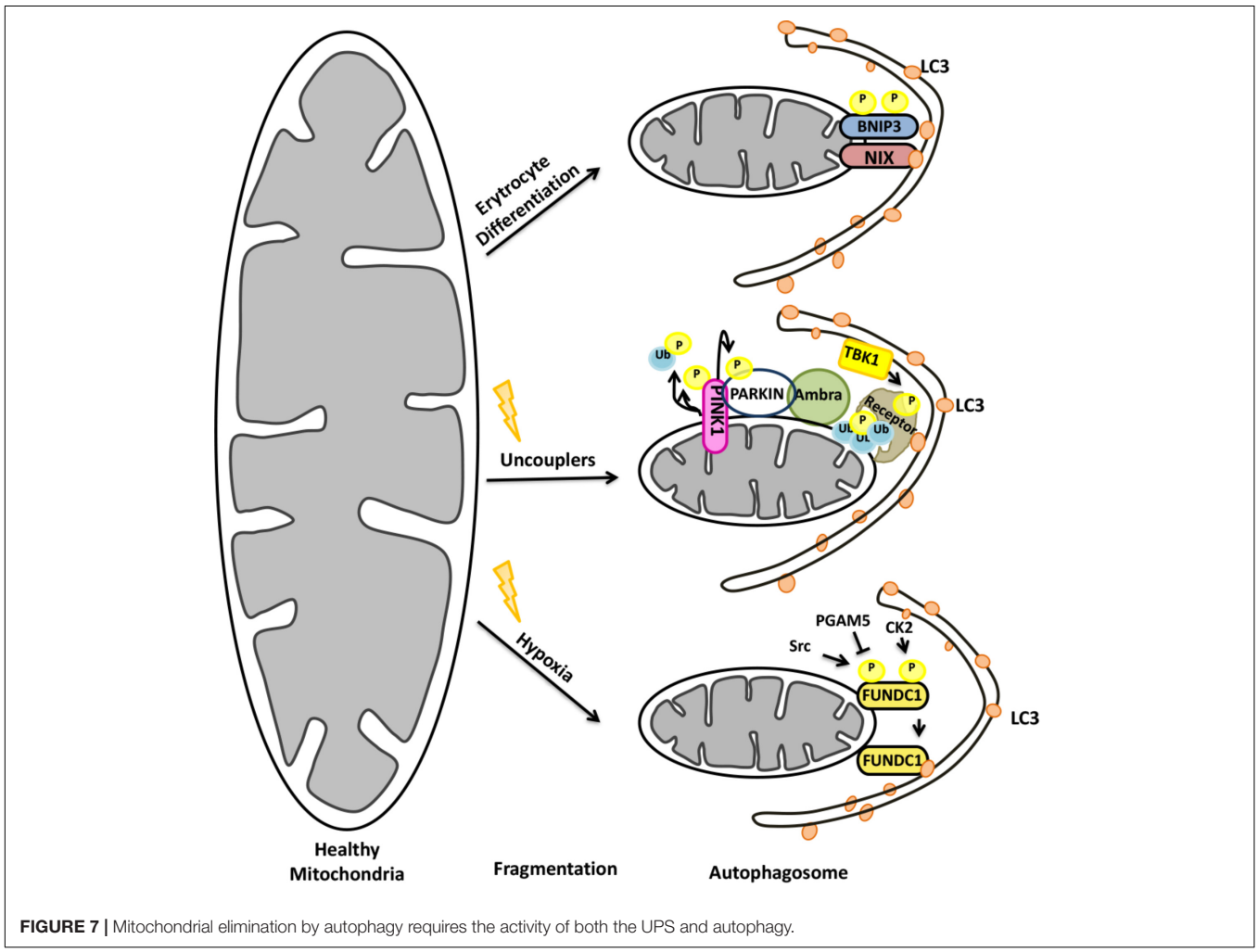


FIGURE 7 | Mitochondrial elimination by autophagy requires the activity of both the UPS and autophagy.

autoinhibition, and stimulated its E3 ligase activity (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Trempe et al., 2013; Wauer and Komander, 2013). Interestingly, PINK1 was shown to phosphorylate ubiquitin molecules on mitochondrial resident proteins as well. Ubiquitin phosphorylation correlated with an increase in the amount of mitochondria-localized Parkin, providing a feed-forward mechanism of Parkin recruitment (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014; Shiba-Fukushima et al., 2014).

Several proteins on the mitochondrial outer membrane were identified as Parkin ubiquitylation substrates. The list includes VDAC, TOM proteins, mitofusins etc (Sarraf et al., 2013). Following ubiquitylation some of these targets were shown to be degraded by the proteasome (e.g., mitofusins) and some were not (e.g., VDAC). Degradation of proteins related to mitochondrial integrity promoted fission events that facilitate engulfment of mitochondrial portions by autophagosomes, whereas proteins that are not degraded upon ubiquitylation rather contributed to mitochondrial rearrangements (e.g., aggregation).

The UPS activity was a prerequisite in the preparation of mitochondria for autophagy. Ubiquitylation of mitochondrial targets preceeded the recruitment of the autophagic machinery onto mitochondria (Yoshii et al., 2011). Selective autophagy receptors were shown to bind ubiquitin-labeled proteins on mitochondria and recruit ATG8/LC3 proteins for mitophagy. Serial knock out of putative autophagy receptors showed that NDP52, optineurin (OPTN) and TAX1BP1 were functional mitophagy receptors, and a triple knockout of these proteins completely blocked mitophagy (Lazarou et al., 2015; Shi J. et al., 2015). On the other hand, the autophagy receptor p62 was essential for clustering of damaged mitochondria in perinuclear region of the cells, but not for mitophagy (Narendra et al., 2010; Okatsu et al., 2010).

Ubiquitin modifications on mitochondria might be reversed by the action of DUB proteins. Several DUBs were identified as positive or negative regulators of mitophagy (Dikic and Bremm, 2014; Wang et al., 2015). For example, deubiquitylation of mitochondrial targets by USP15, USP30, and USP35 prevented further progression of mitophagy in a number of cell lines and experimental models (Bingol et al., 2014; Cornelissen et al., 2014; Wang et al., 2015). DUB-mediated deubiquitylation of targets decreased Parkin recruitment onto mitochondria as well (Bingol et al., 2014). USP8-mediated removal of K6-linked ubiquitin chains from Parkin itself affected recruitment of the protein onto mitochondria and therefore mitophagy (Durcan et al., 2014; Durcan and Fon, 2015).

Parkin-Independent Mitophagy

Expression of Parkin is restricted to a few cell types, including dopaminergic neurons. Consequently, Parkin-null animals showed prominent mitophagy defects only in selected brain regions (Lee et al., 2018). Therefore in other cell types and tissues, mitophagy has to proceed in a Parkin-independent manner. Alternative E3 ligases were found to play a role in mitophagy in these contexts.

Mulan (MUL1) is an E3 ubiquitin ligase that resided on the OMM, and it was shown to play a role in Parkin-independent mitophagy in different model organisms, including *Caenorhabditis elegans*, *Drosophila* and mammals (Ambivero et al., 2014; Yun et al., 2014). Mulan stabilized DRP1, led to degradation of MFN2, and interacted with ATG8 family member protein GABARAP (Braschi et al., 2009; Ambivero et al., 2014). Another E3 ligase that was associated with mitophagy was GP78 (Christianson et al., 2012). Over expression of GP78 induced MFN1 and 2 ubiquitylation and degradation, that was followed by mitochondrial fragmentation and mitophagy in cells lacking Parkin (Fu et al., 2013). Synphilin-1-dependent recruitment of the E3 ligase Siah1 to mitochondria resulted in mitochondrial protein ubiquitylation and mitophagy in a PINK1-dependent but Parkin-independent manner (Szargel et al., 2015). Conversely, another OMM E3 ligase, MITOL (MARCH5), was reported to ubiquitylate FIS1, DRP1 (Yonashiro et al., 2006) and MFN2 (Nakamura et al., 2006), yet inhibited hypoxia-induced and Parkin-independent mitophagy through ubiquitylation and degradation of FUNDC1 (Chen et al., 2017). All these findings underline the fact that mitophagy might proceed in cells which do not express Parkin. Further studies are required to unravel the molecular mechanisms of Parkin-independent mitophagy in different tissues and cell types, and reveal the details of the crosstalk between the UPS and autophagy under these conditions.

A Special Type of Mitophagy During Reticulocyte Maturation

During differentiation, in order to increase their capacity to load hemoglobin-bound oxygen, reticulocytes lose their organelles, including mitochondria, and become mature red blood cells (Dzierzak and Philipsen, 2013). During this process, a protein called NIX (also known as BNIP3L) is upregulated (Aerbajinai et al., 2003). NIX is a C-terminally anchored outer mitochondrial membrane (OMM) protein that contains a LC3-interacting region (LIR) at its cytoplasmic N-terminal part. Through its LIR domain, NIX interacted with LC3, enabling engulfment of mitochondria by autophagosomes in reticulocytes (Novak et al., 2010). Characterization of NIX-deficient mice showed that, NIX-deficient Erythrocytes failed to eliminate their mitochondria revealing a critical role for NIX in mitophagy (Schweers et al., 2007; Sandoval et al., 2008) (**Figure 7**). Although NIX-dependent mitophagy was predominantly studied in reticulocytes, NIX-dependent mitophagy might be important for other cell types as well [for example, see (Esteban-Martínez et al., 2017)].

A role for the UPS in NIX/BNIP3L-dependent mitophagy was revealed. NIX/BNIP3L was discovered to be ubiquitylated through a PINK1/Parkin-dependent mechanism. Ubiquitylated NIX/BNIP3L colocalized with selective autophagy receptors, and the process was necessary for mitochondrial stress-induced mitophagy (Ding et al., 2010; Gao et al., 2015; Palikaras et al., 2015). Therefore, the role of NIX/BNIP3L seems to be more general than previously thought and beyond the developmental context, and stress-induced mitochondrial elimination by autophagy might also require NIX/BNIP3L in different cell and organism types.

Pexophagy: Autophagic Removal of Peroxisomes

Autophagy of peroxisomes, pexophagy, is a selective degradation process of peroxisomes during which the UPS and autophagy mechanisms work in collaboration. Peroxisomes are responsible of a number of cellular functions, including fatty acid oxidation, purine metabolism and phospholipid synthesis (Wanders et al., 2016). Several peroxisomal enzymes are involved in redox regulation due to their dual functions in the generation and scavenging of reactive oxygen and nitrogen species. Therefore, peroxisome biogenesis and degradation must be tightly regulated in order to control peroxisome size, number and function (Du et al., 2015; Honsho et al., 2016). Moreover under stress conditions such as hypoxia, oxidative stress, starvation or conditions causing UPS defects, pexophagy is upregulated.

During pexophagy, a number of peroxisomal membrane proteins, including peroxins and PMP70 become ubiquitylated (Kim et al., 2008). PEX2-PEX10-PEX12 complex serves as an E3 ligase at least for two well studied peroxisome proteins, PEX5 and PMP70. Ubiquitylation of peroxisome proteins result in the recruitment of p62 and/or NBR1 autophagy receptors, priming these organelles for autophagic degradation. For example, PEX2 overexpression or amino acid starvation activated the ubiquitylation of PEX5, and another peroxisomal membrane protein, PMP70, and led to peroxisome degradation (Sargent et al., 2016). Moreover in response to oxidative stress, ATM was recruited onto peroxisomes through physical interaction with PEX5 and promote its ubiquitylation. Inactivation of mTORC1 in a TSC2-dependent manner and stimulation of ULK1 phosphorylation by ATM, potentiated pexophagy (Zhang J. et al., 2015; Tripathi et al., 2016; Wang and Subramani, 2017). On the other hand, AAA ATPase complex (PEX1, PEX6, and PEX26) was shown to extract ubiquitylated PEX5 from peroxisomal membranes and regulate pexophagy (Carvalho et al., 2007; Okumoto et al., 2011; Law et al., 2017) (**Figure 8**). Both NBR1 and p62 were shown to be recruited onto peroxisomes during pexophagy. Yet, NBR1 was a major pexophagy receptor in a number of contexts, and p62 increased the efficiency of NBR1-dependent pexophagy through direct interaction with the latter (Deosaran et al., 2013; Zhang J. et al., 2015; Sargent et al., 2016). Altogether, these findings underline the importance of ubiquitylation for the selective degradation of peroxisomes by autophagy.

Autophagic Removal of Ribosomes and Stress Granules

In addition to major cellular organelles, autophagy was implicated in the clearance of ribosomes. Although ribosomes can be degraded in a non-specific manner during non-selective autophagy, a special form of selective autophagy is activated under various stress conditions, and the process is called ribosomal autophagy or ribophagy. On the other hand, mRNA protein complexes that are stalled during translation form stress granules, and their clearance requires both the UPS and autophagy.

Ribophagy was first described in the yeast during nutrient stress, and was shown to involve ubiquitylation of the 60S ribosome protein Rpl25 by the ubiquitin ligase Ltn1/Rkr1 (Kraft and Peter, 2008; Kraft et al., 2008; Ossareh-Nazari et al., 2014). In the mammalian system, in addition to mTOR inhibition, oxidative stress, induction of chromosomal mis-segregation, translation inhibition and stress granule formation were all shown to induce ribophagy (An and Harper, 2018). Ubiquitylation of ribosomes was observed under ER stress-inducing conditions (Higgins et al., 2015). P97/VCP that binds to ubiquitylated proteins and that functions in the delivery of these substrates to proteasome was necessary for ribophagy both in yeast and mammalian cells (Verma et al., 2013; An and Harper, 2018). Yet, individual ribosomal proteins were indeed shown to be a target of the UPS (Wyant et al., 2018). NUFIP1-ZNHIT3 proteins were identified as novel ribophagy receptors that directly connected ribosomes to LC3 and autophagy, yet whether ubiquitylation is a prerequisite for ribophagy needs to be clarified by future studies (Wyant et al., 2018) (**Figure 9**).

Stress granules are composed of actively accumulated non-translating mRNA ribonucleoprotein complexes (Protter and Parker, 2016). Proteins that accumulated in the stress granules, include stalled 40S ribosomal units and various translation initiation factors [e.g., eIF4E, eIF4G, eIF3, eIF2 and poly(A)-binding protein (PABP)] and regulators such as eIF2-α and GCN2 (Kedersha et al., 2005; Mazroui et al., 2007; Farny et al., 2009; Reineke and Lloyd, 2013). G3BP1 and TIA-1 are also among the proteins that contribute to stress granule formation (Kedersha et al., 2000; Tourrière et al., 2003; Waris et al., 2014). Moreover, an interplay between G3BP1 and Caprin1 proteins and the DUB protein USP10 was shown to regulate

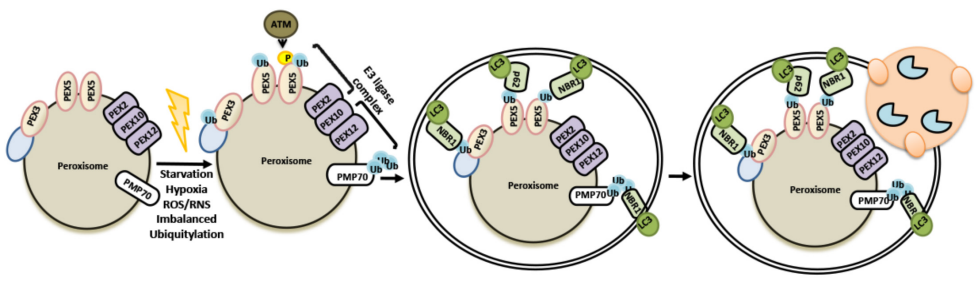


FIGURE 8 | Selective removal of peroxisomes by autophagy utilizes ubiquitylation as signal.

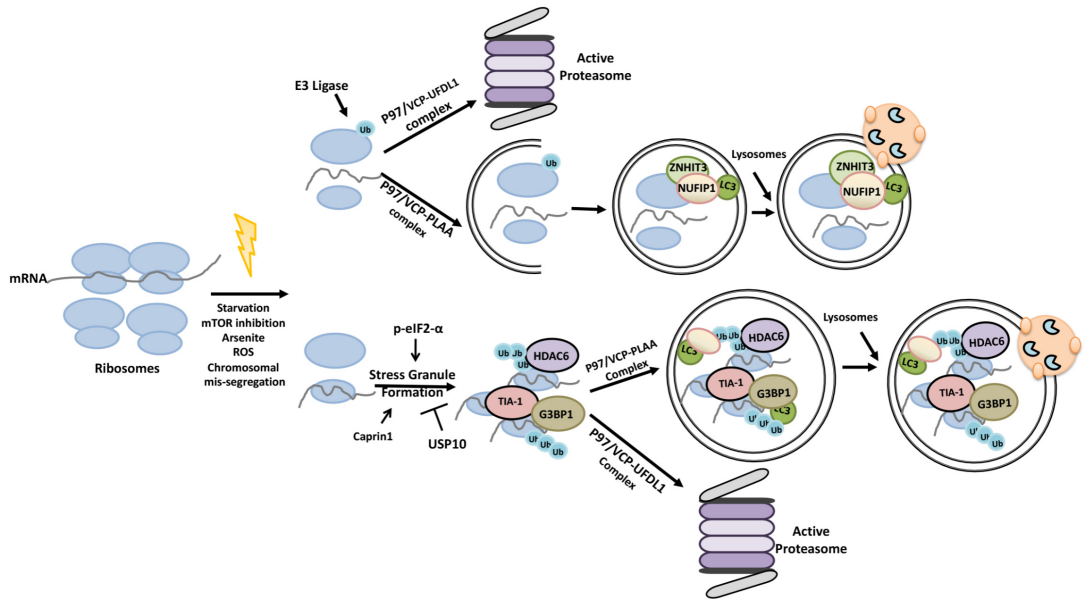


FIGURE 9 | Ubiquitylation primes ribosomes and stress granules for proteasomal degradation and autophagic elimination.

stress granule formation (Kedersha et al., 2016). HDAC6 protein was a component of stress granules as well (Seguin et al., 2014).

Accumulating data indicate that both the UPS and autophagy play a role in stress granüle control and elimination, and the p97/VCP protein was a key component in these processes. For example, inhibition of autophagy or p97/VCP deficiency was linked to decreased stress granule removal (Buchan et al., 2013). Co-factors of p97/VCP determined target selectivity of the protein. In this context, while the association of p97/VCP with the co-factor UFD1L led to the degradation of defective ribosomal products and dysfunctional 60S ribosomes by the UPS (Ju et al., 2008; Fujii et al., 2012; Verma et al., 2013), HDAC6 containing p97/VCP and PLAA associated granules were made a target of ribophagy (Ossareh-Nazari et al., 2010). Therefore depending on the co-factor of choice, p97/VCP has a decisive role in the choice of the degradative pathway through which ribonuclear substrates are eliminated.

Cross Talk Between UPS and Autophagy During Endoplasmic Reticulum Stress

Endoplasmic reticulum (ER) stress is one of the conditions under which both the UPS and autophagy pathways are being activated. Abnormalities in calcium homeostasis, oxidative stress and conditions leading to protein glycosylation or folding defects etc. may result in the accumulation of misfolded and/or unfolded proteins in the ER lumen, a condition known as ER stress. ER stress might be very destructive for cells, therefore ER-specific stress response pathways such as the unfolded protein response (UPR) and the ER-associated degradation (ERAD) pathways were evolved. Both pathways are directly or indirectly connected to the UPS and autophagy.

In mammalian cells, accumulation of unfolded proteins in the lumen of the ER result in the activation of stress responses. Following protein accumulation in the ER, the chaperone protein GRP78/BiP dissociates from the lumen-facing parts of the transmembrane proteins IRE1, ATF-6, and PERK and bind to unfolded proteins in order to assist their refolding. GRP78/BiP release triggers activation of these stress proteins (Bertolotti et al., 2000; Shen et al., 2002). PERK activation leads to the phosphorylation of the α subunit of the translation initiation factor, eIF2α, which inhibits the assembly of the 80S ribosome and cap-dependent protein synthesis, while allowing cap-independent translation of the stress response genes such as ATF4. Activation of IRE1 and ATF6 promotes transcription of other stress response genes. IRE1-mediated processing generates a splice-form of the XBP1 mRNA, resulting in the production of a transcription factor that upregulates chaperones and other relevant genes. GRP78/BiP dissociation results in the transfer of ATF6 to Golgi where cleavage of the protein by S1P and S2P proteases creates an N-terminal ATF6 fragment possessing a transcriptional activity (**Figure 10**). Due to a decrease in the protein load in the ER and an increased folding capacity, the UPR facilitates recovery from stress. In case of failure, the UPR sensitizes cells to programmed death mechanisms.

Components of the UPR were subject to active regulation by the UPS. For example, SCF component E3 ligase βTrCP was shown to lead to the ubiquitylation ATF4 following its phosphorylation (Lassot et al., 2001). On one other hand, persistent ER stress induced transcription of E3 ligase Siah1/2 following PERK-ATF4 and IRE1-XBP1 activation. On the other hand, by targeting prolyl hydroxylase PHD3, Siah1/2 was shown to regulate ATF4 hydroxylation and activity (Scortegagna et al., 2014). CHOP stability was regulated by the UPS and p300 and cIAP were responsible for CHOP ubiquitylation and degradation

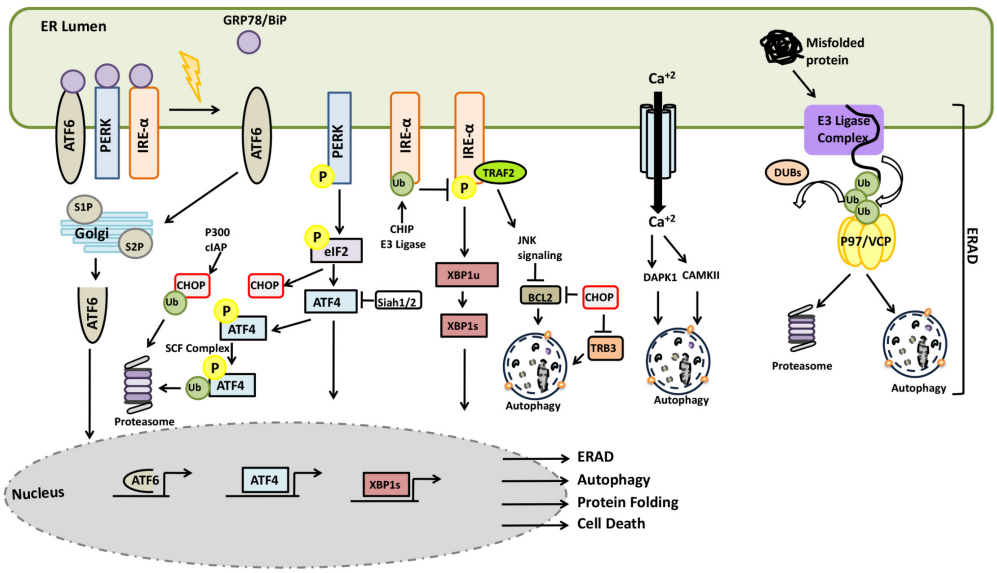


FIGURE 10 | Crosstalk between the UPS and autophagy systems during ER stress and ERAD.

counterbalancing its upregulation during ER stress (Qi and Xia, 2012; Jeong et al., 2014). Another UPR component, IRE1 was identified as a ubiquitylation target of the E3 ligase CHIP during ER stress. Ubiquitylation IRE1 inhibited its phosphorylation, perturbed its interaction with TRAF2, and attenuating JNK signaling (Zhu et al., 2014). Under stress conditions, translation of XIAP, an E3 ligase protein and an inhibitor of apoptosis was downregulated in a PERK-eIF2α-dependent manner. In the same context, ATF4 may promote ubiquitylation and degradation of XIAP, leading to sensitization of cells to ER stress-related cell death (Hiramatsu et al., 2014). Conversely, activation of PERK-eIF2α axis might also show opposing effects through induction of other IAP proteins, cIAP1 and cIAP2, and counter balance cell death induction signals (Hamanaka et al., 2009).

Endoplasmic reticulum stress was shown to trigger autophagy, and ER-related stress response mechanisms were involved in the process. PERK-mediated phosphorylation of eIF2α and resulting ATF4 and CHOP activation, were associated with the transcription of genes such as ATG5, ATG12, Beclin1, ATG16L1, LC3, p62 and TSC2 activator, hence mTOR inhibitor REDD1 (Whitney et al., 2009; B'Chir et al., 2013). Moreover, CHOP downregulated BCL2 binding (Mccullough et al., 2001). TRB3, an AKT inhibitor protein, was also described as a target of CHOP (Ohoka et al., 2005). In addition, IRE1 activation resulted in the recruitment of ASK1 by the adaptor TRAF2 and the outcome was the activation of JNK and p38 kinases (Nishitoh et al., 2002). BCL2 is one of the targets of JNK, its phosphorylation by the kinase resulted in destabilization the inhibitory BCL2-Beclin1 complex, stimulating autophagy (Bassik et al., 2004). On the other hand, in its unspliced form, IRE1 splicing target XBP1, in its unspliced form was shown to target the autophagy activator FOXO1 for degradation by the UPS (Vidal et al., 2012; Xiong et al., 2012).

Endoplasmic reticulum is a major calcium store in cells, and calcium release to cytosol was observed during ER stress. In addition to problems with SERCA refill pumps and leakiness of membranes during stress, upregulation of ERO1-α by CHOP resulted in an IP3-mediated calcium release (Li et al., 2009). Calcium binding protein calmodulin senses the cytosolic increase in the concentration of the ion, and bind to calmodulin-regulated kinases such as CaMKII and DAPK1, modulating their activity. Activated CaMKII was shown to stimulate autophagy through AMPK phosphorylation and activation (Høyer-Hansen et al., 2007). In addition, calmodulin-binding and PP2A-mediated dephosphorylation was necessary for the activation of the autophagy-related kinase DAPK1 (Gozuacik et al., 2008). DAPK1 could directly phosphorylate Beclin1 on the BH3-domain, resulting in the dissociation of Beclin1 from the BCL2-Beclin1 complex and allowing it to stimulate autophagy (Zalckvar et al., 2009).

Proteins that accumulate in the ER are degraded by the ER-associated degradation (ERAD) system. ERAD mediates transport, extraction and ubiquitylation of proteins that cannot be salvaged and target them for degradation in proteasomes. In mammalian cells, ER membrane-resident complexes containing E3 ligases such as HRD1 and GP78, and other regulatory components such as EDEM1, SEL1L, ERManI, and HERP control the ERAD pathway. P97/VCP protein and its co-factors also play a role in the pathway (DeLaBarre et al., 2006; Nowis et al., 2006). Unfolded/misfolded proteins are recognized in the lumen of the ER by chaperone proteins, including BiP/GRP78 and EDEM1, and are then subsequently targeted them to the ERAD pathway. During retrotranslocation of client proteins to cytosol, ubiquitylation is followed by a p97/VCP-assisted extraction. P97/VCP also assists in the delivery of proteins to proteasomes for degradation. DUB proteins, including YOD1, USP13, USP19, and Ataxin-3 were implicated in the control of client protein

ubiquitylation and ERAD substrate modulation (Zhong and Pittman, 2006; Bernardi et al., 2013; Liu Y. et al., 2014; Harada et al., 2016).

ER-associated degradation regulators and therefore ERAD might be controlled by the UPS and autophagy pathways. For example, E3 ligase Smurf1 was found to be downregulated during ER stress, resulting in the accumulation of its direct ubiquitylation target WFS, which is a stabilizer ER-related E3 ligase HRD1 (Guo et al., 2011). Smurf1 was also involved in selective bacterial autophagy (Franco et al., 2017). On the other hand, while the ERAD complex component HERP protein was degraded by the UPS (Hori et al., 2004), EDEM1 and ERManI proteins were eliminated by the autophagy machinery (Le Fourn et al., 2013; Park et al., 2014; Benyair et al., 2015). An ER-localized E3 ligase synoviolin protein was shown to ubiquitylate HERP protein and control its degradation by proteasome (Maeda et al., 2018). Yet, other ERAD-related components, EDEM1 and Derlin2 as well as ubiquitylated EDEM1 proteins colocalized with cytoplasmic aggregates and autophagy receptors p62 and NBR1, they were degraded by selective autophagy (Le Fourn et al., 2013; Park et al., 2014). ERManI, a mannosidase that is responsible for priming ER-resident glycosylated proteins for degradation, was described as an accelerator of the ERAD pathway and clearance of clients by the UPS. But, following proteasome inhibition and subsequent ER stress, ERManI colocalized with LC3 and degraded in an autophagy-dependent manner (Benyair et al., 2015).

All these findings point out to the presence of important junctions and coregulation nodes between the UPS and autophagy in the context of ER stress. Additionally, ERphagy, the autophagy of portions of the ER, was implicated in the recovery from ER stress and control of ER size, but this mechanism was so far described as a ubiquitin-independent process (Schuck et al., 2014).

Transcriptional Mechanisms Connecting the UPS and Autophagy

Several transcription factors that are regulated by the UPS, including p53, NFκB, HIF1α, and FOXO, have been implicated in the control of autophagy. In general, these factors were shown to directly activate transcription of key autophagy genes under stress conditions. Some autophagy proteins such as LC3 are consumed in the lysosome following delivery, and during prolonged stress, cellular levels of these proteins are sustained by mechanisms, including transcription. On the other hand, regulation of the transcriptional activity NRF2 involves a special crosstalk between the two systems. In this section, we will summarize molecular details of transcription regulation by the UPS and autophagy.

P53, a guardian of the genome, is one of the well-known transcriptonal regulators that has a dual role in autophagy depending on its intracellular localization. In the absence of stress, cellular p53 levels are controlled by the E3 ligase HDM2/MDM2 and the UPS. Under stress conditions, p14/p19/ARF protein binds, sequesters and inactivates HDM2/MDM2, stabilizing p53. Accumulating p53 protein activates transcription of several stress- and death-related genes, including autophagy-related genes *PRKAB1*, *PRKAB2, TSC2*, *ATG2, ATG4, ATG7*, *ATG10*,*ULK1*, *BNIP3, DRAM1,* and *SESN2* (Crighton et al., 2006; Feng et al., 2007; Budanov and Karin, 2009; Kenzelmann Broz et al., 2013). On the other hand, a cytosolic form of p53 was shown to inhibit AMPK and activate the mTOR pathway. In this context, non-genotoxic stress by autophagy-inducing agents such as rapamycin, tunicamycin and nutrient deprivation favored HMD2/MDM2-dependent p53 degradation by the UPS (Tasdemir et al., 2008a,b). Interestingly, HMD2/MDM2 stability and activity were also regulated by E3 ligases SMURF1/2 which in turn affected the stability of p53. SMURF1/2-mediated ubiquitylation was shown to increase MDM2-MDMX heterodimerization, decreasing autoubiquitylation of MDM2, therefore stabilized the protein (Nie et al., 2010). Additionally, another E3 ligase, NEDD4-1 was shown to control MDM2 stability and p53 activation (Xu et al., 2015). In addition to MDM2, another E3 ligase, PIRH2, was able to ubiquitylate p53 to control its cellular stability (Shloush et al., 2011).

NF-κB is a well studied transcriptional regulator of autophagy. As a result of its association with IκB, NF-κB is found in an inactive state in the cytosol. In response to agonists, IκB was reported to be ubiquitylated and subsequently degraded by the UPS. Regulation of NF-κB by external signals involved phosphorylation of IκB by upstream kinases of the IKK complex (IKKα, IKKβ, and IKKγ/NEMO). Phosphorylated IκB recruits the E3 ligase SCF-βTRCP, followed by its degradation in the proteasome (Orian et al., 2000). After IκB degradation, NF-κB was then free to migrate to the nucleus of the cell, and induce transcription of target genes, including Beclin1 and p62, and induce autophagy (Copetti et al., 2009; Ling et al., 2012).

Another level of regulation involved TNF-α receptor-associated protein complexes. Binding of TNF-α to TNFR1 led to the recruitment of TRADD and RIPK1 to the receptor, promoting TRAF- and cIAP-mediated K63 and/or K11 linked ubiquitylation of the RIPK1. Ubiquitylated RIPK1 could recruit NEMO and TAB-TAK1 complex for IKK activation and hence NF-κB stimulation. Additionally, RIPK1 could also be modified by A20 through addition of K48-linked poly-ubiquitin chains, sending the kinase for proteasomal degradation (Kravtsova-ivantsiv et al., 2015).

However, in some contexts, TNF-α-induced NF-κB activation was reported to inhibit autophagy (Djavaheri-Mergny et al., 2006). TNF-α-induced activation of IKKα or IKKβ could stimulate phosphorylation of TSC1/2 and activate mTOR, leading to a similar inhibitory outcome (Lee et al., 2007; Dan and Baldwin, 2008). Furthermore in some contexts, RIPK1 silencing activated autophagy under both basal and stress conditions (Yonekawa et al., 2015). On the other hand, RIPK1 itself was reported to be a target of p62-mediated selective autophagy (Goodall et al., 2016). Moreover, autophagy was responsible for the degradation of NF-κB activator NIK and IKK complex subunits, indicating the presence of a tight cross-regulation of the NF-κB pathway by the UPS and autophagy (Qing et al., 2007).

Another transcription factor that was controlling the autophagic outcome was HIF1α. Hypoxia induced HIF1α transcriptionally regulated various hypoxia response genes,

including *GLUT1* (Chen et al., 2001), *NOX2* (Yuan et al., 2011), and *PDK1* (Kim et al., 2006) as well as autophagy genes, including *BNIP3*, *BNIP3L*, *ATG5,* and *BECN1* to stimulate autophagy, mitophagy, and pexophagy (Zhang et al., 2008; Bellot et al., 2009; Walter et al., 2014). HIF1α itself was regulated in a UPS-dependent manner. Under normoxia, hydroxylation of HIF1α specific prolyl hydroxylases (PHDs) hydroxylated HIF1α (Jaakkola et al., 2014) served as a recognition signal for UbcH5, an E2 enzyme and von Hippel-Lindau protein (the pVHL), E3 ligase complex containing Elongin B and C, Cullin-2, and Rbx1 allowing K48 linked ubiquitination of HIF1α and its proteasomal degradation (Ohh et al., 2000; Lee et al., 2015). In contrast, during hypoxia, PHDs were inhibited and HIF1α stabilized. SCF E3 ligase complex was also a regulator of HIF1α stability in response to GSK3β-mediated phosphorylation of the protein (Cassavaugh et al., 2011; Flugel et al., 2012). Another E3 ligase facilitating HIF1α degradation was HAF (also known as $SART1_{800}$). Unlike pVHL, HAF-mediated ubiquitylation of HIF1α was not depending on the oxygen levels, providing an alternative HIF1α regulation mechanism (Koh et al., 2008). Stability of PHD proteins were also controlled by the UPS. For example, SIAH1/2 was shown to direct PHDs for proteasomal degradation under hypoxic stress (Nakayama et al., 2004). Moreover several DUBs were implicated in HIF1α regulation, including USP20 (Li et al., 2002b), USP28 (Flugel et al., 2012), and USP33 (Li et al., 2002a).

FOXO family of transcription factors (FOXOs) were associated with various cellular pathways, including autophagy (Zhao et al., 2007). The activity of FOXOs were regulated by their phosphorylation status and following activation, FOXOs translocated to the nucleus and triggered the expression of a number of genes associated with different stages of the autophagy pathway, including *ATG4*, *ATG12*, *BECN1*, *ULK1*, *PIK3C3*, *MAP1LC3,* and *GABARAP* (Mammucari et al., 2007; Zhao et al., 2007; Sanchez et al., 2012). There are several connections between FOXOs and autophagy. Activation of the AKT pathway inhibited FOXO3 activity, led to a decrease in LC3 and BNIP3 expression, therefore blocked autophagy (Stitt et al., 2004; Mammucari et al., 2007). On the other hand, AMPK activation led to the phosphorylation of FOXO3a and ULK1, inducing *MAP1LC3*, *GABARAP,* and *BECN1* expression and subsequent autophagy activation (Sanchez et al., 2012). Another FOXO family protein FOXK1/2, a negative regulator of FOXO3, was associated with a decrease in autophagy by removing Sin3A/HDAC complex from histone H4 to diminish its acetylation. In this context, nuclear localization of FOXK1/2 was mTOR-dependent and showed an inhibitory effect on autophagy gene expression under basal conditions (Bowman et al., 2014). Moreover, JNK deficiency in neurons increased autophagic activity through FOXO1-mediated BNIP3 upregulation and Beclin1 disassociation from BCL-XL (Xu et al., 2011). Another example of a link between FOXOs autophagy involved ATG14. Liver specific knockout of FOXOs resulted in the downregulation of ATG14 and this event was associated with high levels of triglycerides in the liver and serum of mice (Xiong et al., 2012). Additionally, GATA-1 shown to directly regulate FOXO3-mediated activation of LC3 genes to facilitate autophagic activity (Kang et al., 2012).

Phosphorylation of FOXO proteins by various protein kinases, including AKT, IKK, and ERK, affected their ubiquitylation by E3 ligases and their stability (Huang and Tindall, 2011). For instance, AKT-mediated phosphorylation of FOXO1 provided a signal for its recognition by the SKP protein, an SCF E3 ligase complex component, followed by FOXO1 ubiquitylation and degradation (Huang et al., 2005). COP1 was also identified as an E3 ligase that regulated FOXO protein stability. COP1 ubiquitylated FOXO1 and promoted its proteasomal degradation. This type of regulation might be important in the glucose metabolism of hepatocytes, and possibly in autophagy modulation under this conditions (Kato et al., 2008). Another FOXO regulating E3 ligase was MDM2 that was reported to be responsible for FOXO1 and FOXO3A ubiquitylation and degradation (Fu et al., 2009). MDM2-mediated ubiquitylation was activated by the phosphorylation of FOXOs by AKT. Due to its role in p53 regulation, MDM2 could be part of a more complex regulatory mechanism which might link the UPS, transcriptional regulation and autophagic activity.

NRF2-KEAP1-P62 pathway was defined as another major oxidative stress response mechanism involving an interplay between the UPS and autophagy. NRF2 is a transcription factor, and when activated, is upregulated antioxidant and metabolic enzymes, including *TXNRD1* (Suvorova et al., 2009), *HMOX1* (Reichard et al., 2007), *GPX2* (Banning et al., 2005), *GBE1, PHK1* (Banning et al., 2005), and downregulated proinflammation-related genes such as *IL6*, *IL1B* (Kobayashi et al., 2016). KEAP1 is an adaptor protein of the E3 ligase Cullin-3 and plays a role in substrate recognition. Under normal conditions, transcription factor NRF2 was found in association with KEAP1-Cullin-3 E3 ligase complex, that catalyzed its ubiquitylation, rendering it a substrate for proteasomal elimination by selective autophagy (Ishimura et al., 2014). Competition resulted in the migration of free NRF2 to the nucleus and transactivation of stress-related cytoprotective genes (Kobayashi et al., 2004; Komatsu et al., 2010). Additionally, the NRF2–KEAP1 pathway provides a positive feedback loop for autophagy. P62 was characterized as a direct transcriptional target of activated NRF2 (Jain et al., 2010). Moreover, KEAP1 regulation by p62 was modulated by the E3 ligase TRIM21. NRF2 activation was negatively affected by TRIM21-mediated K63-linked ubiquitylation of p62 (Pan et al., 2016).

Autophagy-UPS Crosstalk in Diseases

Crosstalk between autophagy and the UPS may change character under disease conditions, contribute to the pathogenesis of diseases and even affect their outcome. Degenerative diseases and cancer are examples of diseases that illustrate the interplay between the UPS and autophagy in the clearance of misfolded abnormal proteins (Juenemann et al., 2013).

For example, Huntington Disease is caused by poly-glutamine extensions in a protein called Huntingtin (Htt), leading to abnormal organization and eventual aggregation of the protein. Htt protein was shown to be ubiquitylated via K48- or K63-linked ubiquitin chains (Bhat et al., 2014). Mutant Htt clearance depended on both the UPS and autophagy in different experimental settings. Mutant Htt aggregates were largely cleared

by K63-dependent autophagy mechanisms (Renna et al., 2010; Menzies et al., 2015). On the other hand, overexpression of K48-specific E3 ligase Ube3a, resulted in a UPS-dependent degradation of mutant proteins. Yet, cellular levels of E3 ligase was shown to decline in an age-dependent manner. Therefore, in elderly people, accumulation of K63-linked polyubiquitylated proteins might tip the balance toward clearance of protein aggregates by autophagy. A similar UPS switch was also observed in a CHIP-dependent manner (Jana et al., 2005; Bhat et al., 2014).

Another example involves the ERAD protein p97/VCP. Mutant forms of the protein were associated with a rare syndrome that mainly affects muscles, bones and the brain (Inclusion Body Myopathy with the Paget's Disease of Bone and frontotemporal Dementia, IBMPFD). Moreover, p97/VCP mutations were detected in a fraction of patients suffering from familial forms of Parkinson's Disease or from Amyotrophic Lateral Sclerosis (ALS) (Johnson et al., 2010). As mentioned in the previous sections, p97/VCP is important for the extraction of misfolded ER proteins as well as their delivery to proteasomes. Moreover, p97/VCP was proposed to play a role in autophagosome maturation and autolysosome formation (Tresse et al., 2010). We recently showed that some of the disease-related mutations of p97/VCP (namely P137L and G157R) resulted in the aggregation of the protein itself. Mutant p97/VCP proteins formed complexes with wild-type counterparts and led to further accumulation of ubiquitylated proteins upon ER stress, indicating that the ERAD system was negatively affected by the mutant (Bayraktar et al., 2016). Indeed, ERAD co-factor and ubiquitin binding capacity of the mutant p97/VCP was decreased (Erzurumlu et al., 2013). Yet, autophagy was still functional under these conditions, and could significantly eliminate these aggregates (Bayraktar et al., 2016). Therefore, preferential elimination of mutant proteins by autophagy might tip the balance in favor of wild-type proteins and restore disease-related loss of cellular functions including UPS-related mechanisms.

The role of the crosstalk between the two systems is also prominent in the cancer context. For example, the P53-regulated and cancer-related protein EI24, was introduced as a critical link between the UPS and autophagy (Devkota et al., 2012). EI24 controlled the stability of E3 ligases TRIM41, TRIM2, and TRIM28 by the regulation of their autophagic degradation (Devkota et al., 2016; Nam et al., 2017). Cellular levels of other E3 ligases, namely MDM2 and TRAF2, were also regulated by EI24-controlled degradation, modulating p53 and mTOR pathways, respectively, and influencing cancer formation and progression (Devkota et al., 2016).

Deregulation and/or mutations of proteins that function in the autophagy and/or the UPS were observed in some cancer types, resulting in the modification of individual pathways and possibly affecting the crosstalk between the two systems. Changes include, modulation of levels of E3 ligases such as MDM2 (Haupt et al., 2017), SMURF1 (Fukunaga et al., 2008; Kwon et al., 2013), SCF components (e.g., βTrCP), point mutations of NEDD4 (Amodio et al., 2010), COP1 (Marine, 2012), FBXW7 (Korphaisarn et al., 2017), and mutations in autophagy related proteins Beclin1 (Laddha et al., 2014), LKB1 (Ji et al., 2007), ATG5 (Takamura et al., 2011), ATG4C (Marino et al., 2007) as well as deletions of genes of proteins, such as Beclin1 (Liang et al., 1999; Qu et al., 2003), AMPK (Li et al., 2015) and UVRAG (He et al., 2015). Under these circumstances, dynamic and complex changes in the regulation of the degradative pathways should have dramatic effects that contribute to cancer-related alterations in the proteomic landscape of cells.

Autophagy-UPS crosstalk emerges as a critical factor that determines the success of disease treatment, chemotherapy is one striking example. For instance, proteasome inhibition by the chemotherapy agent bortezomib resulted in the accumulation of misfolded proteins and induced compensatory autophagy in cancer cells (Obeng et al., 2006). Under these circumstances, autophagic activity protected cancer cells from bortezomib-induced cell death, and inhibition of autophagy improved the outcome of chemotherapy. These dual autophagy-UPS targeting approaches also gave promising results in clinical trials (Vogl et al., 2014).

Several companies are now developing drugs that modulate the UPS or autophagy [for example, (Huang and Dixit, 2016)]. Concepts and data that were discussed above and elsewhere indicate that, depending on the disease type and treatment strategy, the crosstalk between the UPS and autophagy should definitely be taken into account in these efforts.

CONCLUSION AND PERSPECTIVES

Autophagy and the ubiquitin proteasome systems are major degradation systems in mammalian cells that allow recycling of cellular contents ranging from soluble proteins to intracellular organelles. Although their mode of action and their requirements for substrate recognition are different, there are several overlaps and interconnections between the UPS and autophagy pathways.

A prominent component of the crosstalk is the ubiquitin protein itself and ubiquitylation. Indeed, ubiquitin is a common signal for both the UPS and autophagy. It was proposed that, ubiquitin chain type could determine the pathway of choice for protein degradation. K48-linked ubiquitylation was introduced to be a signal for the UPS, whereas K63-linked ubiquitylation directed proteins for autophagosomal degradation (Herhaus and Dikic, 2015). Yet, a number of independent studies provided evidence that both ubiquitylation types could lead to autophagic degradation of substrates (Wandel et al., 2017). Moreover, recent studies underline the importance of ubiquitin phosphorylation as an event that increased the affinity of autophagy receptors for their targets during selective autophagy (Kane et al., 2014; Koyano et al., 2014). Additionally, non-ubiquitin modifications (e.g., acetylation, sumoylation, neddylation etc.) were shown to affect protein degradation as well (Hwang and Lee, 2017). Therefore, a barcode of ubiquitin and other modifications seem to prime proteins for one or the other degradation pathway and determine their fate. As another level of regulation, deconjugating enzymes such as DUBs may counteract or redirect proteins for different degradation systems.

E3 ligases emerged as important components of the UPS-autophagy switches. For example, Cullin-3

(Pintard et al., 2004), SMURF1 (Ebisawa et al., 2001), MDM2 (Shi and Gu, 2012) E3 ligases directed proteins to degradation by the UPS, whereas the role of Parkin (Chan et al., 2011), LRSAM1 (Huett et al., 2012), and CHIP (Shin et al., 2005) in priming proteins for autophagic degradation was observed in several studies. On the other hand, the same E3 ligase that might be able to generate different ubiquitin linkages under different conditions and on different substrates (Chan et al., 2011), the switch between degradative pathways being controlled by specific E3 ligase adaptors, post-translational modifications on target proteins as well as other unknown factors. A prominent example is the Parkin protein. During mitophagy although some of the proteins that are ubiquitylated by Parkin are degraded, other ubiquitylated proteins contribute to mitochondrial clustering and recognition by autophagy receptors. To date, factors or modifications that determine the substrate selectivity of Parkin are unknown.

Another example of UPS-autophagy switch involves the p97/VCP protein. While binding of the co-factor PLAA to p97/VCP resulted in the autophagic degradation of ubiquitylated clients of the protein, binding of UFDL1 as a co-factor favored degradation by the UPS. Moreover, p97/VCP was also associated with aggregate formation in collaboration with some autophagy receptors.

Signaling switches involved in the regulated activation of one or the other system was shown to modify cellular responses to stress. For example, NRF2 degradation by the UPS was controlled through p62-mediated KEAP1 elimination by autophagy (Jain et al., 2010). Prevention of HIF1α degradation by the UPS, resulted in the expression of stress response genes, including autophagy genes, led to autophagy activation. In another example, the UPS activity was required for NF-κB activation and NF-κB-mediated autophagy gene upregulation. Yet, autophagic degradation of NF-κB activators NIK and IKKs provided a negative feedback loop in the control in this context (Qing et al., 2007). Therefore, modification of cellular signaling pathways by degradative systems might modulate upstream signals that control autophagy and/or the UPS, and affect their activation and amplitude.

Degradation of the components or regulators of one system by the other system was also reported. For example, proteasomes were defined as substrates of selective autophagy (Marshall et al., 2015). Conversely, various autophagy proteins were ubiquitylated and degraded by the UPS in a regulated manner. Therefore, checks and balances between the two systems exist, and these control mechanisms possibly allow remodeling of the cellular proteome under different conditions.

Compensation mechanisms are also operational between the two systems. Inhibition of the UPS generally upregulated autophagy, whereas failures in the autophagy system were associated with increased UPS activity, although inefficient compensation and failure in both systems were also observed under certain conditions (Korolchuk et al., 2009a,b). Moreover, alternative protein degradation pathways, such as CMA and microautophagy might come into play under these conditions as well. Nevertheless, depending on the character of the target to be degraded, compensation mechanisms were less or more effective. For example, large aggregates and whole organelles should be cleared by autophagy, but defective ribosomal products that could not be accumulated in stress granules were shown to be directed for proteasomal degradation. Therefore for cellular homeostasis and for proper functioning of cells, ideally both systems should be fully operational.

Data obtained so far demonstrate that crosstalk and communication between autophagy and the UPS generally rely on non-specialized and even indirect links. Yet, there might exist so far unknown specialized proteins providing coordination and co-regulation of the two systems. Furthermore, regulation through direct protein-protein interactions between known system components is another possibility. Therefore, dedicated communication proteins or pathways between the degradation mechanisms may be present, allowing better and faster coordination in case of need. Further studies are required to unveil the nature of these putative proteins, interactions and pathways.

An emerging theme in the regulation and coordination of autophagy and the UPS involves non-coding RNAs and their intricate networks. A growing list of microRNAs as well as long non-coding RNAs were implicated in the control of autophagy (Tekirdag et al., 2016) as well as the UPS (Wu and Pfeffer, 2016; Chang et al., 2018). MicroRNAs have the advantage of affecting the level of multiple proteins at once, and they are able to rapidly reshape cellular signaling mechanisms and pathways. Therefore, non-coding RNA networks possibly contribute to the co-regulation of these degradative systems. Intriguingly, deregulation of non-coding RNA levels contribute to the progression of diseases such as cancer. Future studies on non-coding RNAs will reveal their relevance in the autophagy-UPS crosstalk under physiological and pathological conditions.

Overall, coordination, interconnection and crosstalk mechanisms between the UPS and autophagy exist at various levels. In addition to ubiquitin and ubiquitylation, several proteins and signaling pathways were implicated in the communication and mutual regulation of the two systems. Considering the importance of protein catabolism for cellular and organismal homeostasis and health, a better understanding of individual systems as well as the interconnections and crosstalks between them will be most rewarding from both a basic science perspective and with regards to clinical management of diseases involving protein quality control problems.

AUTHOR CONTRIBUTIONS

NK and DG wrote the manuscript and did critical reading. NK prepared the illustrations in the manuscript.

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Initial Steps in Mammalian Autophagosome Biogenesis

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During the last decade, autophagy has been pointed out as a central process in cellular homeostasis with the consequent implication in most cellular settings and human diseases pathology. At present, there is significant data available about molecular mechanisms that regulate autophagy. Nevertheless, autophagy pathway itself and its importance in different cellular aspects are still not completely clear. In this article, we are focused in four main aspects: (a) *Induction of Autophagy*: Autophagy is an evolutionarily conserved mechanism induced by nutrient starvation or lack of growth factors. In higher eukaryotes, autophagy is a cell response to stress which starts as a consequence of organelle damage, such as oxidative species and other stress conditions. (b) *Initiation of Autophagy*; The two major actors in this signaling process are mTOR and AMPK. These multitasking protein complexes are capable to summarize the whole environmental, nutritional, and energetic status of the cell and promote the autophagy induction by means of the ULK1-Complex, that is the first member in the autophagy initiation. (c) *ULK1-Complex:* This is a highly regulated complex responsible for the initiation of autophagosome formation. We review the post-transductional modifications of this complex, considering the targets of ULK1. (d)*The mechanisms involved in autophagosome formation*. In this section we discuss the main events that lead to the initial structures in autophagy. The BECN1-Complex with PI3K activity and the proper recognition of PI3P are one of these. Also, the transmembrane proteins, such as VMP1 and ATG9, are critically involved. The membrane origin and the cellular localization of autophagosome biogenesis will be also considered. Hence, in this article we present an overview of the current knowledge of the molecular mechanisms involved in the initial steps of mammalian cell autophagosome biogenesis.

Keywords: autophagy regulation, mTOR, AMPK, ULK1, VMP1

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There are three types of autophagy, processes where cytoplasmic components are delivered to lysosomes for degradation: microautophagy/endosomal microautophagy (Li et al., 2012; Galluzzi et al., 2017), chaperone-mediated autophagy (CMA) (Cuervo and Wong, 2014; Kaushik and Cuervo, 2018) and macroautophagy (hereafter mentioned as autophagy). This is the engulfment of cytoplasmic contents by a double membrane vesicle, named autophagosome. The outer membrane of the autophagosome eventually fuses with the lysosome, where the inner vesicle is delivered (**Figure 1**). Here we present a brief overview of the mechanisms involved in the initial steps of mammalian cell autophagosome biogenesis.

INDUCTION OF AUTOPHAGY

The main task of autophagy is to deal against poor nutrient environments. In superior eukaryote cells, mTOR, which is a serine/threonine kinase, checks the presence of growth factors and nutrients. In presence of amino acids (mainly leucine, glutamine and arginine), mTORC1 maintains the autophagy inhibition. When nutrients are no longer available, the inhibition of mTORC1 releases the 'brake' and autophagy is eventually induced (Carroll et al., 2016). Growth factors negatively regulate the autophagy by activation of mTOR. Activation of the insulin receptor induces the phosphorylation of TSC2, avoiding the TSC1/2 complex formation and the mTORC1 inhibition (Haeusler et al., 2018). Other growth factors induce the RAS pathway, which activates the ERK1/2 dimer that inhibits the TSC1/2 complex and phosphorylates RAPTOR activating mTORC1 and suppressing autophagy (Carriere et al., 2011).

AMPK is a key serine/threonine kinase that is activated in low energy conditions (Egan et al., 2011). Then, AMPK activates the autophagosome formation by mean of direct and indirect ways. Furthermore, AMPK can be activated by CaMKKB in the ER-overloaded response (Hoyer-Hansen et al., 2007). The unfolded protein response, by mean of IRE1α, PERK and ATF6, is also an autophagy triggering event, enhancing LC3 conjugation (Ding et al., 2007; Kouroku et al., 2007).

During quick and intense oxygen fluctuations, autophagy is induced by mTORC1-dependent pathways and/or by ER stress. (Papandreou et al., 2008; Rouschop et al., 2010). In moderate but chronic hypoxia, autophagy is triggered mainly by HIF1α and PKCδ-JNK1 pathways (Mazure and Pouyssegur, 2010). HIF1α is the major transcription factor involved in cell response to hypoxia (Brocato et al., 2014). Among the genes transcribed by HIF1α is BNIP3 which disrupts the Bcl2-BECN1 interaction releasing BECN1 to be part of the autophagy process (Zhang et al., 2008), and VMP1, which interacts with BECN1 and is required for autophagosome formation (Ropolo et al., 2007; Rodriguez et al., 2017). Regarding to the PKCδ pathway, this kinase activates JNK1 that in turn phosphorylates Bcl2 to release it from BECN1 (Pattingre et al., 2009).

Oxidative stress induces autophagy in order to recycle damaged mitochondria (and other damaged organelles), and eliminate proteins aggregates (Ureshino et al., 2014). NRF2 is bound to antioxidant response elements promoting the transcription of p62, a cargo receptor for autophagy (Puissant et al., 2012). FOXO3 induces the expression of LC3 (an ATG protein that is described below) and BNIP3 (Mahalingaiah and Singh, 2014). Finally, ROS inhibit ATG4-mediated LC3 delipidation, that takes place immediately after formation of the autolysosome, conferring stability to LC3 and favoring its recruitment to the autophagosome (Scherz-Shouval et al., 2007).

INITIATION OF AUTOPHAGY

Independently of the induction agent, in canonical autophagy, the initiation of autophagosome biogenesis is managed by the kinases mTOR and AMPK. In fact, through the association with RAPTOR, DEPTOR, PRAS40 and mLST8, mTOR constitutes the complex 1 [mTORC1]. At basal conditions, mTORC1 is stimulated by the small GTPase Rheb. In turn, mTOR triggers cell growth and diverse anabolic processes such as lipids, proteins and nucleotides synthesis (Lamb et al., 2013; Klionsky and Schulman, 2014). On the other hand, active mTORC1 abolishes most of catabolic processes including the autophagy (Lamb et al., 2013; Klionsky and Schulman, 2014; **Figure 1B**). Therefore, mTOR inhibits autophagy, by several phosphorylations on the first complex of the pathway (see further), when optimal nutrients concentration is available.

During starvation, Rheb is inhibited by the TSC1/2 heterodimer removing the activation stimulus on mTOR (Huang and Manning, 2008). This inhibition of mTORC1 decreases its influence on autophagy and as a consequence, the mechanism of autophagosome biogenesis is triggered (Carroll et al., 2016; **Figure 1E**). Moreover, the inactivation of mTORC1 allows that the dephosphorylated TFEB translocates to the nucleus (Puertollano et al., 2018) where it induces the transcription of ATG genes, such as UVRAG, WIPI, MAPLC3B, SQSTM1, Vps11, Vps18, and ATG9B. TFEB also promotes the lysosomal function in the cell (Settembre et al., 2011).

AMPK is a heterotrimeric complex composed by a catalytic α subunit and two regulatory subunits, β and γ (Egan et al., 2011). Since AMPK is activated in low energy conditions, this kinase inhibits anabolic processes, and induces catabolic pathways, such

Abbreviations: AMBRA1, Activating molecule in BECN1-regulated autophagy protein 1; AMPK, AMP-activated Kinase; AP-4, Adaptor protein 4; ATF6, Activating transcription factor 6; ATG, Autophagy related gen or protein; Bcl-2, B-cell lymphoma 2; BECN1, Coiled-Coil Moesin-Like BCL2-Interacting Protein; BH3, Bcl-2 homology 3; BiP, Binding immunoglobulin protein; BNIP3, BCL2 interacting protein 3; CaMKKB, Calcium/calmodulin-dependent protein kinase kinase 2; COPII, Coat complex protein II; CTAGES5, Cutaneous T-cell linphoma-associated antigen 5; CUL3, Cullin-3; DAPK, Death-associated protein kinase; DEPTOR, DEP domain containing mTOR-interacting protein; DFCP1, Double FYVE containing protein 1; EP300, Histone acetyltransferase p300; ERK1/2, Mitogen-activated protein kinase; Esyt, Extended synaptotagmin; FIP200, FAK family-interacting protein of 200 kDa (also known as RB1CC1); FOXO3, Forkhead box protein O3; GSK3, Glycogen synthase kinase 3; HIF1α, Hypoxia-inducible factor 1 alpha; HORMA, Hop/Rev7/Mad2 domain; IDR, Intrinsically disordered region; IRE1α, Inositol-requiring enzyme 1 alpha; JNK, c-Jun N-terminal kinases; KAP1, E3 SUMO-protein ligase TRIM28; KHLH20, Kelch-like protein 20; LC3, Microtubule-associated proteins 1A/1B light chain 3B (also known as MAP1LC3B); LIR, LC3-interacting region; LKB1, Serine/threonine-protein kinase STKB1; MIT, Microtubule interacting and trafficking domain; mLST8, mammalian Letal with SEC13 protein 8; NEDD4, Neural precursor cell expressed developmentally down-regulated protein 4; NEDD4L, Neural precursor cell expressed developmentally downregulated gene 4-like; NRF2, Nuclear factor erythroid 2-related factor 2; PDK1, 3-phosphoinositide-dependent protein kinase 1; PERK, Proline-rich receptor-like protein kinase; PI3K, Phosphatidylinositol 3-kinase; PI3P, Phosphatidylinositol 3-phosphate; PKCδ, Protein kinase C delta type; PRAS40, Proline-rich Akt substrate of 40 kDa; PROPPIN, β-propeller that bind polyphosphoinositides; RAB, Ras-related protein; RAPTOR, Regulatory-associated protein of mTOR; Rheb, Ras homolog enriched in brain; ROS, Reactive oxygen species; SERCA, sarco/endoplasmic reticulum Ca2+-ATPase; SIRT1, Sirtuin 1; SQSTM1, Sequestosome 1 (also known as p62); STX17, Sintaxin 17; SUMO, Small ubiquitin-like modifier; TFEB, Transcription factor EB; TIP60, 60 KDa Tat-Interactive Protein; mTOR, mammalian Target of Rapamycin; TRAF6, Tumor necrosis factor receptor (TNFR)-associated factor 6; TRAPPIII, transport protein particle (TRAPP) III complex; TSC1/2, Tuberous sclerosis 1/2; ULK1, unc-51-like kinase 1; UVRAG, UV radiation resistance associated protein; VMP1, Vacuole membrane protein 1; Vps, Vacuolar protein sorting; WIPI, WD repeat domain phosphoinositide-interacting protein.

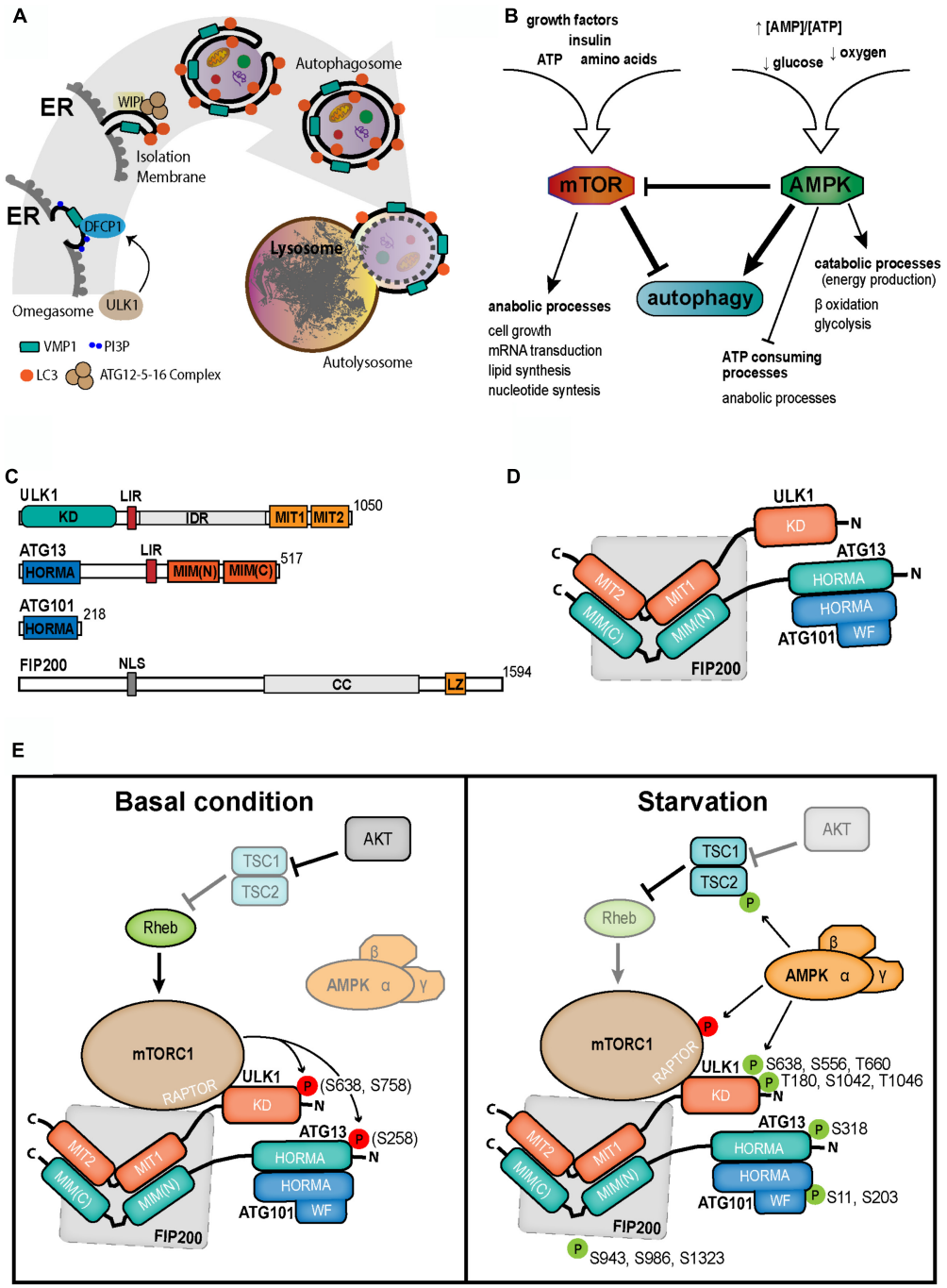


FIGURE 1 | (A) Schematic overview of autophagy. UKL1 activation leads to autophagosome biogenesis. On the ER surface, the transmembrane protein VMP1 recruits a PI3K complex. The consequent PI3P subdomain is recognized by DFCP1 on the omegasome structure. Then, in the isolation membrane, WIPI proteins recruit the ATG5-ATG12-ATG16 complex which in turn make possible the lipidation of LC3 on the membrane. The formation of autophagosome, a double membrane vesicle, allows the carrying of cargo to lysosome. Eventually, cargo is degraded in the resulted autophagolysosome. ER, endoplasmic reticulum; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol (3,4,5) triphosphate (PI3P). **(B)** Diagram of interrelationship among the cellular energetic and metabolic regulators, mTOR and AMPK, and the autophagy. **(C)** Representative scheme of the ULK1 complex proteins. Upper right number in each scheme shows the length of the amino acid chain. Described domains are showed for each protein. **(D)** Possible structure and interrelationship among the ULK1 complex proteins, suggested from available data. KD, kinase domain; LIR, LC3-interacting region; IDR, intrinsically disordered region; MIT, microtubule interacting and trafficking domain; HORMA, HOP1, REV7, and MAD2 domain; MIM, MIT-interacting motif; NLS, nuclear localization signal; CC, coil-coil region; LZ, leucine zipper; WF, WF finger motif. **(E)** Regulation of the autophagy initiation complex ULK1 by mTOR and AMPK at basal (left) and starvation (right) conditions.

as autophagy (Egan et al., 2011; Zhang et al., 2013; **Figure 1B**). AMP binding allows LKB1 to phosphorylate AMPK (Thr172) (Xiao et al., 2007; Zhang et al., 2013), which in turn directly and indirectly activates the autophagosome formation as is explained in the next sections.

ULK1 COMPLEX

ULK1 is so far the first complex in the core molecular machinery involved in the biogenesis of autophagosomes. This complex is composed by the serin/threonin protein kinase ULK1, ATG13, FIP200, and ATG101. Activated ULK1 is capable of triggering series of phosphorylations that enable the nucleation process and autophagosome biogenesis. At N-terminal ULK1 is the kinase domain followed by a disordered region that is postulated as highly regulated. On the opposite side, there are two MIT domains in tandem that compose a globular structure (Noda and Fujioka, 2015). ULK1 structure was characterized in complex with ATG13. On the C-terminal of ATG13 there are two MIT-interacting motifs in a helical region for recognition-interaction with the ULK1 MIT domains (Noda and Fujioka, 2015; Qi et al., 2015). Additionally, both proteins, ULK1 and ATG13, have a LIR domain for interaction with LC3 family members. ATG101, the smallest member of the complex, is essential for autophagy (Mercer et al., 2009). ATG101 is almost fully composed by a HORMA domain with direct interaction with the HORMA domain at the N-terminus of ATG13. ATG101 stabilizes ATG13 and ULK1 (Mercer et al., 2009; Suzuki et al., 2015) and seems to recruit downstream molecules through its WF finger motif (Suzuki et al., 2015). The last member of ULK1-complex is FIP200, that is the largest molecule involved in this complex (Hara et al., 2008; **Figures 1C,D**).

ULK1 complex is regulated by the two major key proteins related to nutritional and energetic sensing, mTOR and AMPK (He and Klionsky, 2009). Under growth factors stimulation and nutrient availability, the activated mTORC1 interacts with ULK1 through RAPTOR and phosphorylates several sites of ULK1 (Ser757/5637 in mouse, Ser758 in human) (Alers et al., 2012) and Atg13 (Ser258 in mouse) subunits (Kim et al., 2011; Puente et al., 2016). Then, ULK1 complex remains inactivated and autophagy repressed. AMPK induces ULK1-mediated autophagy by three strategies: 1- AMPK phosphorylates TSC2 at Ser1345 enhancing the activity of this mTORC1 inhibitor (Inoki et al., 2003). 2- AMPK is able to inhibit mTORC1 activity directly by phosphorylation of Raptor in Ser792/722 (Gwinn et al., 2008; Egan et al., 2011). 3- AMPK interacts with and phosphorylates ULK1 in Ser317/777 for its activation (Kim et al., 2011; **Figure 1E**).

Another pathway for ULK1 autophagy activation has been proposed: AMBRA1 may act as a bridge between ULK1 and the ubiquitin ligase E3 TRAF6 (Nazio et al., 2013; Grumati and Dikic, 2018). TRAF6-mediated poly ubiquitination, K63 type branched ubiquitin, potentiates autophagy activation by promoting stabilization and self-association of ULK1. This event initiates a positive loop, where ULK1 phosphorylates AMBRA1 enhancing TRAF6-mediated ULK1 ubiquitination (Nazio et al., 2013; Grumati and Dikic, 2018). Further, growth factors withdrawal might induce the activation of TIP60 by GSK3-mediated phosphorylation at Ser86. TIP60 is an acetyltransferase that induces the activation of ULK1 by acetylation of Lys162/606 enhancing the triggering of autophagy (Lin et al., 2012).

THE MECHANISMS INVOLVED IN AUTOPHAGOSOME BIOGENESIS

Once activated, ULK1 is able to phosphorylate several substrates. Among them, there are two initial complexes, the ULK1 complex itself and the PI3KC3 complex 1 (PI3KC3-C1). In the first complex, ULK1 phosphorylates to itself (Thr180/1046, Ser1042) (Bach et al., 2011), and the other members of the complex, Atg13 (Ser318/203), FIP200 (Ser943/986/1323) and ATG101 (Ser11/203) (Lin and Hurley, 2016; Orhon and Reggiori, 2017; **Figure 1E**). In the second complex, ULK1 potentiates the PI3K activity of the catalytic subunit Vps34, by the phosphorylation of two members of the complex, BECN1 (Ser14) and ATG14L (Ser29), resulting in the increment of PI3P production (Russell et al., 2013). Following to ULK1 complex activation, the transmembrane protein VMP1 interacts with the BH3 domain of BECN1 through its ATG domain, recruiting the PI3KC3-C1 to the autophagosomal membrane (Molejon et al., 2013).

There are two main PI3KC3 complexes in autophagosome biogenesis. The complex 1 is composed by BECN1, ATG14L, Vps15 and Vps34, which is a key component in autophagosome initiation. The other complex, PI3KC3-C2, is related to autophagosome maturation and endosomal trafficking and is composed by the same members except for the regulatory protein ATG14L which is replaced by UVRAG. Structurally, the PI3KC3-C1 is stabilized in pairs, BECN1/ATG14L and Vps15/Vps34 (Stjepanovic et al., 2017). Upon autophagy induction, BECN1 recruitment induces the complex assembly, through the adaptor ATG14L, where the WD domain of Vps15 organizes the proteins into the complex allowing the activity of Vps34 (Stjepanovic et al., 2017). Moreover, the KAP1-mediated SUMOylation of Vps34 enhances the interaction of this protein with the rest of the complex (Yang et al., 2013). As it was commented before, ULK1-mediated phosphorylation of BECN1, ATG14L and Vps34 potentiates PI3K activity in this complex. The tumor suppressor DAPK, a calcium/calmodulin serine/threonine kinase, also contributes to the PI3KC3-C1 recruitment to the autophagosome membrane. This kinase phosphorylates BECN1 on its BH3 domain interfering with the BECN1-Bcl-xL association and releasing BECN1 (Zalckvar et al., 2009). This effect is reaffirmed by TRAF6 which ubiquitinates BECN1 on the same region (Shi and Kehrl, 2010). Recently, it has been proposed that Vps34 activity may be switched on/off by an EP300-dependent acetylation/deacetylation on K771, as another regulation of the PI3KC3-C1 (Su and Liu, 2017; Su et al., 2017).

The cascade of subsequent activations of ULK1 and PI3KC3-C1 complex members is limited by a series of degradative

processes. The deubiquitinase A20 (DUB A20) controls BECN1 participation on autophagosome formation by elimination of poly ubiquitin chain in the BH3 domain placed by ATF6 E3 ligase. Beyond that regulation, the E3 ligases NEDD4 and NEDD4L induce degradation of key members in ULK1, and Vps34 complexes respectively (Platta et al., 2012; Nazio et al., 2016). BECN1 is poly ubiquitinated with K11-linked ubiquitin chain by NEDD4 to be eliminated in the proteasome. Similar activity is carried out by NEDD4L on ULK1 targeting this protein with K27- and K29-linked ubiquitin chains. In both cases, the proteasome-mediated elimination of those proteins causes the destabilization of its respective complexes. In a redundant way of labeling for degradation, the poly ubiquitination with K48-linked ubiquitin chains on ULK1, BECN1, and Vps34 is catalyzed by the complex CUL3-KHLH20 (Liu and Chen, 2016).

The Omegasome and the Isolation Membrane

The local enrichment of PI3P in ER-subdomains acts as the signal for the nucleation of several autophagy-related proteins in a structure named omegasome that resembles the Greek letter omega (Ktistakis and Tooze, 2016). The first protein which recognizes the PI3P is DFCP1. DFCP1 possesses a diffuse pattern over the ER, mitochondria and Golgi but it is rapidly mobilized to the PI3P spots by the recognition of this phospholipid with the two FYVE motifs of its structure. Although it is a marker of omegasome, little is known about its role during the initial steps of autophagosome biogenesis. Additionally, the DFCP1 depletion does not seem to interfere with the progression of autophagy.

The rising omegasome leads to extension of a sack-like structure named isolation membrane or phagophore. WIPI2b, a member of the PROPPIN family, recognizes the local PI3P by the FRRG motif of its WD40-repeat β-propeller on the isolation membrane (Nascimbeni et al., 2017a). The process continues with two ubiquitin like systems: ATG12 and LC3. Cytoplasmic ATG12 is covalently attached to a C-terminal glycine of ATG5. This catalytic reaction resembles the ubiquitination process where ATG7 and ATG10 are subrogated to E1 and E2 enzymes, respectively (Klionsky and Schulman, 2014). ATG5-ATG12 complex is highly important, since it functions as E3 enzyme for LC3 conjugation to phosphatidylethanolamine (PE) on the autophagosomal membrane. This process seems to be mediated by ATG16L, which is composed by a WD40-repeat β-propeller domain localized in the C-terminal sequence. At N-terminal sequences, ATG16L possesses a binding domain that allows the interaction with ATG5 to eventually form the ATG12-ATG5-ATG16L complex (Wilson et al., 2014). The middle sequence of ATG16L expands a coil-coil (cc) dimerization domain that induces the formation of ATG16L dimers (Wilson et al., 2014). Then, WIPI2b is recognized by a region of ATG16L, between the cc-dimerization domain and the WD40-repeated β-propeller domain. Consequently, the ATG12-ATG5-ATG16L complex is recruited to the isolation membrane. LC3 plays a central role in autophagy being involved in vesicle elongation, maturation, fusion of autophagosome-lysosome and even as an adaptor to cargo recognition (Nakatogawa et al., 2007; Lee and Lee, 2016). LC3 shows a diffuse pattern distributed over the cytoplasm and into the nucleus (known as LC3-I) in basal conditions. Upon autophagy triggering, LC3 is deacetylated in the nucleus by SIRT1 (Huang et al., 2015) and is cleaved in cytoplasm by ATG4B, which eliminates the C-terminal arginine residue to expose a glycine (Satoo et al., 2009; Maruyama and Noda, 2017). In an ubiquitin-like reaction, the exposed glycine is combined to form a thioester bound, first with ATG7 (E1-like enzyme) and then with ATG3 (the E2-like enzyme) (Satoo et al., 2009; Maruyama and Noda, 2017). ATG3 is recognized by ATG12 of the ATG12-ATG5-ATG16L complex which has been already recruited to isolation membrane through WIPI2b. The ATG12-ATG5-ATG16L complex functions as the E3 enzyme leading the formation of an amide bound with the amine headgroup of PE (Noda et al., 2013; Otomo et al., 2013; Dooley et al., 2015). The lipidated LC3 (LC3-II) is present at the isolation membrane and on the autophagosome, in both sides of the membrane. The arrival of autophagosome to the lysosome is a fusion dependent mechanism of the HOPS complex, through STX17 (Jiang et al., 2014), and RAB7 (Gutierrez et al., 2004). Since LC3 is present in both membranes of autophagosome, once exposed to lysosomal hydrolases, there is a pool of LC3 that is degraded with cargo. However, the LC3 localized in the external membrane is cleaved from the PE, by ATG4B, and then recycled. (Noda et al., 2013; Otomo et al., 2013; Dooley et al., 2015).

Autophagosome Biogenesis in Non-Canonical Autophagy

Furthermore, of which is explained above, autophagy is able to follow unconventional pathways. ER-stress or glucose influx after starvation in NIH3T3, can induce autophagy independent of mTOR inhibition and where AMPK activation is not essential (Corona Velazquez and Jackson, 2018). Moreover, the glucose influx in mouse embryonic fibroblast can trigger autophagy independent of ULK1/2. Starved chicken DT40 cells show an autophagy dependent of ATG13-FIP200 interaction but independent of ULK1. Similar behavior is observed in some viral infection, such as coronaviruses, HBV or Poliovirus, which induce a non-degradative ULK1-independent form of autophagy. Even more interesting is that the oleate fatty acid can induce an autophagy mechanism that lacks of PI3P synthesis, since it cannot be inhibited by knocking-down of BECN1, Vps34, or ATG14. These examples suggest that autophagy is flexible and the pathways in autophagosome biogenesis may adapt to different situations depending on the inductor and the biological context (Corona Velazquez and Jackson, 2018).

Autophagosome Initiation Site

It is accepted that the initial structure related to autophagy is located on the ER. The data suggest that ULK1 complex translocates to phosphatidylinositol-enriched ER-subdomains and then, the membrane structure is fed by ATG9A-containing vesicles (Nishimura et al., 2017). Then, autophagosomes are formed in highly active ER-subdomains where lipidic interchange between ER and other cytoplasmic organelles occurs.

TABLE 1 | Main molecules involved in the initial steps of mammalian autophagosome biogenesis.

Protein	Complete name	Autophagy related function	Reference
mTOR	Mammalian target of rapamycin	Members of mTOR complex 1 (mTORC1): Autophagy inhibition by phosphorylation of ULK1 complex	Lamb et al., 2013
RAPTOR	Regulatory-associated protein of mTOR		
DEPTOR	DEP domain containing mTOR-interacting protein		
PRAS40	Proline-rich AKT1 substrate 40		
mLST8	Mammalian lethal with SEC13 protein 8		
AMPK	AMP-activated protein kinase	Autophagy activation by ULK1, mTORC1, and TSC2 phosphorylation	Egan et al., 2011
p62	Sequestosome-1 (SQSTM1 gene)	Autophagy cargo receptor	Puissant et al., 2012
ULK1	Unc-51-like kinase 1	Members of ULK1 complex	Bach et al., 2011; Russell et al., 2013
ATG13	Autophagy-related protein 13		
FIP200	FAK family interacting protein of 200 kDa		
ATG101	Autophagy-related protein 101		
BECN1	Beclin 1	Members of PI3KC3-C1/2	Ktistakis and Tooze, 2016
Vps15	Serine/threonine-protein kinase VPS15		
Vps34	Phosphatidylinositol 3-kinase VPS34		
ATG14L	Autophagy-related protein 14L	Member of PI3KC3-C1	
UVRAG	UV radiation resistance associated protein	Member of PI3KC3-C2	
KAP-1	E3 SUMO-protein ligase TRIM28	SUMOylation of Vps34	Yang et al., 2013
DAPK	Death-associated protein kinase	BECN1 phosphorylation	Zalckvar et al., 2009
CUL3	Cullin-3	Poly ubiquitination of ULK1, Vps34, and BECN1	Liu and Chen, 2016
KLHL20	Kelch-like protein 20	Substrate-binding subunit of CUL3 ubiquitin ligase. Recognition of ULK1, Vps34, and BECN1 as substrates	Liu and Chen, 2016
VMP1	Vacuole Membrane Protein 1	Recruitment of PI3KC3-C1 by interaction with BECN1 /autophagosomal membrane	Ropolo et al., 2007
EP300	EP300-interacting inhibitor of differentiation 300	Vps34 acetylation	Su et al., 2017
DFCP1	Double FYVE-containing protein 1	Omegasome marker	Ktistakis and Tooze, 2016
WIPI2b	WD40-repeat phosphoinositide-interacting protein	Isolation membrane marker	Nascimbeni et al., 2017a
ATG12	Autophagy-related protein 12	Member of ATG12-ATG5-ATG16L complex: E3 like function in LC3 conjugation to phosphatidylethanolamine	Klionsky and Schulman, 2014
ATG5	Autophagy-related protein 5		
ATG16L	Autophagy-related protein 16L		
ATG7	Autophagy-related protein 7	E1 in LC3 lipidation and ATG12-ATG5 conjugation	
ATG10	Autophagy-related protein 10	E2 in ATG12-ATG5 conjugation	
ATG3	Autophagy-related protein 3	E2 like function in LC3 lipidation	Satoo et al., 2009
LC3	Microtubule-associated proteins 1A/1B light chain 3B	Vesicle maturation/cargo recognition	Lee and Lee, 2016
SIRT1	NAD-dependent deacetylase sirtuin-1	LC3 deacetylation	Huang et al., 2015
ATG4B	Autophagy-related protein 4B	Clevage of C-terminal Gly of LC3	Maruyama and Noda, 2017
ATG9A	Autophagy-related protein 9 A	Isolation membrane extension	Feng and Klionsky, 2017
Esyt 1, 2, 3	Extended synaptotagmin-1, 2, 3	ER-PM contact sites	Nascimbeni et al., 2017b
AP-4	Adaptor protein 4	Isolation membrane extension	Mattera et al., 2017
Sar1	Sar1	COPII coat: participation in autophagosome biogenesis	Karanasios et al., 2016
Sec 13, 23, 24, 31			
Rab11A	Ras-related protein Rab-11A	Recycling endosomes	Puri et al., 2018
AMBRA1	Activated in BECN1-regulated autophagy protein 1	ULK1 ubiquitination	Nazio et al., 2013
TRAF6	TNF receptor (TNFR)-associated factor 6	ULK1 and BECN1 ubiquitination	Grumati and Dikic, 2018

Two sites of autophagosome biogenesis have been recently demonstrated: The ER-plasma membrane contact site (ER-PM) and the ER-Mitochondria contact site (Hamasaki et al., 2013; Nascimbeni et al., 2017b). VMP1 is a key player in the biogenesis of autophagosomes that remains in the autophagosomal membrane (Grasso et al., 2011). VMP1-BECN1 interaction allows the recruitment of PI3KC3-C1 to the ER-PM contact site by the interaction with the proteins Esyt 1, 2, and 3 (Nascimbeni et al., 2017b). Moreover, VMP1 was suggested to also regulate the ER-mitochondria contact site during autophagy and to be involved in the release of the initial autophagosome vesicle by activation of SERCA pump (Tabara and Escalante, 2016; Zhao et al., 2017). The transmembrane protein ATG9A is in Golgi and endosomal system, in early and late endosomes with a minimal percentage of recycling ones (Feng and Klionsky, 2017). In starvation, the TRAPPIII complex, related to ER-Golgi vesicular trafficking, mobilizes ATG9A vesicles to the sites of nascent autophagosomes (Shirahama-Noda et al., 2013). The adaptor protein AP-4 is required for this event, since it mediates the trafficking of ATG9A from *trans*-Golgi network to the site of autophagosomes maturation (Mattera et al., 2017). This event would potentiate the expansion of the isolation membrane. Nevertheless, the contribution of this membrane by the ATG9A vesicles is not enough to explain the growth of the membrane itself. Moreover, ATG9 seems to take a distinctive role in different systems. In contrast to mammals, yeast ATG9 has a fundamental role at very early steps in the pre-autophagosomal structure. On the other hand, in plants, the depletions of *Arabidopsis* ATG9 still allows formation of autophagosomal structures supplemented with ATG8 (LC3 ortholog) suggesting divergent regulation and mechanisms of this types of vesicles (Zhuang et al., 2017).

Ribosomes-free regions specialized in ER-Golgi communication are present in the rough ER. Vesicles arise targeted to the Golgi from these areas, described as ER-exit sites (ERES). These vesicles are supplemented by the proteins Sar1, Sec23, Sec24, Sec13 and Sec31, that constitute the COPII coat (Zahoor and Farhan, 2018). Before reaching Golgi, the COPII-coated vesicles go through an intermediated structure named ER-Golgi intermediate compartment (ERGIC) (Ben-Tekaya et al., 2005). The function of these structures is not completely understood, but they might participate in the autophagosome biogenesis. An impairment of these compartments causes an autophagy downregulation (Karanasios et al., 2016; Zahoor and Farhan, 2018).

Data suggest that the bulk contribution for the growth of the autophagosome membrane comes from the ER-Golgi vesicular trafficking. During starvation, the FIP200-CTAGES5 interaction induces the remodeling and enlargement of ERES positives for Sec12 (Ge et al., 2017). This allows the production of COPII-coated vesicles that are released to contribute to autophagosome formation. Moreover, ULK1 phosphorylates Sec23A, a member of the COPII multiprotein complex. This event is related to morphological variations on ERES during starvation and might turn the secretory machinery from anabolic to catabolic state.

A recent work shows a previously unexpected key role of Rab11A-positive membranes in autophagosome biogenesis (Puri et al., 2018). They demonstrated that WIPI2 relies, beyond the recognition of PI3P, in the interaction with Rab11A for recruitment of ATG16L. Also, the authors suggest a model where isolation membrane is represented by Rab11A-positive membrane, likely to be recycling endosomes. In this context, Rab11A-positive membranes constitute the platform for autophagosome formation initial steps.

CONCLUSION AND PERSPECTIVES

The initial molecular steps in autophagosome biogenesis are determined by three mains complexes: ULK1 complex; PI3KC3-C1; and ATG16L1–ATG5–ATG12 which eventually favors LC3 lipidation in the growing isolation membrane. LC3 family seems to play a relevant role in cargo recognition, autophagosome closure and fusion with lysosomes. However, while the initial molecular steps seem to be essential and well-known in canonical autophagy, the subsequent events in mammalian autophagosome biogenesis are less characterized. Moreover, the wide spectrum of autophagy-related events and the number of molecules involved (**Table 1**) leads to the concept that different pathways might account for diverse types of autophagy and may reveal different functions of autophagy in physiological and pathological cellular processes. Furthermore, the meaning of different origins and composition of the autophagosomal membrane, such as those supplied by ATG9A and COP-II vesicles (Feng and Klionsky, 2017), are still not fully understood.

Moreover, autophagosome biogenesis is regulated by a variety of signaling pathways through posttranslational modification, such as phosphorylations, ubiquitinations, SUMOylations and acetylation, that may account for diverse conditions, functions or selectivity. Furthermore, this molecular regulation, that are eminently druggable, may be relevant in the development of therapeutic strategies of autophagy modulation for complex pathologies such as cancer (Galluzzi et al., 2015) or neurodegenerative diseases (Zare-Shahabadi et al., 2015).

Although there are many aspects still unclear on mammalian autophagosome biogenesis, future findings that shed light on this sophisticated intracellular process can be taken for granted.

AUTHOR CONTRIBUTIONS

DG did the literature search, wrote the first draft of the manuscript and designed all the figures. FR wrote a session of the first draft of the manuscript and assisted with the edited version. MV edited and added to the draft of the manuscript and figures and revised the final version of the manuscript.

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Identification of Kinases and Phosphatases That Regulate ATG4B Activity by siRNA and Small Molecule Screening in Cells

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Autophagy protease ATG4B is a key regulator of the LC3/GABARAP conjugation system required for autophagosome formation, maturation and closure. Members of the ATG4 and the LC3/GABARAP family have been implicated in various diseases including cancer, and targeting the ATG4B protease has been suggested as a potential therapeutic anti-cancer strategy. Recently, it has been demonstrated that ATG4B is regulated by multiple post-translational modifications, including phosphorylation and de-phosphorylation. In order to identify regulators of ATG4B activity, we optimized a cell-based luciferase assay based on ATG4B-dependent release of Gaussia luciferase. We applied this assay in a proof-of-concept small molecule compound screen and identified activating compounds that increase cellular ATG4B activity. Next, we performed a high-throughput screen to identify kinases and phosphatases that regulate cellular ATG4B activity using siRNA mediated knockdown and cDNA overexpression. Of these, we provide preliminary evidence that the kinase AKT2 enhances ATG4B activity in cells. We provide all raw and processed data from the screens as a resource for further analysis. Overall, our findings provide novel insights into the regulation of ATG4B and highlight the importance of post-translational modifications of ATG4B.

Keywords: ATG4B, siRNA, small molecule, kinase, phosphatase, cDNA, screen, AKT2

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INTRODUCTION

Autophagy is a cellular process central to multiple aspects of health and disease. A key function of autophagy is to mediate lysosomal degradation of cellular material through the formation of an autophagosome, a double-membrane structure that engulfs cytoplasmic material, seals it from the surrounding cytoplasm and delivers it to the lysosome. The formation of an autophagosome is governed by a number of ATG (AuTophaGy-related) proteins that are conserved from yeast to mammalian cells (Tsukada and Ohsumi, 1993).

A key step in the formation of an autophagosome is the conjugation of microtubule-associated protein 1 light chain 3 (LC3) and gamma-aminobutyric acid receptor-associated protein (GABARAP) proteins to the autophagosomal membrane. LC3/GABARAP proteins are synthesized

in the cell as an inactive form (pro-LC3/GABARAP) that require activation through C-terminal proteolytic cleavage by the ATG4 family of proteins to generate LC3/GABARAP-I.

It is thought that ATG4 mediates two key processing steps of LC3/GABARAP, the proteolytic processing prior to lipidation and insertion of lipidated LC3/GABARAP-II in the autophagosomal membrane, and the de-lipidation of LC3/GABARAP-II, leading to recycling of processed LC3/GABARAP-I. There are four members of the ATG4 family in mammalian cells that are partially redundant in substrate processing, but have also distinct specificities. ATG4B, the main isoform of the ATG4 family of proteins is regulated by different types of post-translational modifications, including ubiquitination (Kuang et al., 2012), O-GlcNAcylation (Jo et al., 2016), *S*-nitrosylation (Li et al., 2017), capase mediated proteolysis (Betin and Lane, 2009; Betin et al., 2012), redox mechanisms (Scherz-Shouval et al., 2007; Qiao et al., 2015; Heintze et al., 2016) and phosphorylation (Yang et al., 2015; Huang et al., 2017; Pengo et al., 2017; Sanchez-Wandelmer et al., 2017; Ni et al., 2018). It is not well understood how ATG4B hydrolase activity toward its two substrates pro-LC3 and LC3-II could be differentially regulated, but recently it has been pointed out that post-translational modifications may control the ATG4B proteolytic and de-lipidation activity. It has been shown that local phosphorylation by ATG1/ULK1 at the forming autophagosome inhibits ATG4 activity in yeast (Sanchez-Wandelmer et al., 2017) and ATG4B in mammalian cells (Pengo et al., 2017), whereas de-phosphorylation by PP2A renders ATG4B active in the cytoplasm of cells. Other phosphorylation events may also contribute to such regulation, since AKT1 and MST4 are capable of phosphorylating ATG4B (Huang et al., 2017; Ni et al., 2018), although the spatio-temporal context of this has not yet been defined.

A role for ATG4B in cancer has been proposed, including chronic myeloid leukemia (Rothe et al., 2014), osteosarcoma (Akin et al., 2014), colorectal cancer (Liu et al., 2014), prostate cancer (Mouratidis et al., 2014), breast cancer (Bortnik et al., 2016) and pancreatic adenocarcinoma (Yang et al., 2018). The rationale that ATG4 proteins might be therapeutic targets mostly stems from the fact that these proteins are highly over-expressed in some cancer types compared to non-cancerous cells (Costa et al., 2016) and genetic inhibition of ATG4B either through siRNA or use of a dominant negative form of the gene show some benefit in chronic myeloid leukemia (Rothe et al., 2014), breast cancer (Bortnik et al., 2016) and pancreatic carcinoma (Yang et al., 2018).

Multiple efforts are underway to develop biochemical assays to monitor ATG4B activity and thus identify compounds targeting ATG4B (Kurdi et al., 2017). Assay types include the use of enzymatic reporter genes, such as the phospholipase A2-linked substrate approach (Ni et al., 2015), amino-methylcoumarin (AMC)-type esters of LC3 substrates, BRET-based assays (Woo et al., 2014), and gel electrophoresis assays (Cleenewerck et al., 2016).

We have previously developed a cell-based system to monitor cellular ATG4B activity that utilizes the non-conventional secretion of a small luciferase (Ketteler et al., 2008; Luft et al., 2014). Key advantages of this assay are that it is very sensitive, non-invasive and highly quantitative (Ketteler and Seed, 2008). This assay has supported significant discoveries that helped to understand the post-translational regulation of ATG4B. These include the identification of the ubiquitin ligase RNF5 as a key regulator of ATG4B stability (Kuang et al., 2012), the O-GlcNAc modification of ATG4B to increase its proteolytic activity (Jo et al., 2016), and the regulation of ATG4B activity by phosphorylation (Yang et al., 2015; Pengo et al., 2017). Here, we present a small molecule and siRNA screen to identify regulators of ATG4B activity in cell-based assays. The identified compounds are effective to overcome cancer-associated defects in LC3A processing and are valuable tool compounds for further development and understanding of ATG4 biology. Furthermore, we have identified a number of kinases that modulate ATG4B-mediated LC3 processing that were not previously known to have this function.

MATERIALS AND METHODS

Cell Lines

HeLa cells expressing mCherryLC3 were obtained from Dr. Ramnik Xavier (Massachusetts General Hospital, Boston). Retroviral supernatants of HEK293T cells transfected with pMOWS-ActinLC3dNGLUC, GagPol and VSV-G were obtained by calcium phosphate precipitation as described (Ketteler et al., 2002) to generate stable HeLa ActinLC3dNGLUC/mCherryLC3 and HEK293T-ActinLC3dNGLUC cells. Briefly, pBABE-mCherry-GFP-LC3 or pMOWS-ActinLC3dN was transfected with VSV-G and GagPol into HEK293 cells using calcium phosphate transfection. Supernatants were harvested and filtered through 0.45 μm filters, supplemented with 8 μg/ml final concentration of polybrene (Sigma) and added to target cells for overnight incubation. Transduced cells were then passaged and selected with puromycin. All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Life Technologies), L-Glutamine and Penicillin/Streptomycin.

Plasmids

Expression plasmids pGEXATG4B and pGEXATG4B^{C74S} were created as described previously (Pengo et al., 2017). pGEXATG4B$^{\Delta 1-24}$ (mutation by deletion of 24 amino acid residues from the N-terminus), pGEXATG4B^{S34A}, pGEXATG4B^{S34D} and pGEXATG4B^{S121A} were created by PCR using pGEXATG4B as a template and primers Δ1–24 forward (5′-CCC GTT TGG ATA CTG GGT AGA AAA TAC AGC-3′) and Δ1–24 reverse (5′-GAA TTC CGG GGA TCC CAG GGG C-3′), primers S34A forward (5′-GCT ATT TTC ACA GAA AAG GAC GAG-3′) and S34A reverse (5′-GTA TTT TCT ACC CAG TAT CCA AAC-3′), primers S34D forward (5′-GAT ATT TTC ACA GAA AAG GAC GAG-3′) and S34D reverse (5′-GTA TTT TCT ACC CAG TAT CCA AAC-3′), primers S121A forward (5′-GCT TAC TAC TCC ATT CAC CAG ATA-3′) and S121A reverse (5′-GTC CTT CCT GTC GAT GAA TGC GTT-3′), and primers S262A forward (5′- GCA GCC

CAC TAC TTC ATC GGC TA-3′) and S262A_reverse (5′- GTT GGG CTT CCC TCC GAT GAC-3′), respectively. All PCR were performed at 30 cycles using Pyrobest DNA polymerase (Takara, R005A). The PCR products were phosphorylated, ligated and transformed into *Escherichia coli* DH5α for selection of correct plasmids. The following constructs were described elsewhere: pMOWS-ActinLC3dN (Ketteler et al., 2008), pGEXCLK2cd (Prak et al., 2016), pEAK12-ActinLC3A-R70H-dNGLUC (Costa et al., 2016), and pEAK12-GFP (Ketteler et al., 2008). The ATG4B promoter construct ATG4B-FLUC was obtained from Switchgear Genomics (#S711306). The sequence of the inserted promoter region from ATG4B is shown in **Supplementary Figure S1**. The vector map can be found on the company's website[1]. Transcriptional activation of ATG4B promoter was measured by monitoring Renilla luciferase in cell lysates according to the manufacturer's instructions (Promega).

Compounds

The Chemibank compound collection was obtained from David Selwood[2]. The ranges of some of the molecular properties are as follows: molecular weight between 126 and 600, AlogP between −3.5 to 6, hydrogen bond donors between 0 and 6, hydrogen bond acceptors between 0 and 12, rotable bonds between 0 and 15 and number of rings between 1 and 8. The library has hit-like properties (rule of 6) and falls just outside the Lipinski's rule of five. A total of 30,000 compounds was stored as a 10 mM DMSO stock solution under nitrogen (5% O_2) and low humidity (5%) at room temperature and in the dark (Roylan San Francisco storage pod). For screening, compounds were transferred to assay plates using the Labcyte Echo 520 at a final concentration of 10 μM with a final DMSO concentration of 0.2%. Hit compounds were re-purchased from Asinex (Delft, Netherlands), or Life Chemicals (Ukraine). Bafilomycin A, DTT, H_2O_2, *N*-acetyl cysteine and rapamycin were obtained from Sigma.

Small Molecule Screening

High-Throughput Screening was performed in 384-well plates (Greiner). First, compound was added to the plates using the Echo 520 (Labcyte). Next, HeLa-ActinLC3dNGLUC cells (20,000/well) were dispensed onto the compounds using the Thermo Fisher Multidrop and cultured for 24 h at 37°C. Supernatants (5 μl) were harvested and dispensed into black 384-well plates. Native coelenterazine (Cambridge Bioscience, #BT10110) in GLUC buffer (0.1% disodium phosphate, 5% glycerol, 150 mM sodium bromide, 1 mM EDTA, 25 mM Tris-HCL pH8 and 2 mM ascorbic acid) at a final concentration of 10 μg/ml was injected immediately prior to analysis using the Envision II (PerkinElmer) plate reader. For Z' factor calculation, the following formula was used:

$$Z' = 1 - (3x(STD_{pos} + STD_{neg})/|mean_{pos} - mean_{neg}|)$$

with STD_{pos} = standard deviation of the positive control and STD_{neg} = standard deviation of the negative control. For cell-based assays, we accept values that are higher than 0.3 for the Z' factor.

siRNA and cDNA Screening

Stable HEK293T-ActinLC3dNGLUC were sent for STR profiling and confirmed as HEK293T cells. Cells were counted and 5,000 cells were seeded into 384-well and incubated overnight at 37°C and 5% CO_2. The siRNA library for human kinases and human phosphatases (Sigma MISSION, **Supplementary File S1**) consists of 3 siRNA oligonucleotides per gene in a 96-well format where the outer columns 1 and 12 were used for controls. First, the 3 siRNAs for each gene were pooled using the automated Tecan Freedom Evo liquid handler. The siRNA pools were then transfected at a final concentration of 55 nM with lipofectamine 2000 (Invitrogen) using an automated protocol on the Tecan Freedom Evo in 384-well plates. Briefly, 5 μl of the siRNA stock solution (100 μM) was mixed with 0.5 μl lipofectamine and 50 μl Optimem for 20 min at RT. Ten μl of this mixture was added to cells in the 384-well plate to a total volume of 50 μl and incubated for 48 h at 37°C. After 48 h, 5 μl of supernatant was transferred using the Tecan Freedom Evo to black 384 multi-well plates and 25 μl substrate of native coelenterazine was added prior to reading luminescence in the PerkinElmer Envision II. Substrate was added using the injectors of the PerkinElmer Envision II to ensure equal times from addition of substrate to measurement in all wells. The cDNA kinome library (**Supplementary File S2**; Thermo Fisher) was transfected at 100 ng/well in HEK293T cells stably expressing the ActinLC3dNGLUC reporter and luciferase release was monitored after 24 h.

Statistical Analysis

The primary screening data was analyzed using CellHTS2 (Boutros et al., 2006). Relative luciferase light units were normalized across the plate and the *B* scores were calculated to determine Z scores of each individual compound. All error bars shown unless otherwise indicated are calculated as standard deviations from the mean of the replicates. Statistical significance was calculated using a two-sided paired *T*-Test (Microsoft Excel). In **Figure 3C**, a one-way ANOVA with Tukey's multiple comparison test was applied to calculate significances. The graph was drawn in GraphPad Prism.

Luciferase Release Assay

The luciferase release assay was described previously (Ketteler et al., 2008). Native coelenterazine was prepared as 1 mg/ml stock solution in acidified Methanol and diluted 1:100 in PBS or GLUC assay buffer. Typically, five μl of supernatant was harvested and mixed with 25 μl coelenterazine in 384-well plates or 50 μl of coelenterazine in 96-well plates. All experiments were performed in triplicates except the siRNA screen that was done in quadruplicates.

[1] www.switchgeargenomics.com

[2] www.ucl.ac.uk/chemibank

Cell Viability Assay

Cellular Viability was assessed using the Cell Counting Kit (CCK8, Sigma). Briefly, 5 μl of CCK8 solution was added in 50 μl PBS to the cells and incubated for 60 min at 37°C prior to measurement of absorbance at 450 nm in the Envision II.

Protein Purification

Recombinant proteins were purified from bacteria as described previously (Prak et al., 2016). Protein expression and purification of LC3B-GST, ATG4B, and ATG4B mutant C74S was done as described previously (Pengo et al., 2017). Protein expression and purification of ATG4B mutants Δ1–24, S34A and S34D were done the same way as that of ATG4B. GST was removed from GST-tagged ATG4B and GST-tagged ATG4B mutants using PreScission Protease (GE Healthcare, 27-0843-01). All recombinant proteins were stored at −80°C in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 33% glycerol and 1 mM dithiothreitol (DTT).

In vitro Phosphorylation Assays

In vitro radioactive assays were performed by incubating 100 ng recombinant ATG4B diluted in assay buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 20 μM cold ATP, 0.16 μM ATP [γ-^{32}P] Perkin-Elmer NEG502A100UC) in the presence of recombinant AKT2 (Sigma-Millipore) at 30°C for the indicated time. The reaction was stopped by adding 5X SDS Loading Buffer and boiling for 5 min. Samples were loaded on NUPAGE Acrylamide gel (Invitrogen, NP0321BOX). Gels were stained with InstantBlue Protein Stain (Expedeon, ISB1L) before drying on filter paper and measuring incorporated radioactivity by exposing on photographic film (Bio-Rad).

LC3B-GST Cleavage Assay and Analysis of Enzyme Kinetics for ATG4B and Its Mutants

The cleavage assay was done at 37°C in a reaction volume of 20 μl containing 1 mg/ml LC3B-GST and 0.004 mg/ml ATG4B wide type and mutants Δ1–24, S34A and S34D in assay buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DTT) for 0.4–10 min. The reaction was stopped by adding the same volume of 2X SDS Loading Buffer and boiling for 5 min. The sample were analyzed on a 4–20% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, 456 1096) and Coomassie Brilliant Blue Staining. The images of the gels were scanned and the intensity of each protein band were quantified using Fiji[3] using the analyse >gel built-in function. The percentage of the substrate that remain at each reaction time point (% of remaining substrate, *y*-axis) equal to optical density (OD) LC3-GST/(OD LC3-GST + OD GST + OD LC3-I) × 100% was plotted versus the reaction time (s, *x*-axis) and the curves were then fitted using the non-linear regression method in R software, from which the time needed to catalyze amount of substrate were derived.

[3]http://fiji.sc/Fiji

To analyze the enzyme kinetics for ATG4B and its mutants, purified ATG4B and ATG4B mutants at 45.15 nM except mutant C74S at 11.3 μM were incubated with twofold serial dilutions of LC3B-GST from 39 to 2.4 μM in assay buffer A in a reaction volume of 20 μl at 37°C. The incubation time was 6 min for all except mutant C74S was incubated for 5 h. The reaction was stopped by adding the same volume of 2X SDS Loading Buffer and boiling for 5 min. The samples were subjected to a 4–20% Mini-PROTEAN® gel and the intensity of protein bands were analyzed the same way as that of the cleavage assay above. The initial velocity (μM/min, *y*-axis) was calculated as the concentration of GST produced, which was plotted versus the concentration of substrate LC3B-GST before reaction (μM, *x*-axis). The curves were then fitted using the non-linear regression method in R software, from which the V_{max} and K_m (Michaelis constant) for each enzyme-substrate reaction were derived. The k_{cat} (catalytic constant) was determined diving V_{max} by the enzyme concentration. The catalytic efficiency is defined as k_{cat}/K_m (inverse molar liter per second).

RESULTS

Optimization of a Cell-Based ATG4B Sensor for High-Throughput Screening

In order to set up a screen for small molecule regulators of ATG4B, we used the previously described luciferase release assay (Ketteler et al., 2008). This assay relies on non-conventional release of Gaussia luciferase (GLUC) from cells upon ATG4B-dependent cleavage of an ActinLC3B2dNGLUC reporter construct (**Figure 1**). The amount of luciferase in supernatants correlates with cellular ATG4B activity, making this a very simple quantitative assay. We have recently confirmed that non-conventional release of GLUC from cells is not dependent on autophagosome formation, since ATG5 knockout cells are able to release GLUC from cells (Luft et al., 2014). Thus, this assay is suitable for screening for modulators of ATG4B-mediated LC3 cleavage.

First, we tested various autophagy-modulating compounds for response in the luciferase release assay (**Figure 2**). In line

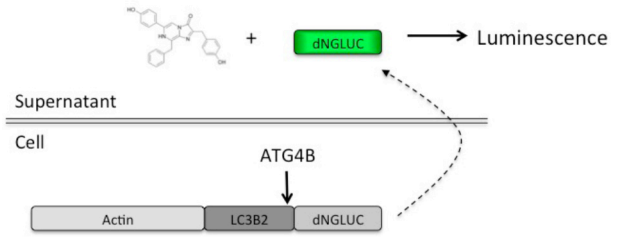


FIGURE 1 | Schematic of the luciferase release assay. A fragment of Gaussia luciferase is linked to full-length LC3B2 and β-actin inside cells. Upon cleavage of LC3B2 by ATG4B, Gaussia luciferase (dNGLUC) is released into the supernatants and can be monitored as a correlate of cellular ATG4B activity. The structure of native coelenterazine is shown.

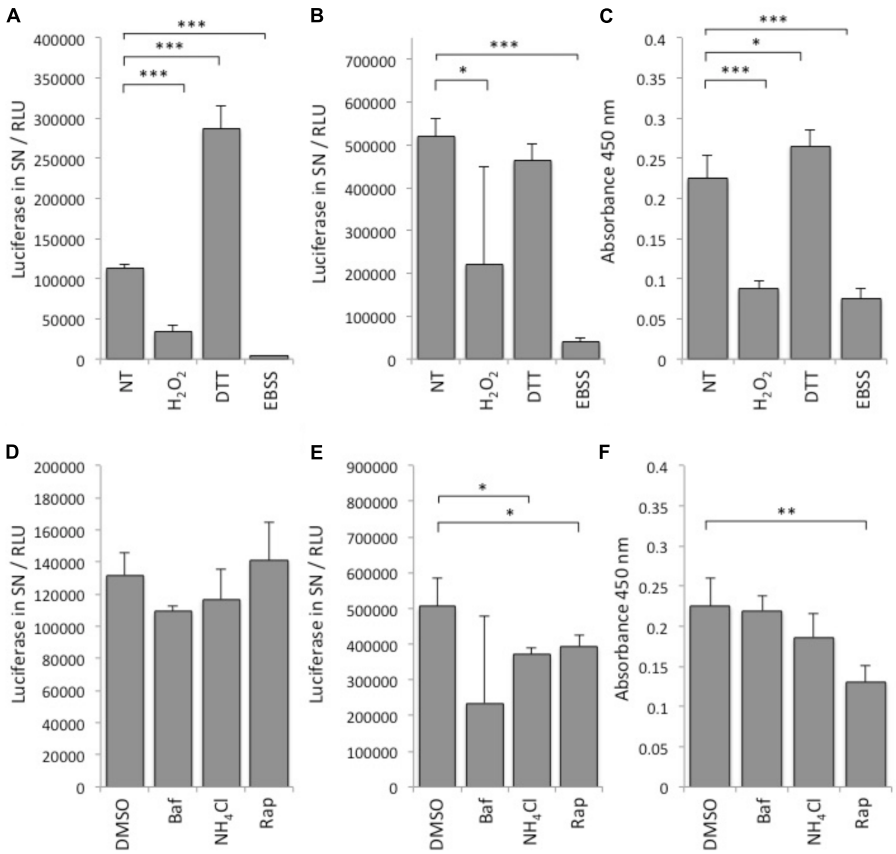


FIGURE 2 | Performance of the luciferase release assay. **(A)** Luciferase activity in relative light units (RLU) was monitored in supernatants (SN) of HEK293T cells transfected with ActinLC3dNGLUC and treated with the indicated reagents or left (NT, not treated). **(B)** HEK293T cells transfected with dNGLUC (without ActinLC3 linker) were treated as in **(A)** and luminescence released into supernatants was measured as an indicator of general dNGLUC secretion. **(C)** Cell viability was measured using the cell-counting kit 8 (CCK-8, Sigma). The same cells as in **(A)**, were subjected to the CCK-8 cell viability assay by adding the cell viability reagent for 60 min before reading the absorbance at 450 nm in the PerkinElmer Envision II. Lower absorbance values reflect low cell viability. **(D)** HEK293T cells were transfected with ActinLC3dNGLUC and treated with the indicated reagents or DMSO as control. **(E)** HEK293T cells transfected with dNGLUC (without ActinLC3 linker) were treated as in **(D)** and luminescence released into supernatants was measured in the PerkinElmer Envision II. **(F)** Cell viability was measured as in **(C)** after treatment with the indicated reagents. Baf, bafilomycin A; Rap, rapamycin. All results displayed are from three independent experiments and statistical significance was determined using a two-sided paired *T*-Test (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$). Error bars represent standard deviation.

with previous observations that ATG4B activity is highly redox-dependent (Scherz-Shouval et al., 2007), we found that treatment of cells with DTT can strongly activate the reporter, whereas treatment with H_2O_2 reduced reporter activity (**Figure 2A**). To assess whether the treatment affected general dNGLUC secretion, we expressed dNGLUC, which is constitutively released independent of ATG4B. We observed that DTT did not modulate secretion of dNGLUC and H_2O_2 moderately reduced secretion (**Figure 2B**). This decrease in dNGLUC secretion was due to strongly reduced cell viability upon treatment with H_2O_2 whereas treatment with DTT only mildly affected cell viability (**Figure 2C**). Overall, these results suggest that redox regulation directly affects cellular ATG4B activity. Other autophagy modulating treatments such as NH_4Cl or bafilomycin A1 had very little impact on cellular ATG4B activity (**Figure 2D**), confirming that the luciferase release assay specifically measures ATG4B activity and not general autophagy activity or flux. Starvation by EBSS resulted in a decrease in luciferase secretion from the ActinLC3dNGLUC construct, but at the same time a decrease in general dNGLUC secretion and cell viability (**Figures 2A–C**) as well,

TABLE 1 | Ratios of luciferase released from cells expressing the ActinLC3BdNGLUC reporter normalized by cell viability.

Treatment	Ratio Luc/viability
DMSO	1.00 ± 0.26
DTT	1.84 ± 0.32
H_20_2	0.66 ± 0.24
Baf	0.85 ± 0.10
EBSS	0.09 ± 0.04
NH_4Cl	1.07 ± 0.35
Rapamycin	1.84 ± 0.59

*Data calculated from values in **Figure 2**. Displayed are mean values with standard deviation.*

suggesting that the decrease in luciferase release is due to reduced cell viability. Treatment with rapamycin had no strong effect on luciferase release from the ActinLC3dNGLUC reporter, but reduced both overall dNGLUC secretion and cell viability (**Figures 2D–F**). Upon calculating the ratio of luciferase release over cell viability, rapamycin was confirmed as inducer of ATG4B activity (**Table 1**). In conclusion, the ATG4B luciferase release assay specifically detects cellular ATG4B activity such as redox-sensitive mechanisms. One caveat in using this assay is that effects on cellular viability can reduce the net release of luciferase from the reporter, but such effects can be normalized by assessing the cellular viability in parallel.

Next, we established that the assay is amenable to high-throughput screening by determining the Z' factor, a good surrogate for assessing the robustness of an assay (Zhang et al., 1999). In the absence of potent small molecule inhibitors or activators of ATG4B, we used Brefeldin A as a well characterized inhibitor of dNGLUC secretion (Luft et al., 2014). As shown in **Supplementary Figure S2**, Brefeldin A resulted in a robust reduction in secreted dNGLUC from cells. We determined the Z' factor as 0.46, which was within a suitable range for cellular screening. We screened a collection of 30,000 compounds from UCL Chemibank[4] in 384-well format in triplicates. The raw

[4]www.ucl.ac.uk/chemibank

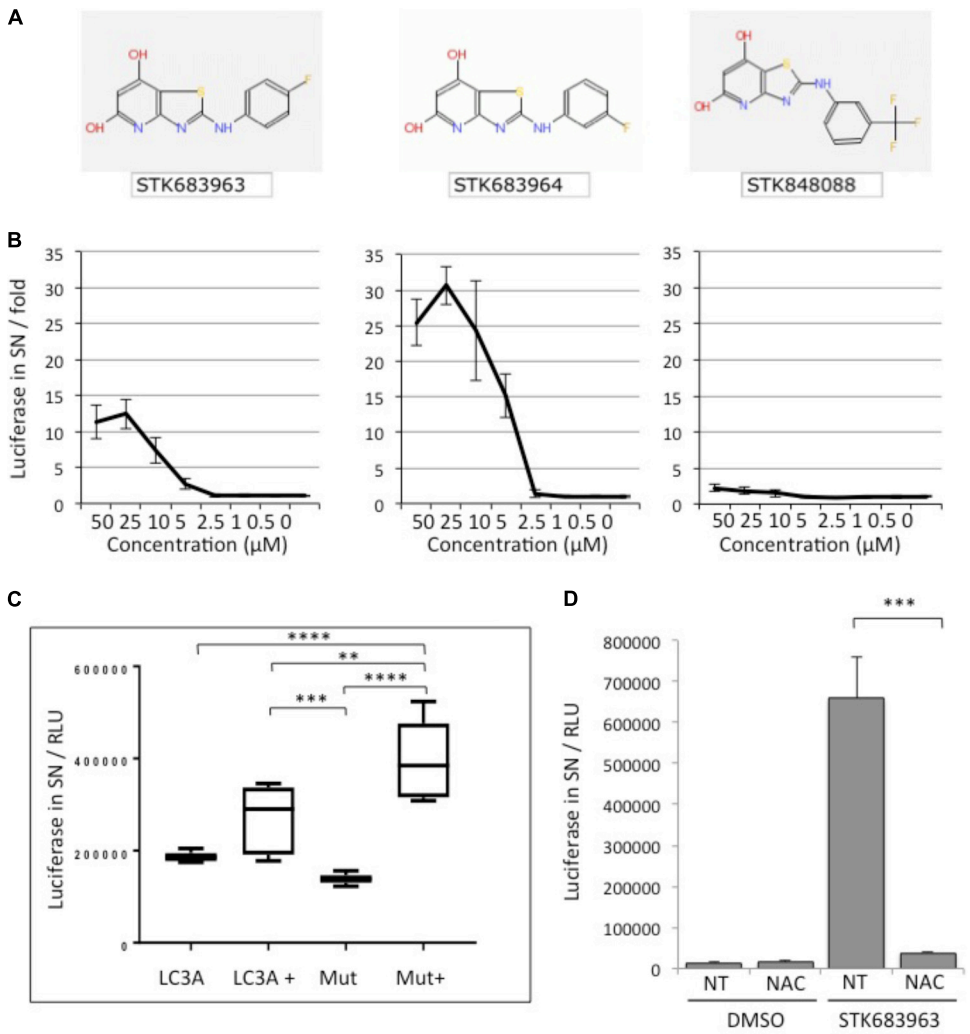


FIGURE 3 | STK683963 is a novel activator of ATG4B. **(A)** Structures of hit compound STK683963 and its analogs STK683964 and STK848088. **(B)** HeLa-ActinLC3dNGLUC cells were treated with the hit compound STK683963 and two analogs (STK683964, STK848088) overnight at the indicated concentrations and luciferase activity released into supernatants was monitored. Data expressed as fold of DMSO control. **(C)** HEK293T cells were transfected with ActinLC3AdNGLUC or ActinLC3A-R70H-dNGLUC and treated overnight with DMSO or STK683963. Luciferase release was measured in the PerkinElmer Envision II. A one-way ANOVA with Tukey's multiple comparison test was applied to calculate significances (****$p < 0.0001$ and **$p < 0.01$). **(D)** HeLa cells stably expressing ActinLC3dNGLUC were treated with STK683963 in combination with *N*-acetyl cysteine (NAC) and luciferase release was monitored after an overnight incubation. NT, not treated. All results displayed are from three independent replicates and statistical significance was determined using a two-tailed paired *T*-Test (***$p < 0.001$ and *$p < 0.05$). Error bars represent standard deviation.

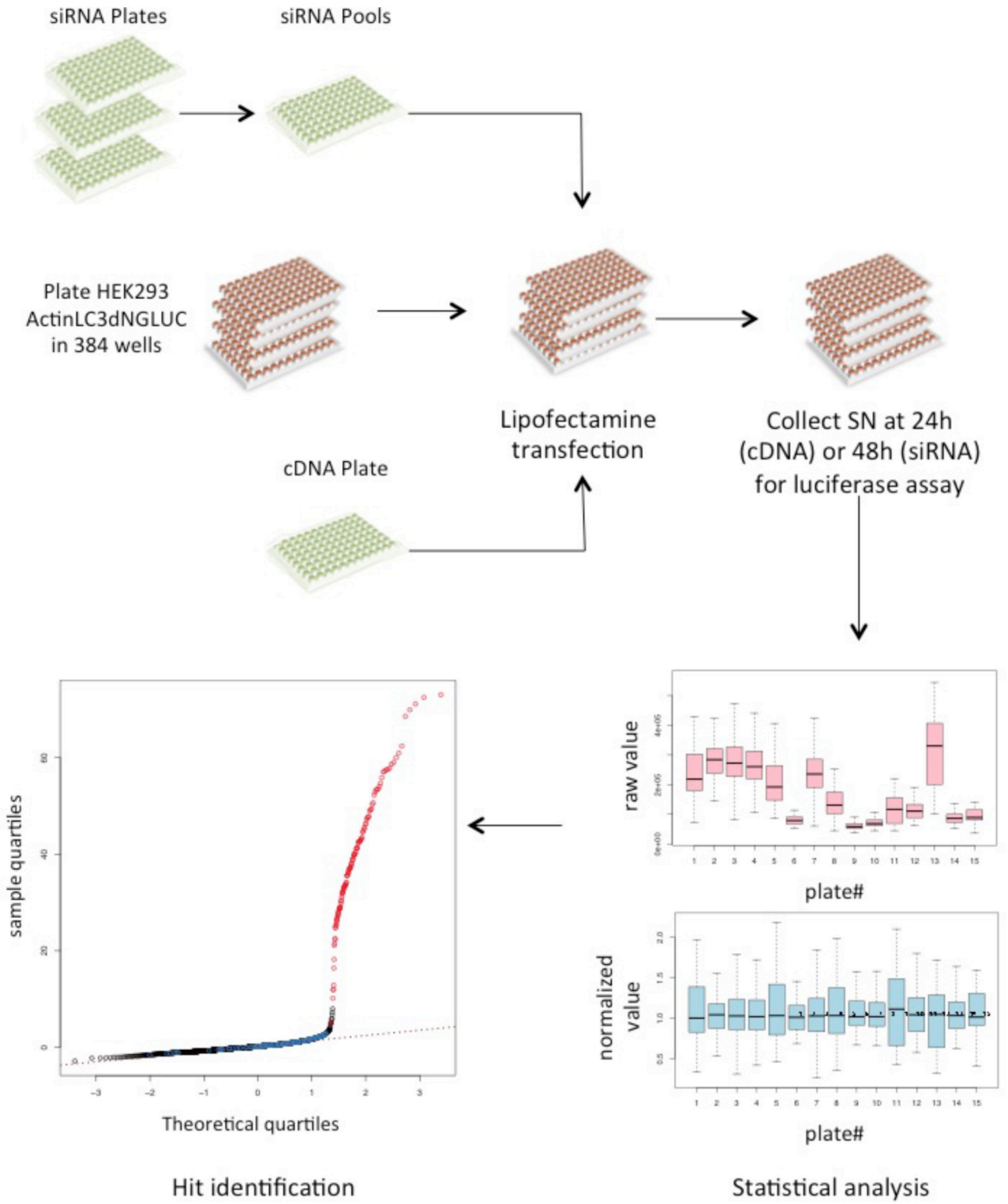


FIGURE 4 | A cDNA and siRNA screen to identify kinases and phosphatases that regulate ATG4B activity. Schematic of the screening workflow. For each gene, three different siRNA oligonucleotides were pooled together in each well and a transfection mix was dispensed into 384-well plates, where each quadrant corresponds to one 96-well layout. For cDNA transfection, the library was re-arrayed from 96-well plates into 384-well plates and transfected onto the reporter cell line. A total of 15 siRNA plates (11 kinase and 4 phosphatase plates) were screened in quadruplicates and the cDNA library was screened in triplicates. Supernatants (SN) were collected 48 h after transfection and measured on the PerkinElmer Envision II. All raw values were put into CellHTS2 for statistical analysis and hits were identified based on ranking of the normalized values.

luminescence values in the supernatants were normalized to the plate median and a B score analysis was applied to account for possible edge effects (**Supplementary Figure S2**).

A strong activator of cellular ATG4B activity identified in the screening was the compound STK683963 (**Figure 3A**). STK683963 strongly up-regulates the luciferase release reporter in a dose-dependent manner (**Figure 3B**) after 24 h. The analogous compound STK683964 showed slightly higher luciferase values in a similar concentration range in activating ATG4B, whereas another analog STK848088 did not. The effect of STK693963 on increasing ATG4B activity is most likely indirect since the increase in luciferase was only seen after overnight treatment and not at earlier time points. Also, STK693963 had no effect on *in vitro* ATG4B-mediated cleavage of a LC3B-GST reporter (data not shown). One possibility was that the compound might enhance ATG4B transcription. However, STK683963 does not activate ATG4B transcript expression since we could not observe an increase in the transcriptional activation of an ATG4B-promoter-luciferase construct (**Supplementary Figure S3**), while the positive control, Biochanin A, resulted in an increase in luciferase expression. STK683963 had no effect on viability of HeLa cells (**Supplementary Figure S4**). Next, we tested whether STK683963 can overcome LC3A deficiency that is associated with the R70H cancer mutation (Costa et al., 2016). When LC3A R70H was inserted in the ActinLC3dNGLUC reporter to monitor cleavage of this mutant, we observed that ATG4B-mediated processing was mildly reduced (**Figure 3C**). However, treatment with STK683963 activated LC3A R70H processing, suggesting that it can be used to enhance LC3 processing deficiencies in some conditions. In order to identify a possible mechanism of action for STK6983963, we investigated whether it might act on the redox mechanism of ATG4B. Thus, we treated cells with *N*-acetyl cysteine (NAC), a reducing agent that was previously shown to affect LC3 processing (Heintze et al., 2016). We found that STK683963 strongly activated luciferase release, which was completely blocked in the presence of NAC (**Figure 3D**). Thus, we propose that STK683963 acts as a mediator of redox-regulation of ATG4B in cells and is a strong activator of cellular ATG4B activity.

siRNA Screening to Identify Regulators of ATG4B Activity

Having established that the luciferase reporter is amenable to large-scale screening, we reasoned that this assay is well suited to identify regulators of ATG4B activity in cells. We therefore screened siRNA libraries targeting the human kinome and human phosphatome and a cDNA overexpression library of human kinases in HEK293T cells stably expressing the luciferase release construct (**Figure 4**). By reverse transfection, we seeded HEK293T-ActinLC3dNGLUC cells into 384-well plates on top of the siRNA transfection mix using an automated workflow. As a negative control, cells were left untransfected, and as a positive control, we transfected cDNA expressing ATG4B in the last column. The cells were incubated for 48h to achieve knockdown of the target genes, prior to harvesting the supernatants and analysis of luciferase activity in the PerkinElmer Envision II plate reader. Raw values were normalized to plate median and ranked by *Z* score. The robustness of the screen was assessed in four replicates, and overall standard deviations showed that the results were highly reproducible. The code used in R package for analysis is shown in **Supplementary File S3**. The complete set of results from the siRNA screen displaying activators and inhibitors is shown in **Supplementary File S4**. Several strong inhibitors of ATG4B activity were observed (knockdown of these genes resulted in an increase of luciferase activity). These include VRK1, TYK2, TRIB1, STK11, GUCY2B, and CAMK2D with a significant *Z* score above 4 (**Table 2**). Interestingly, CAMK2D was previously reported in another siRNA screen as inhibitor of LC3 puncta formation (Szyniarowski et al., 2011), in agreement with our results and suggesting it may control pro-LC3 processing. The strongest inhibition was seen upon knockdown of PAK1 (Z score = −2.85). Overall, a higher number of genes resulting in activation upon knockdown than inhibition were identified.

AKT2 Activates ATG4B-Mediated LC3 Processing

In parallel to the siRNA screen, we also performed a cDNA overexpression screen using the luciferase reporter assay (**Supplementary Figure S5**). For cDNA expression screening, we combined the four 96-well plates of the human kinome library into one 384-well plate and transfected this in triplicates in HEK293T-ActinLCdNGLUC cells. We identified a number of activators and inhibitors of ATG4B-mediated luciferase release (**Supplementary File S5**). We were particularly interested in AKT2 since AKT1 has previously been shown to be involved in autophagy and mitophagy (Ni et al., 2018; Soutar et al., 2018). First, we transfected AKT2 in the ActinLC3dNGLUC reporter cell line, concomitantly with ULK1, a kinase we recently identified as a negative regulator of ATG4B activity and measured luciferase release (Pengo et al., 2017). As expected, ULK1 significantly reduced ATG4B-mediated LC3 processing, whereas AKT2 overexpression activated the luciferase reporter (**Figure 5A**). In order to determine potential phosphorylation sites in ATG4B, we used the scansite algorithm to search for AKT2 target sites

TABLE 2 | Hits from siRNA screening.

Gene name	*Z* Score
VRK1	7.97
TYK2	7.22
TRIBl	6.66
STK11	5.89
GUCY2D	5.87
CAMK2D	5.18
SNRK	5.07
PPP1R9B	4.89
AKAP13	4.78
MAST3	4.03

The top 10 inhibitors, where knockdown increases luciferase release from cells are shown and their Z score after normalization calculated from the median of four replicates. For full details including statistics, see ***Supplementary File S4****.*

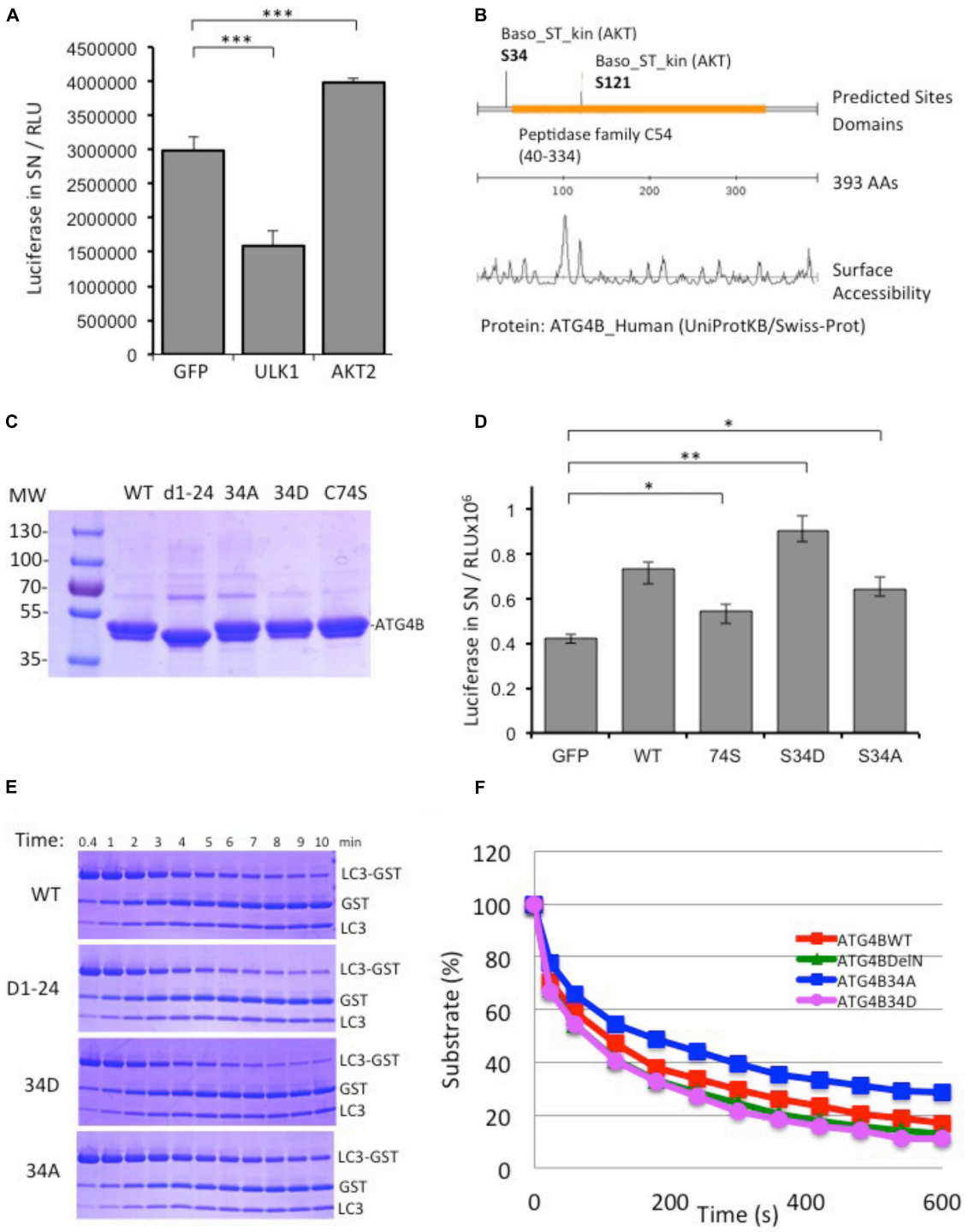


FIGURE 5 | AKT2 activates ATG4B. **(A)** HEK293T cells stably expressing ActinLC3dNGLUC were transfected with cDNA for ULK1 or AKT2 and luciferase activity was monitored as described. ULK1 strongly inhibited luciferase release, while AKT2 activated the reporter. Results displayed are from three independent replicates and statistical significance was determined using a two-sided paired *T*-Test (***$p < 0.001$). **(B)** Prediction of AKT2 phospho-target sites from Scansite (www.scansite.mit.edu). The ATG4B protein sequence (UniProt ID Q9Y4P1) was used as input and prediction was performed at low stringency. Serine 34 and Serine 121 were predicted as potential phosphorylation sites for AKT2. **(C)** Coomassie gel of the purified proteins. Equal amounts of the proteins were used as input in the following experiments. **(D)** HEK293T cells stably expressing ActinLC3dNGLUC were transfected with pEAK12-GFP as a control, or wild-type (wt) ATG4B and its mutants (C74S, S34D, and S34A). Luciferase activity in supernatants was monitored as described (**$p < 0.01$ and *$p < 0.05$). **(E)** GST-LC3 cleavage assay to determine time kinetics for 0.004 mg/ml of ATG4B and mutants (without GST-tag) to catalyze 50% of 1 mg/ml substrate LC3B-GST (see section "Materials and Methods"). **(F)** The catalytic kinetics were determined after incubation of the purified enzyme with LC3B-GST at 37°C from three different experiments and % of remaining substrate is shown.

(Obenauer et al., 2003). Scansite can generate predictions of protein residues that are phosphorylated by protein kinases based on data derived from experimental peptide arrays. Two sites in ATG4B were predicted as potential AKT2 target site, Serine 34 and Serine 121 (**Figure 5B**). One of these sites, Serine 34 was previously reported as a target of AKT1-mediated phosphorylation (Ni et al., 2018). Therefore, we generated mutants of ATG4B that either cannot be phosphorylated (S34A) or that mimic constitutive phosphorylation (S34D) (**Figure 5C**) and investigated the consequence of S34A and S34D mutation on cellular ATG4B activity. Indeed, ATG4B S34A showed reduced ATG4B activity in the luciferase release assay, although higher than the catalytic mutant C74S, whereas S34D showed higher activity than WT ATG4B (**Figure 5D**), in line with a potential role for AKT in positively regulating the activity of ATG4B.

Additionally, we saw that both an N-terminal deletion mutant (Δ1–24) and the S34D mutant exhibited increased LC3 substrate cleavage *in vitro* using the LC3-GST assay (**Figure 5E**). The calculation of the K_m values of mutants compared to ATG4B WT was 2.15, 1.74, 1.86, and 2.55 $\times$ 10^{-5} for ATG4B WT, Δ1–24, S34D and S34A, respectively (**Figure 5F** and **Table 3**). Overall, these results suggest that post-translational modification of Serine 34 in ATG4B might influence the LC3B processing kinetics.

Next, in order to assess whether ATG4B can be phosphorylated by AKT2, we performed an *in vitro* kinase assay (**Figure 6**). In addition to ATG4B S34A, we also generated two other mutants, ATG4B S121A and S262A (**Figure 6A**). We detected a phosphorylation signal in the presence of AKT2 but not in the presence of another control kinase (CLK2) indicating that AKT2 can phosphorylate ATG4B *in vitro* (**Figures 6B,C**). However, phosphorylation was also evident in the ATG4B S34A mutant, suggesting that there are other phosphorylation sites in ATG4B. Indeed, when assaying the S121A and S262A mutants, we observed a strong decrease in phosphorylated ATG4B S121A and S262A, indicating that these sites might be targets for AKT2, at least *in vitro* (**Figure 6C**). Overall, our results suggest that multiple sites in ATG4B can be phosphorylated by AKT2, all potentially contributing to the regulation of activity.

DISCUSSION

The autophagy machinery has the delicate task to co-ordinate the initiation and formation of an autophagosome under basal conditions and upon stresses that induce autophagy. How these events – from initiation to fusion with the lysosome – are controlled is only poorly understood. It has recently been suggested that the spatio-temporal control may be regulated by post-translational modification of specific autophagy proteins (Pengo et al., 2017; Sanchez-Wandelmer et al., 2017). Therefore, we set out to get a better understanding of the post-translational regulation of ATG4B, one of the key enzymes that co-ordinate the processing of LC3/GABARP family of proteins.

Multiple factors that regulate ATG4B activity are starting to emerge. A key regulator of ATG4 activity is redox-regulation, which was initially shown to modulate the de-lipidation reaction (Scherz-Shouval et al., 2007). It is now well established that oxidation of a cysteine residue in proximity to the catalytic site reduces LC3 processing. How this redox regulation is established in cells is less well understood, but it is thought that sub-cellular areas of increased ROS production may specifically affect local ATG4 activity. In line with this, thioredoxin and NADPH regulating enzymes such as Ribose-5-phosphate isomerase have been linked to ATG4-mediated LC3 processing (Perez-Perez et al., 2014, 2016; Heintze et al., 2016). It is thus not surprising that compounds that modulate redox signaling may affect cellular ATG4B activity. However, no small molecule compound activator of ATG4B has been identified to date. Here, we have shown that STK683963 and analogs are strong activators of cellular ATG4B activity. We only observed activation after overnight treatment and not at earlier time points (data not shown), suggesting that the compound may not act directly on ATG4B, but rather through indirect mechanisms. Furthermore, treatment with NAC – a reducing agent – blocked the activation of cellular ATG4B activity, hypothesizing that STK683963 primarily acts through a redox-regulated mechanism. The identification of this ATG4B enhancing compound provides a very useful tool that may have applications in conditions where reduced ATG4 activity or reduced LC3 processing has been observed (Costa et al., 2016).

In addition to redox mechanisms, phosphorylation of ATG4 family members is emerging as an important step in the regulation of cellular autophagy. The first evidence for this concept came from the observation that two residues, Ser383 and Ser392, were phosphorylated in cells, but the underlying kinase responsible has not been identified (Yang et al., 2015). In addition, multiple kinases are known to directly regulate and/or bind to ATG4B: ULK1/ATG1 mediated phosphorylation reduces ATG4B activity in mammalian cells (Pengo et al., 2017) and ATG4 activity in yeast (Sanchez-Wandelmer et al., 2017). AKT1 can bind to and phosphorylate Ser34 in ATG4B (Ni et al., 2018), but the effects on ATG4B activity have not been fully addressed. Recently, MST4 has been shown to phosphorylate Ser383 (Huang et al., 2017). Overall, these findings point to a complex regulation of ATG4B activity by kinases, and it is possible that such phospho-regulation may be dependent on the sub-cellular localisation of the kinase (Sanchez-Wandelmer et al., 2017).

High-throughput screens to identify regulators of autophagy have previously been performed. The screens published to date utilize siRNA libraries in phenotypic assays, studying the formation of the autophagosome either through immunostaining or a fluorescent protein reporter linked to LC3 (Chan et al., 2007; Lipinski et al., 2010a,b; Szyniarowski et al., 2011; McKnight

TABLE 3 | Enzyme kinetics of *in vitro* cleavage assay.

	K_m (M)	STDV	K_{cat}/K_m (1/M.S)	STDV
WT	2.14957E-05	3.21287E-07	73758.29226	1955.269708
Δ1-14	1.74178E-05	4.47812E-06	86959.80316	1099.428911
34A	1.86258E- 05	4.51334E-06	65214.59464	7583.879041
34D	2.54884E-05	6.56644E-06	83324.94999	5185.885677

The indicated ATG4B mutants were incubated with GST LC3B substrate and subjected to in vitro cleavage as described in the "Materials and Methods" section.

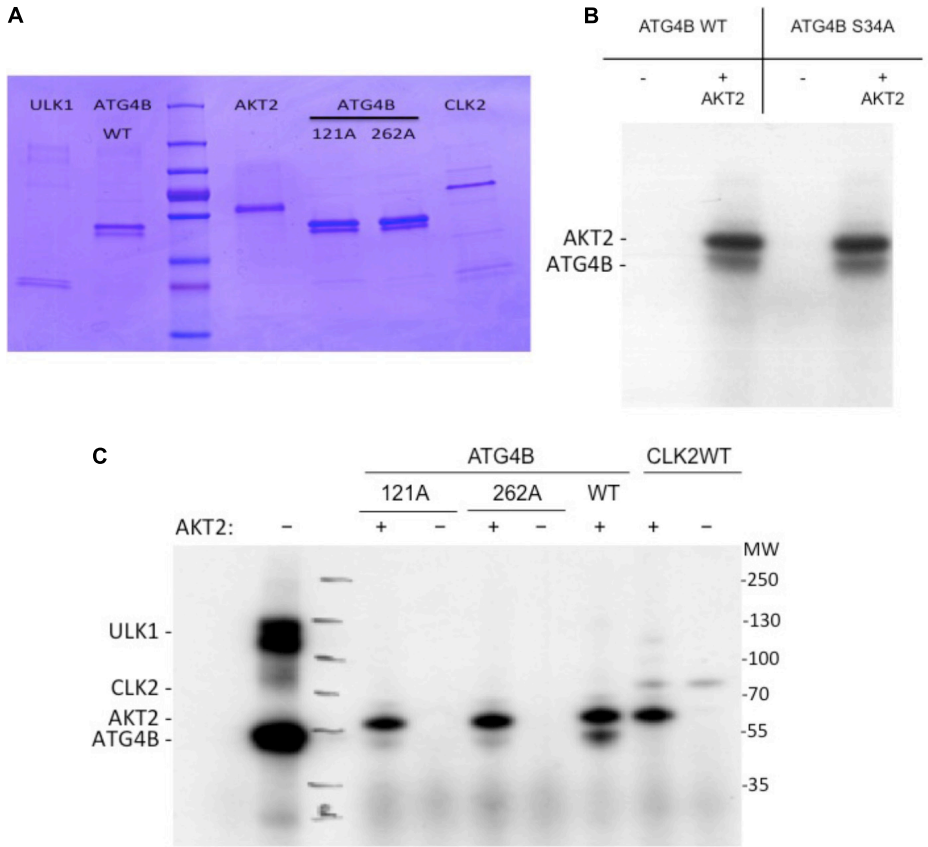


FIGURE 6 | *In vitro* kinase assay. **(A)** Coomassie gel showing the purified proteins used in the *in vitro* kinase activity assays. **(B)** Recombinant ATG4B or ATG4B S34A was incubated with recombinant AKT2 and ATP γ-^{32}P and incorporation of labeled γ-^{32}P was measured by auto-radiography. The upper band corresponds to AKT2 auto-phosphorylation and the lower band corresponds to ATG4B. **(C)** Recombinant ATG4B wild-type (WT), S121A or S262A were incubated with (+) or without (–) recombinant AKT2 and incorporation of labeled γ-^{32}P was measured by auto-radiography. On the left side, ULK1 mediated phosphorylation of ATG4B was included as control. On the right side, CLK2 (CLK2 catalytic domain with GST-tagged (Prak et al., 2016)) was included as another protein control to show that in the absence of ATG4B, AKT2 resulted in auto-phosphorylation.

et al., 2012). Here, we present the first siRNA- and cDNA-based screen that interrogates the function of ATG4B, using a luciferase-based readout. We have identified multiple kinases and phosphatases that regulate ATG4B activity. In particular, AKT2 is a novel gene that activates ATG4B, and promises to be an interesting candidate for future studies. The AKT family of proteins are known to regulate autophagosome formation and mitophagy (Soutar et al., 2018), and AKT1 has recently been shown to directly phosphorylate ATG4B at Ser34 (Ni et al., 2018). However, it has not been assessed whether this phosphorylation resulted in an increase or decrease of ATG4B activity. In agreement, we identified AKT2 as an activator of ATG4B in our cDNA expression screen. Of note, the two homologs AKT1 and AKT3 were not present in the cDNA library that we used. We noted that a S34A mutant displayed reduced ATG4B activity, while a S34D phospho-mimetic mutant showed an increase in ATG4B activity (**Figure 5**). We identified other potential AKT2-mediated phosphorylation sites within ATG4B at serine 121 and serine 262. Our assays do not rule out the phosphorylation of serine 34, since this may be masked by the two other sites in our assay. However, at this point we cannot attribute the AKT2-mediated activation of cellular ATG4B activity to a single phosphorylation site within ATG4B. Overall, our findings point to a complex level of regulation by the AKT family of protein kinases, which will require further investigation.

In summary, we provide here a dataset from small molecule, siRNA and cDNA screening that identified novel inhibitors and activators of cellular ATG4B activity and we share this data with the community for further investigations.

AUTHOR CONTRIBUTIONS

NP, KP, JC, CL, AA, and JF performed experiments and analyzed data. CG, AC, and DS provided material, expertise, and technical help. JK-V analyzed large datasets and provided bioinformatics expertise. NP and RK designed the study. RK wrote the manuscript and analyzed data. All authors read and contributed to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00148/full#supplementary-material

FIGURE S1 | Sequence of the ATG4B promoter in the pLightSwitch vector (Switchgear Genomics, CA).

FIGURE S2 | Small molecule screen for inhibitors and activators of cellular ATG4B acPvity. **(A)** HeLa cells stably expressing ATG4B (4B) displayed a strong activation of luciferase release into supernatants (SN) that was reduced in cells treated with Brefeldin A (BA). A Z' factor for ATG4B + BA compared to ATG4B + DMSO was calculated for samples in a 384-well plate format and determined as 0.46. **(B)** Distribution of hits in the small molecule screen. Activators of ATG4B are shown on the left of the graph, and inhibitors on the right. **(C)** Distribution of raw value counts for each plate (triplicates of each plate, left panel) and values normalized to plate median (right panel). Over time, it was noted that the reporter cells showed an increase in basal luciferase release. We therefore decided to generate a new stable cell line after plate 44 that was more robust. Results displayed are from three independent replicates and statistical significance was determined using a two-sided paired T-Test (***$p < 0.001$).

FIGURE S3 | STK683963 does not activate the ATG4B-luciferase promoter construct. HEK293T cells were transfected with pLightSwitch-ATG4B luciferase and renilla luciferase activity was measured after 24 h. Biochanin A (Bioch) and Genistein (Genist) significantly up-regulater promoter-dependent luciferase, whereas STK683963 has no effect on ATG4B promoter activity. RLU, relative light unit. Results displayed are from three independent replicates and statistical significance was determined using a two-tailed paired T-Test (**$p < 0.01$, *$p < 0.05$).

FIGURE S4 | HeLa-ActinLC3dNGLUC cells were treated with the indicated concentrations of STK683964 overnight and cell viability was determined using the cell counting kit 8 (CCK8). No obvious effect on viability was observed at the indicated concentrations.

FIGURE S5 | cDNA expression screen. **(A)** Heatmap of normalized luciferase values in the 384-well plate. The four 96-well plates of the cDNA human kinome library was pooled into one 384-well plate and 100 ng/well was transfected in HEK293T-ActinLC3dNGLUC cells. Positive (ATG4B transfection) and negative (untransfected) controls were included in the last two columns of the plate. **(B)** Distribution of samples relative to positive (red color) and negative controls (blue color). **(C,D)** The raw values were normalized to plate median and a B score calculation was applied. The distribution of positive and negative controls is shown in the left and right panel. The Z' factor for this replicate was 0.41.

FILE S1 | Information about the human kinase and human phosphatase siRNA libraries, including gene names and sequences for all siRNA oligonucleotides.

FILE S2 | Information about the human kinase cDNA library.

FILE S3 | R code used for statistical analysis of siRNA screening data.

FILE S4 | Raw and normalized data from siRNA screening.

FILE S5 | Raw and normalized data from cDNA overexpression screening.

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REVIEW
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Selective Autophagy and Xenophagy in Infection and Disease

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Autophagy, a cellular homeostatic process, which ensures cellular survival under various stress conditions, has catapulted to the forefront of innate defense mechanisms during intracellular infections. The ability of autophagy to tag and target intracellular pathogens toward lysosomal degradation is central to this key defense function. However, studies involving the role and regulation of autophagy during intracellular infections largely tend to ignore the housekeeping function of autophagy. A growing number of evidences now suggest that the housekeeping function of autophagy, rather than the direct pathogen degradation function, may play a decisive role to determine the outcome of infection and immunological balance. We discuss herein the studies that establish the homeostatic and anti-inflammatory function of autophagy, as well as role of bacterial effectors in modulating and coopting these functions. Given that the core autophagy machinery remains largely the same across diverse cargos, how selectivity plays out during intracellular infection remains intriguing. We explore here, the contrasting role of autophagy adaptors being both selective as well as pleotropic in functions and discuss whether E3 ligases could bring in the specificity to cargo selectivity.

Keywords: xenophagy, ubiquitination, p62, NDP52, OPTN, TAX1BP1, inflammation, DUBs

INTRODUCTION

Autophagy, a basal cargo degradation process, is responsible for elimination of superfluous and unwanted cytoplasmic materials including misfolded proteins and aggregates, damaged organelles and other macromolecules including lipids and carbohydrates in the cells. While basal autophagy is important for maintaining homeostasis by providing energy substrates to the cell, this process also gets induced by various environmental cues, including stress (osmotic, nutritional, serum starvation) and pathogen stimulation. The complexity of this seemingly simple process of cargo degradation began to be unraveled following the discovery of *ATG* family of genes in yeast cytoplasm to vacuole targeting (cvt) pathway (Nakatogawa et al., 2009). Since then, mammalian orthologs of yeast *Atg* genes have been found which perform similar functions but in a more sophisticated manner. The step-wise process of cargo tagging, autophagosome formation and targeting them to the lysosomes for degradation has been fairly well studied (Levine et al., 2011; Deretic, 2012; Feng et al., 2014; Mizushima, 2018; Yu et al., 2018).

In selective autophagy, specific cargos are first tagged by ubiquitination, following which they get recognized by the autophagy adaptor molecules for subsequent targeting to autophagosomes for degradation. The tagging and targeting of cargos imparts selectivity to the degradation process that is referred to as selective autophagy, which is different from bulk degradation of the packaged cargo that occurs in a non-selective manner. The selective cargos could be misfolded proteins, damaged organelles like mitochondria and peroxisomes or intracellular pathogens like *Mycobacterium* spp., *Salmonella* spp., *Listeria* spp. amongst others (Gatica et al., 2018). Based on the cargo being delivered for degradation, selective autophagy has been classified into mitophagy (degradation of damaged mitochondria), pexophagy (peroxisomes), lipophagy (lipid droplets), glycophagy (glycogen), ribophagy (ribosomes), ER-phagy (ER) and xenophagy (intracellular pathogens) (Gatica et al., 2018). Considering the diversity of potential targets autophagy could degrade, it is plausible that they are implicated in regulating diverse physiological processes including cellular homeostasis, inflammation as well as fate of intracellular infection. Nutrient recycling is one of the earliest discovered functions of autophagy, which helps maintain cellular homeostasis by extracting energy from catabolic substrates during energy requirement in diverse stress conditions including bacterial infections. This key homeostatic function of autophagy can be exploited by bacterial pathogens to source nutrients for their own survival, adding another dimension on how autophagy could impact bacterial survival during infections (Chaston and Goodrich-Blair, 2010).

Interestingly targeting of both intracellular cargos as well as intracellular pathogens rely on the core autophagic tagging, recognition and degradation machinery. Ubiquitination of cargos is among the first steps in targeting them toward autophagic degradation, which may be akin to an intracellular "eat-me" signal (Boyle and Randow, 2013; Shaid et al., 2013). Ubiquitinated cargos are subsequently recognized by autophagy adaptors (also called autophagy receptor proteins), which then tag them to phagophores, the nascent autophagic membranes, subsequently maturing into autophagosomes (Shaid et al., 2013; Stolz et al., 2014). It is therefore widely appreciated that shared components of autophagic machinery get involved irrespective of whether it is for the homeostatic purposes or for the cellular defense mechanisms. While each of the different possible autophagic cargos and the corresponding selective machinery involved have been extensively studied (Gatica et al., 2018), majority of these studies mostly rely on a particular kind of cargo in isolation. Under physiological conditions, especially during intracellular infections, however, different arms of autophagic machinery must work in conjunction considering the intertwining of homeostatic and defense functions of autophagy (Chandra and Kumar, 2016). How cargo tagging, recognition and degradation works in specific manner when multiple potential targets for autophagic degradation are present inside the cell, remains obscure. Specifically, during intracellular infections, where xenophagy occurs alongside selective autophagy for intracellular cargos, how ubiquitin ligases and autophagy adaptors discriminate among such cargos within same cell, remains unexplored. In this review, we try to bring together the selectivity and redundancy in the roles of different regulators in terms of cargo tagging, recognition and autophagy-mediated degradation during cellular homeostatic and defense functions.

AUTOPHAGY AS A HOMEOSTATIC AND ANTI-INFLAMMATORY CELLULAR PROCESS

Inflammation is a stress-mediated cellular response, elicited by infections or tissue damages and triggered by either cell-intrinsic or extrinsic factors (Chovatiya and Medzhitov, 2014). The surveillance machinery for initiating inflammatory responses involves pattern recognition receptors (PRRs), which recognizes pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) for pathogenic and cell-intrinsic factors respectively. Generally, signaling through PRRs eventually lead to formation of a large multi-molecular complex called inflammasome (Saitoh et al., 2009). The indication that autophagy could be involved in regulation of inflammation emerged first through a GWAS study on patients with Crohn's disease (CD), an inflammatory disorder of the gut, where SNP in an autophagy-related gene *ATG16L1* show strong association with the disease susceptibility (Hampe et al., 2007). The physiological consequence of ATG16L1 function in regulating inflammation is evident in *ATG16L1* knockout animals, which show dramatically enhanced production of pro-inflammatory cytokines like IL-1β and IL-18 (Saitoh et al., 2008). Likewise, several studies report that increased inflammation during aging actually reflects loss of the autophagic capabilities resulting in accumulation of damaged, depolarized mitochondria (Green et al., 2011). These studies together highlight the homeostatic functions of autophagy. Other than aging, mitochondrial depolarization also acts as the trigger for activation of NLRP3 inflammasome. Accumulation of damaged mitochondria, and the resulting increase in cellular redox-stress upon inhibition of autophagy and mitophagy activates NLRP3 signaling leading to inflammasome activation (Zhou et al., 2011). Intriguingly, mitochondria are not the only cell organelle, which can directly impact inflammation. There are close associations reported between endoplasmic reticulum, peroxisomes, protein aggregates and inflammation (Schrader and Fahimi, 2006; Zhang and Kaufman, 2008; Gebbink et al., 2009; Garg et al., 2012). Interestingly, autophagy can selectively target each one of these organelles/cargos for degradation through reticulophagy or ER-phagy, pexophagy and aggrephagy, respectively. Thus autophagy, by virtue of its degradative capabilities, serves as a key anti-inflammatory pathway by selectively degrading components, which could potentially trigger inflammation.

Although, inflammation is the prime innate immune host response against any pathogen attack (Mogensen, 2009), prolonged inflammation may cause severe tissue damage (Saitoh et al., 2009; Qian et al., 2017; Takahama et al., 2018). Therefore, its regulation is important to check the prolonged effects of inflammation, including severe tissue damage and

cell death. There is strong co-relation between autophagy and inflammation where on one hand autophagy supports the survival of inflammatory cells including macrophages, lymphocytes and neutrophils (Qian et al., 2017) and at the same time, it also controls the secretion of pro-inflammatory cytokines from innate immune effector cells (Qian et al., 2017). A positive role of autophagy has been described in suppressing mitochondrial reactive oxygen species (mtROS) and production of IL-1β, IL-18 in a p62, also called sequestosome 1 (SQSTM-1) dependent manner in response to rapamycin-induced autophagy in macrophages (Ko et al., 2017). Similar mechanism is also in display during pro-inflammatory stimulation of macrophages, where classically activated macrophages (M1 or the inflammatory sub-type) shows decline in autophagy, which allows these cells to acquire the inflammatory potential (Matta and Kumar, 2015). This process, schematically shown in **Figure 1**, to a large extent, is dependent on AKT mediated shift toward aerobic glycolysis (Matta and Kumar, 2015). This is also true during Toll Like Receptor (TLR) stimulation of Dendritic Cells (DCs) or macrophages, where inhibition of autophagy, due to loss of ATG16L1 or ATG7, causes massive pro-inflammatory cytokine signaling including IL-1β and IL-18 (Saitoh et al., 2008). Similarly, in the diabetic model of macrophages, autophagy inhibition exhibits generation of reactive oxygen species (ROS) and pro-inflammatory cytokines including IL-1β (Dai et al., 2017). An interesting mechanism of the anti-inflammatory effector function of autophagy revealed recently show that loss of autophagy protein ATG16Ll in macrophages results in the accumulation of adapter protein TRIF, which otherwise gets targeted for degradation through selective autophagy adaptors p62 and Tax-1 binding protein 1 (TAX1BP1). The study further shows knocking down of TAX1BP1 also help enhance pro-inflammatory cytokine signaling, further establishing the role of selective autophagy in limiting inflammation (Samie et al., 2018).

Priming of macrophages with IFNγ helps controlling *Mycobacterium tuberculosis* via increased maturation of phagosomes in IRGM mediated manner (Singh et al., 2006). IRGM is a strong mediator of inflammation and regulates secretion of IL-1β, IL-4, IL-6, IFN-γ, amongst others in a TLR-mediated pathway (Yang et al., 2014). It also mediates generation of mtROS and may recruit autophagic machinery after PAMP recognition in infected macrophages, where *M. tuberculosis* is targeted to autophagosomes in a selective manner (Chauhan et al., 2015). At the same time, classical activation of macrophages in normal or hypoxic condition trigger bacterial killing by inhibiting autophagy, which results in mitochondrial depolarization and ROS generation in AKT dependent manner (Matta and Kumar, 2015, 2016). In the context of *Pseudomonas aeruginosa* activation of NLRC4 inflammasome by mitochondrial damage is checked by mitophagy *in vitro* as well as *in vivo* (Jabir et al., 2015; Harris et al., 2017). Capturing of inflammasome subunits by autophagy is one of the important feature besides mitophagy to suppress inflammation (Harris et al., 2017). In case of gram negative bacterial infection including *Salmonella,* non-canonical inflammasome recognizing LPS promotes inflammation and activation of caspase-11 (Kayagaki et al., 2013). In contrast, selective autophagy is induced when autophagy adaptor Nuclear dot protein 52 kDa (NDP52) recognizes ubiquitin chains on the bacteria, aiding in limiting caspase-11 (Takahama et al., 2018). Similar role of autophagy-mediated control of mitochondrial depolarization, mtROS production and inflammation is on display during Vesicular stomatitis virus (VSV) infection (Tal et al., 2009). Thus, selective autophagy effectively serves as a master regulator in limiting inflammation. Moreover, it is also evident that control of inflammation by autophagy represents the homeostatic arm of selective autophagy, since it is the loss of homeostasis that eventually results in inflammation.

Yet another dimension of cellular homeostasis where autophagy plays a critical role involves extracting energy from catabolic substrates during energy requirement in several stress conditions including bacterial infections. Autophagy-mediated degradation of macromolecules help rebuild new structures and increase basic nutrient pool in the cell. To counteract pathogenic infections, hosts can develop stringent environment to limit the nutrient availability to starve the pathogens (Zhang and Rubin, 2013). For example availability of Fe^{2+} to the pathogens can be restricted in the hosts by molecules like NRAMP and transferrin (Porcheron et al., 2013). Similarly restricting availability of amino acids helps host control bacterial infections (Zhang and Rubin, 2013). While amino acid restrictions imposed by hosts is an important contributor to immunity against pathogens, pathogens could also exploit autophagy to source nutrients. For example in *Leishmania mexicana* autophagy is involved in transferring macromolecules to parasitophorous vacuoles (Schaible et al., 1999). Similarly, Large cell variant form of *Coxiella burnetii* acquire nutrients when autophagosomes fuse with the replicative vacuoles of the bacterium. Similarly, *Chlamydia trachomatis* escapes amino acid limitation in the host by converting itself into an aberrant body form (having less nutritional requirement) from replicating reticulate bodied form (Zhang and Rubin, 2013). *Legionella* with the help of its virulence factor ank B obtain free amino acid which are transferred with the help of host SLC1A5 transporter to the *Legionella* containing vacuole (LCV) containing vacuole. *M. tuberculosis* on the other hand, when challenged by host through tryptophan depletion via IDO expression, is capable of synthesizing tryptophan on its own while residing in the phagocytic vacuole (Zhang and Rubin, 2013). *Francisella tularensis* utilizes non- essential amino acids with ATG-5 independent autophagy (Steele et al., 2013). Interestingly, deprivation of essential nutrients by hosts have helped pathogens to evolve into auxotrophs for up to 10 amino acids thereby remarkably limiting the ability of nutritional immunity of the host (Steele et al., 2015).

Taken together, it is evident that the homeostatic functions of autophagy turns out to be more helpful for bacteria, whether via controlling inflammation or by providing nutrients for the bacteria. This is in contrast from the autophagy-mediated degradation of bacterial pathogens where autophagy works against the pathogen survival. To understand the distinction between the homeostatic arm and anti-bacterial (defense) arm of autophagy, it is important to explore the

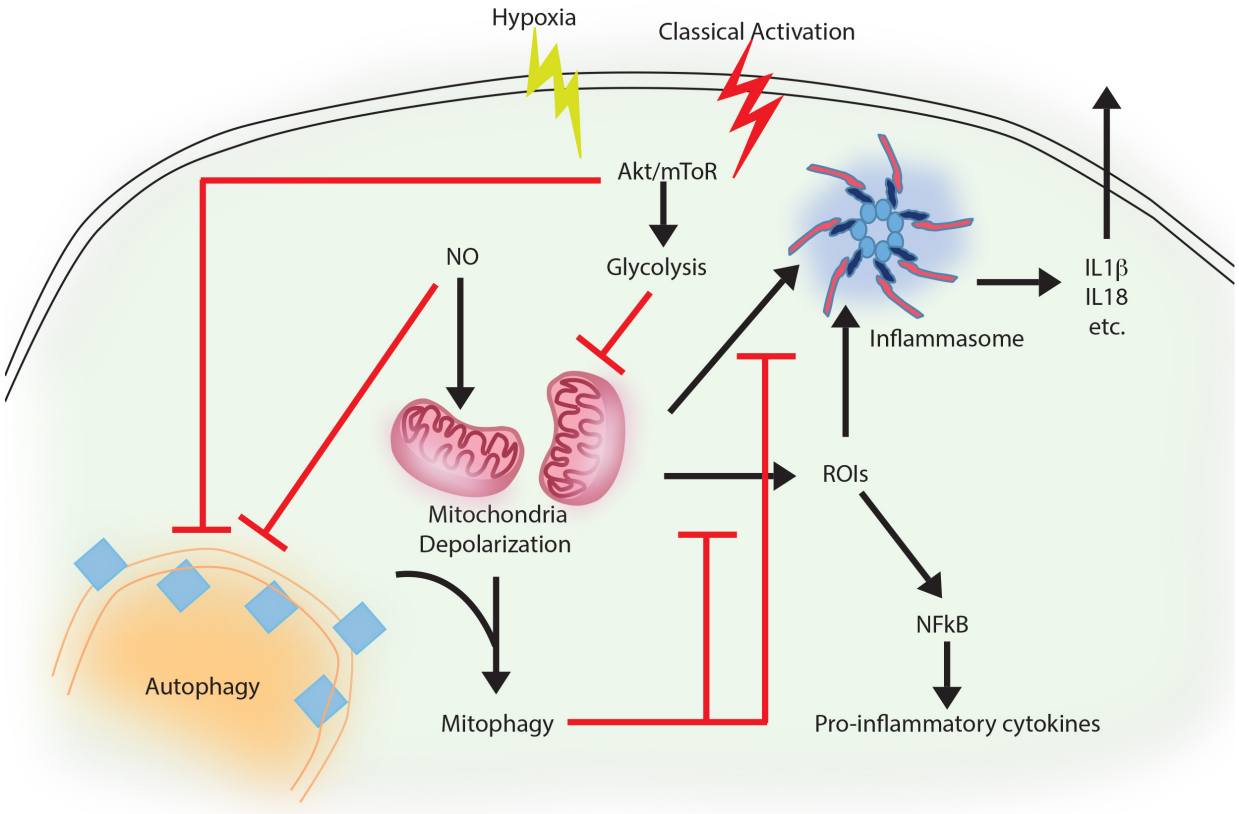


FIGURE 1 | Autophagy as an anti-inflammatory process. Activation of macrophages upon IFNγ and LPS treatment results in classically activated macrophages, which are more phagocytic, microbicidal and inflammatory in nature. During classical activation, autophagy is inhibited in a NO-dependent manner. This coincides with mitochondrial depolarization and accumulation of such depolarized mitochondria due to lack of mitophagy (since the core machinery for autophagy and mitophagy are same). Depolarized mitochondria are source of heightened reactive oxygen intermediates (ROIs) in the activated macrophages and also activates NLRP3 mediated inflammasome pathway and secretion of cytokines like IL1β and IL18. Inhibition of autophagy occurs in mToR/Akt dependent manner and inhibition of Akt signaling can alleviate the autophagy levels and control mitochondrial depolarization by directing damaged mitochondria toward mitophagy. Together, inhibition of autophagy in these cells helps achieve the pro-inflammatory phenotype, establishing the anti-inflammatory function of autophagy. Similar events also occur when macrophages are exposed to hypoxia, with the exception of NO production, which does not occur under hypoxia.

mechanisms of selective xenophagy regulation as discussed below.

BACTERIAL EFFECTORS IN SELECTIVE AUTOPHAGY (XENOPHAGY) DURING INFECTIONS

Several bacterial effectors are known to regulate selective autophagy through myriad mechanisms. Rapid detection of PAMPs during pathogenic infections is crucial for mounting a strong inflammatory response and control of infection. Therefore, how selective autophagy exhibiting clear anti-inflammatory functions help during bacterial infection? In addition to its homeostatic and anti-inflammatory functions, autophagy can also directly tag bacteria for lysosomal degradation (Gutierrez et al., 2004). This indeed was the earliest understanding that led to the characterization of autophagy as a defense mechanism (Deretic, 2006). Several bacterial effectors are known to impact autophagy regulation in the infected host cells, briefly summarized in **Table 1**. In case of *Salmonella*, it is known that effectors from Type III secretion system (T3SS) help its escape from *Salmonella*-containing vacuole (SCV) and exposes them to cytosolic PRRs, which results in ubiquitination and recruitment of selective autophagy adaptors like Optineurin (OPTN) and p62 (Herhaus and Dikic, 2017). Recent studies have revealed that *M. tuberculosis* releases its DNA into the cytosol that can be recognized by the Stimulator of IFN genes (STING)-dependent cytosolic sensing pathway, which further aids in marking the bacteria with ubiquitin, and delivering it to the autophagic machinery through the selective autophagic receptors p62 and NDP52 (Watson et al., 2015). This particular process is dependent on the non-conventional secretory system, ESAT-6 secretion system (ESX1) of *M. tuberculosis*, which is also known for its role in mycobacterial virulence. Interestingly, the ESX1 machinery and its effectors like ESAT-6 are known to block the maturation stage of xenophagy selectively (Chandra et al., 2015). In case of *Shigella flexneri*, the virulence factor VirG (also known as IcsA), that is also a ligand for the autophagy protein Atg5, is involved in inducing autophagy (Ogawa et al., 2005),

TABLE 1 | Bacterial effectors in autophagy and inflammation.

	Bacterial effectors in autophagy		
Bacteria	**Ubiquitination/Deubiquitination**	**Autophagosome targeting/Maturation**	**Bacterial effectors in inflammation**
Shigella flexneri		IcsB Binds to VirG, ligand for ATG5, inhibits phagophore recruitment (Ogawa et al., 2005) VirA Inactivates Rab1 and inhibits the autophagosome formation (Dong et al., 2012)	IpaH9.8 Targets NEMO for degradation (Ashida et al., 2010 IpaH 7.8 Activation of NLRC4 inflammasome (Suzuki et al., 2014) OspG Prevents IkBα degradation, (Kim et al., 2005)
Salmonella spp.	SseL Removes ubiquitin aggregates on SCV (Mesquita et al., 2012)	SseF and SseG Impairs autophagy initiation via disrupting Rab1 signaling (Feng et al., 2018)	AvrA and SseL Prevents degradation of IkBα, (Rytkonen and Holden, 2007; Ye et al., 2007) SpvD Interferes with the nuclear translocation of p65 and thus NF- αB signaling, (Rolhion et al., 2016)
Listeria spp.	Inlk Mask the bacterial surface by recruiting MVP, prevents ubiquitination (Dortet et al., 2011) ActA Recruits Arp2/3 complex and Ena/Vasp, prevents ubiquitination (Yoshikawa et al., 2009)	PlcA and Plc B Inhibits pre autophagosomal maturation (Mitchell et al., 2015)	InlC Interacts with IKKα and decreases phosphorylation of IkB, (Gouin et al., 2010)
Mycobacterium tuberculosis		Eis Inhibits autophagy by mediating Akt/MTOR pathway via activation of IL-10 (Duan et al., 2016) PtpA Targets VPS33B and V-ATPases to prevent maturation of phagosome (Bach et al., 2008; Wong et al., 2011) ESAT-6 Prevents Rab5 to Rab7 conversion on autophagosomes, inhibiting maturation (Chandra and Kumar, 2016)	PtpA Partially inhibits NF-B by targeting TAB3 (Wang et al., 2015) ESAT-6 Activates NLRP3/ASC inflammasome Mishra Bibhuti et al., 2010)
Legionella pneumophila	SdeA DUB domain contact the ubiquitin chains of bacteria (Sheedlo et al., 2015)	RavZ Prevents the lipidation of LC3 (Choy et al., 2012) *Lp*Sp1, Reduces levels of sphingosine in the cell and further delays autophagic response (Rolando et al., 2016)	

whereas during *Listeria monocytogenes* infection, its toxin listeriolysin (LLO) is responsible for targeting the bacteria to the autophagosomes (Meyer-Morse et al., 2010). Why should intracellular bacteria have virulence mechanisms involving induction of autophagy, a potentially disastrous outcome for the pathogens? It turns out, although intracellular pathogens get targeted via xenophagy for their degradation, they have evolved several mechanisms to inhibit or modulate autophagy at multiple steps in order to survive better in the cell. The manipulation by the bacteria can be done by limiting ubiquitination, by inhibiting the formation of Microtubule-associated protein 1A/1B-light chain 3 (LC3) and Phosphatidylethanolamine (PE) conjugate on the autophagosome membrane or at the stage of autophagosome maturation. For example, *S. flexneri* is able to escape xenophagy by secreting its effector IcsB, which binds competitively to its surface protein VirG, thereby, inhibiting the bacterial recruitment to the phagophore (Ogawa et al., 2005). Additionally, *S. flexneri* effector protein VirA inhibits the activity of Rab1, which is required for early phagosome formation from the ER (Dong et al., 2012). In contrast, *L. monocytogenes*

escapes autophagic recognition by ActA protein, which recruits the Arp2/3 complex and Ena/VASP to the bacterial surface for preventing its ubiquitination and autophagic recognition (Yoshikawa et al., 2009). Another virulence factor Inlk of *L. monocytogenes* helps to mask its cell surface by binding to host cytoplasmic Major vault protein (MVP) to block ubiquitination and avoid xenophagy (Dortet et al., 2011). Phospholipases, PlcA and PlcB from *L. monocytogenes* also inhibit autophagy by blocking the lipidation of LC3 (Mitchell et al., 2015). In case of *Salmonella typhimurium*, more than 30 effector proteins are secreted in to the host cytosol via its T3SS2, leading to the recruitment of Focal adhesion kinases (FAK) to the surface of SCV followed by activation of AKT-mTOR and suppression of autophagy (Casanova, 2017). Additionally, the role of effector protein SseL is quite specific in that it deubiquitinates ubiquitin aggregates on the surface of SCV, thereby decreasing its targeting to the autophagosomes (Mesquita et al., 2012). Another effectors from *Salmonella*, SseF and SseG inhibits autophagosome formation by disrupting Rab1-A signaling (Feng et al., 2018). Although, many bacterial proteins are known to disrupt the autophagy pathway indirectly, RavZ, which is a T4SS effector of *Legionella pneumophila* is the only identified bacterial factor that directly inhibits components of the autophagy pathway. RavZ irreversibly cleaves the amide bond linking LC3 to PE, consequently blocking the ability of phagophores to recognize ubiquitylated cargo (Choy et al., 2012). Interestingly, RavZ is not present in all the strains of *L. pneumophila*, therefore they employ *Lp*Sp1 (Sphingosine-1 phosphate lyase), secreted by the Dot/Icm type IV secretion system, which down regulates host shingolipid levels and causes delay in the autophagic response (Rolando et al., 2016). In addition, the autophagy-related SNARE, syntaxin 17, which is recruited to autophagosomes via IRGM, is degraded by the *L. pneumophila* effector Lpg1137 to suppress autophagy and apoptosis (Arasaki et al., 2017; Kumar et al., 2018). Some bacteria are not targeted to the autophagosomes since they are capable of degrading the adaptor proteins. For instance, in the case of Group A *Streptococcus* (GAS), the effector protein SpeB, which is a cysteine protease, degrades the autophagy adaptors p62, NBR1 and NDP52, thereby escaping xenophagy altogether (Barnett et al., 2013). Manipulation of xenophagy by inhibiting the maturation of autophagosomes is well studied in *M. tuberculosis*. The effectors from *M. tuberculosis* ESX-1, a type VII secretion system, inhibit Rab5 to Rab7 conversion on autophagosomes, thus preventing its maturation (Chandra et al., 2015). Another *M. tuberculosis* effector protein, PtpA, a tyrosine phosphatase, inhibits the phagosome-lysosome fusion by phosphorylating vacuolar protein sorting 33B (VPS33B), which is the regulator of membrane fusion (Bach et al., 2008). In addition to this, PtpA also alters the V-ATPase machinery on the phagosome preventing its maturation (Wong et al., 2011). Enhanced intracellular survival (EIS) protein of *M. tuberculosis* also inhibits autophagy by mediating AKT/mTOR pathway via activation of IL-10 (Duan et al., 2016). Some bacteria, instead of inhibiting autophagy, induces it for their own benefit. In this case, augmentation of overall autophagy, rather than promoting bacterial clearance via xenophagy, facilitates the acquisition of nutrients by the invading bacteria. Bacteria like *Anaplasma phagocytophilum, Yersinia pseudotuberculosis, C. burnetii* and *F. tularensis* may sabotage the host defense mechanism elicited by induction of autophagy and the resulting accumulation of autophagosomes via utilizing the autophagic vesicles as nutrient source for microbial growth (Winchell et al., 2016). It is also possible that increased autophagy, through its anti-inflammatory effects, inadvertently helps bacterial survival.

BACTERIAL EFFECTORS IMPACTING INFLAMMATION

During bacterial infection, PAMPs are recognized by the host PRRs like TLRs and Nod-like receptors (NLRs). PAMPs recognition by these receptors activates a proinflammatory response via two major signaling pathways, that are mediated by MAPKs and NF-κB. Inhibition of these pathways is a crucial strategy for bacterial survival in the cell. Many bacterial proteins, such as type III secretion system effectors, toxins, and extracellular adherence proteins, are known to possess anti-inflammatory abilities that helps the bacteria to bypass the host's response and prolong their survival in the hosts (**Table 1**). The virulence factors from *L. monocytogenes,* LLO and InlB can activate the NF-κB pathway, whereas its effector protein internalin C (InlC) downregulates the same by directly interacting with IKKα, thereby decreasing the phosphorylation of the inhibitory component of NF-κB, IκB (Gouin et al., 2010). The IKK complex has emerged as the main target of many bacterial effectors in controlling inflammation. The E3 ligase like effector IpaH9.8 of *S. flexneri* also targets IKK. IpaH9.8 mimics host E3 ubiquitin ligases and ubiquitinates NEMO, an upstream component of IKK complex, so as to target it for proteasomal degradation and preventing NF-κB activation (Ashida et al., 2010). Additionally, *S. flexneri* secretes two more E3 ligases, IpaH1.4 and IpaH2.5, which indirectly targets IKK by carrying out the proteasomal degradation of Linear ubiquitin chain assembly complex (LUBAC) (de Jong et al., 2016). LUBAC is a multimeric E3 ubiquitin ligase which is responsible for activating IKK and further NF-κB. The *Salmonella* proteins, SseL and AvrA prevents the degradation of IkBα and thereby impairs NF-kB activation (Ye et al., 2007; Le Negrate et al., 2008). Similarly, OspG, the effector protein of *S. flexneri* prevents IkBα degradation (Kim et al., 2005). *M. tuberculosis* protein PtpA partially inhibits activation of NF-κB pathway by targeting TAB3 (Wang et al., 2015). Sop A, type III secretion system effector of *S. typhimurium* is a HECT type E3 ligase and is reported to target host TRIM 56 and TRIM 65. This leads to the modulation of innate immune receptors RIG-1 and MDA-5, which causes pro-inflammatory cytokine production (Kamanova et al., 2016; Fiskin et al., 2017). Recent studies have shown that *S. typhimurium* effector protein SpvD, a cysteine protease binds the nuclear exportin, Xpo2, resulting in the disruption of normal recycling of importin-α from the nucleus, leading to the defect in nuclear translocation of p65, consequently resulting in the inhibition of

NF-κB induced immune responses (Rolhion et al., 2016). During *Yersinia* infection, an acetyltransferase, YopP/J gets translocated into the host cells, thereby acetylating IKK complex as well as MAPK Kinases (MKKs), which prevent their phosphorylation and subsequent inflammatory signaling (Pha and Navarro, 2016). Besides, modulating NF-κB and MAPK signaling, pathogens can also directly restrict or modulate activation of inflammasome. *S. flexneri* utilizes E3 ligase IpaH7.8 to ubiquitylate GLMN protein, which is involved in inhibiting the activation of NLRP3 and NLRC4 inflammasome (Suzuki et al., 2014). *M. tuberculosis* ESAT-6 also potentially activates NLRP3/ASC inflammasome (Mishra Bibhuti et al., 2010). Interestingly, a T6SS effector EvpP from *Edwardsiella tarda* inhibits NLRP3 inflammasome, however, a T3SS effector from the same pathogen activates NLRC4 and NLRP3 inflammasomes (Chen et al., 2017). It is important to note that inflammation and resulting cell death in itself can impact bacterial pathogens survival within the host. Considering autophagy as an anti-inflammatory process, it is possible that bacteria may strive to inhibit inflammation through activation of autophagy. Immune cells like macrophages acquire inflammatory and microbicidal properties by inhibiting autophagy under the influence of pro-inflammatory environment (Matta and Kumar, 2015, 2016). However, knowing that autophagy can also directly target bacterial pathogens for lysosomal degradation, how a possible selectivity may playout during infections that allows bacteria at one hand to escape autophagic targeting while homeostatic arm of autophagy continues unabated is an interesting question. A clue to such selectivity arises from our understanding of the autophagy adaptors, which are critical for selective autophagic targeting of various cargos. Curiously, there are only a handful of autophagy adaptors known so far and their recruitment/role in homeostatic autophagy or xenophagy are very much overlapping, leaving the question open that how selectivity is ensured.

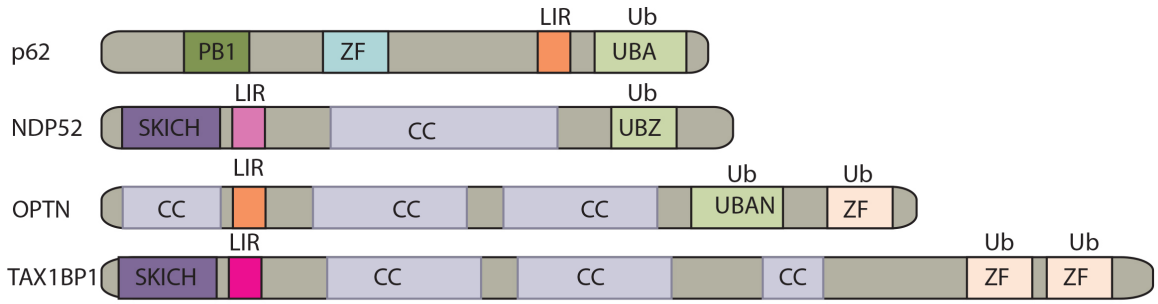
AUTOPHAGY ADAPTORS: SHARED PLAYER FOR SELECTIVE AUTOPHAGY DURING INFECTION AND INFLAMMATION

How different cargos (intracellular or pathogenic) are selectively targeted for autophagic degradation? One of the first steps involves tagging of the cargo by ubiquitin chains through the action of E3 ubiquitin ligases. Each of the cargos destined for autophagic degradation must get ubiquitinated for recognition by autophagy adaptor molecules. Autophagy adaptor proteins serve as a bridge between the cargo to be degraded and the nascent autophagosomes. All adaptor proteins share three common domains – LC3 interacting region (LIR) domain (through which they interact with LC3II decorating the autophagosomes), dimerization or multimerization domain and ubiquitin-binding domain (Behrends and Fulda, 2012). In addition to their role in selective autophagy, these proteins also regulate innate immunity signaling pathways, thus representing a new class of PRRs, the sequestosome-1-like receptors (SLRs) causing inflammation (Deretic et al., 2013). In the sections below, we discuss four key autophagy adaptors p62, NDP52, OPTN and TAX1BP1 in detail including their domains, interacting partners as well as their involvement in regulating autophagy under diverse contexts, which is also summarized in **Figures 2**, **3**.

P62

p62 is among the first mammalian autophagy adaptors initially identified and described. The Phox and Bhem 1 (PB1), LIR, and Ubiquitin associated (UBA) domains of p62 are implied in the degradation of ubiquitinated cargos by selective autophagy. By directly interacting with several E3 ligases, such as TRIM50, TRAF6 and MURF2., which promotes ubiquitination of p62 substrates, it is involved in the formation of inclusion bodies and execute aggrephagy (Rogov et al., 2014). Interestingly, p62 also contributes to pexophagy (Kim et al., 2008; Tripathi et al., 2016) and mitophagy, which is dependent on E3 ligase-"Parkin" (Geisler et al., 2010). The importance of p62 in executing anti-bacterial autophagy or xenophagy is primarily explored in controlling the invading bacteria *S. typhimurium* (Zheng et al., 2009). Other bacterial species such as *S. flexneri* and *M. tuberculosis* are also reported to be selectively targeted in a p62-dependent manner for recruitment and delivery into nascent LC3-positive isolation membranes for autophagic degradation (Mostowy et al., 2011; Franco et al., 2017). In *ex vivo* infection experiments using macrophages, it is shown that p62 co-localizes with *M. tuberculosis* and controls its survival and replication (Sakowski, 2015). True to the above observation, knocking down p62 in macrophages during *ex vivo* infections increases *M. tuberculosis* survival. However, the redundancy in the p62-mediated physiological functions gets evident *in vivo* in mice, where $p62^{-/-}$ mice never show any sickness past 80 days of *M. tuberculosis* infection and effectively controls bacterial replication (Kimmey et al., 2015). Additional modulators of p62, which functions in regulating xenophagy have been reported. For example, TBK1 plays an important role in promoting the xenophagy by activating p62 via phosphorylation of Serine 403 in the UBA domain of p62. UBA domain phosphorylation strongly enhances the activity of p62 and is implicated in the elimination of *M. tuberculosis* via autophagy (Pilli et al., 2012). Additional mechanisms for p62-mediated xenophagy are also reported, like involvement of the lysosomal protein DRAM-1 in recruiting p62 for restricting *M. marinum* infection in zebrafish (Meijer and van der Vaart, 2014).

Considering their critical role in regulating selective autophagy, adaptors like p62 could have direct involvement in controlling inflammation in a selective manner. The structural speckles of p62 are said to be involved in TRAF6 oligomerization resulting in NF-κB activation, and subsequent inflammation (Nakamura et al., 2010). On the contrary, the anti-inflammatory functions of p62 are also well known, for example, it down-regulates inflammation in response to adiponectin after LPS stimulation (Tilija Pun and Park, 2017). p62 is also shown to regulate oxidative stress. Activated TAK1 phosphorylates p62, which induces the interaction of p62 with keap-1 that



Domain	p62	NDP52	OPTN	TAX1BP1
PB1	Promotes homo-oligomerization of p62 resulting in degradation of p62, Important for aggrephagy			
LIR (For LC3 interaction)		Has two putative LIR, a canonical N-terminal SKICH domain and a non-canonical LIR, binds to LC3C	Functions in xenophagy and aggrephagy	Has two putative LIR, a canonical N-terminal SKICH domain and a non-canonical LIR. Recruitment to autophagosomes during non-selective autophagy is mediated by LIR or Ub
UB	UBA domain binds to K63 and K48 polyubiqutin chains (high affinity for K63) Involved in multiple ubiquitinated cargos including soluble proteins, peroxisomes, mid-body rings and *Salmonella* etc.	Binds to K63 polyubiquitin chains. Contains one UBZ domain which binds to ubiquitinated *Salmonella* for its cytosolic clearance	UBAN and UBZ domains; binds to linear ubiquitin chains Involved in aggrephagy, xenophagy	Contains two UBZ domains in comparison to one in NDP52, but share similar function as NDP52 to bind to K63 linked proteins/cargo.
CC		Also functions in ubiquitin binding	Self-interact and oligomerize via coiled coiled domain to perform aggrephagy	SKICH domain and the linker between SKICH and coiled coiled domain mediate interactions with GABARAP family members

FIGURE 2 | Domain structure of autophagy adaptors and their function. The following abbreviations are used for each domain: PB1, Phox and Bem1 domain; CC, coiled-coil domain; LIR, LC3-interacting region; UBA, ubiquitin-associated domain; SKICH, SKIP carboxyl homology domain; ZF/UBZ, Ubiquitin binding Zinc-finger domain; UBAN, ubiquitin binding in ABIN and NEMO domain.

causes subsequent degradation of keap-1 through autophagy (Hashimoto et al., 2016). This results into increased Nrf2 expression, the master regulator of antioxidant gene expression. Since, TAK1 participates in TLR, NLR, IL-1 and stress induced pro-inflammatory signaling, its regulation of p62-Keap-1-Nrf2 axis characterizes a link between inflammation and

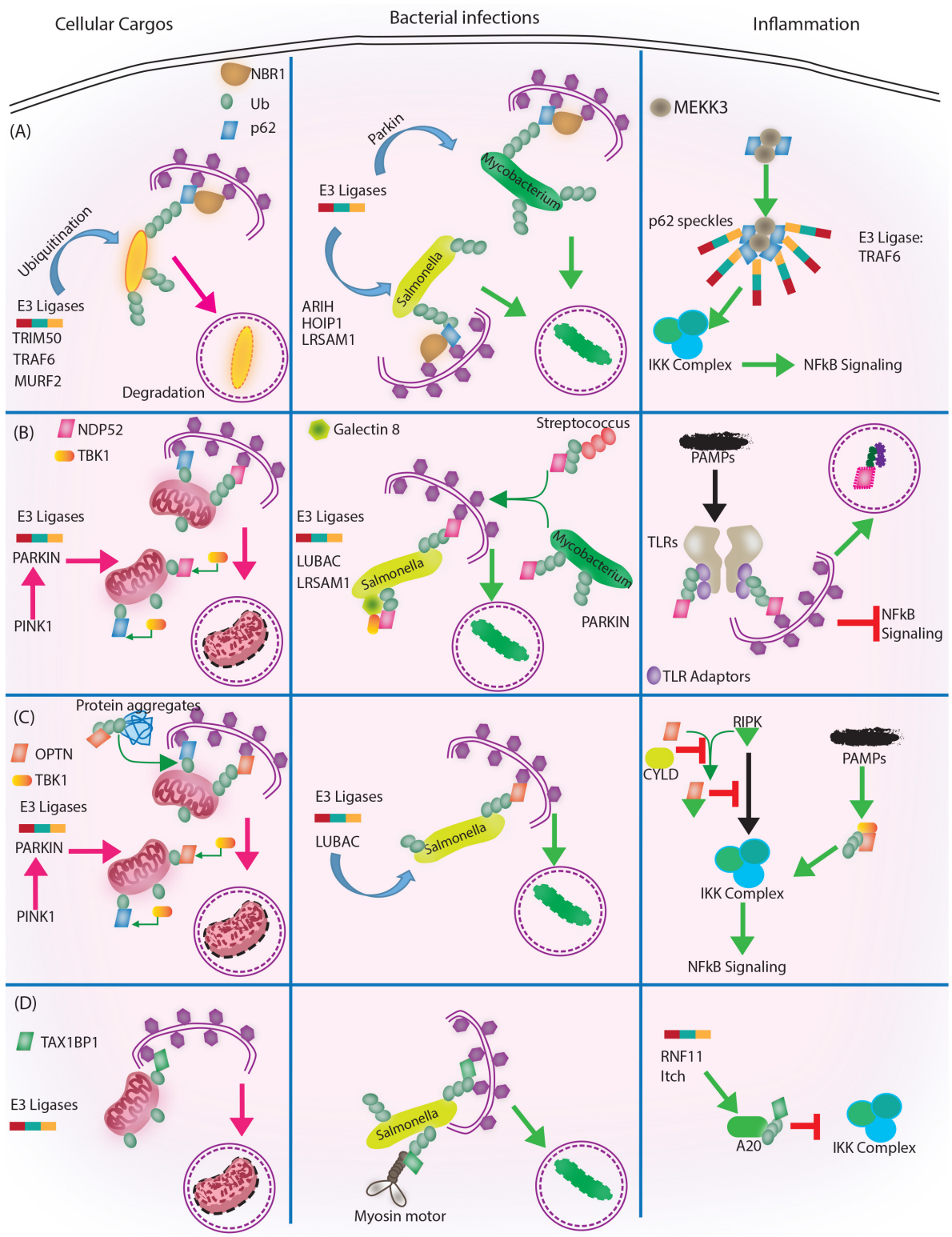


FIGURE 3 | Autophagy adaptors in selective autophagy, xenophagy and inflammation. Roles of different autophagy adaptors are highlighted here in the context of selective autophagy of intracellular cargos, xenophagy and inflammation. **(A)** p62/SQSTM1: E3 ligases TRIM 50, TRAF 6, MURF 2 ubiquitinate the intracellular cargos, followed by their recognition by autophagy adapter p62 and NBR1. Adaptors then target the cargo to autophagosome for subsequent degradation. During

(*Continued*)

FIGURE 3 | Continued
bacterial infection, p62 adaptor protein recognizes targets that are tagged by E3 ligases like ARIH, HOIP1, LRSAM1 (which act on *Salmonella*), after which p62 and NBR1 are recruited to the bacteria and targets *Salmonella* for autophagic degradation. In case of *M. tuberculosis* so far only Parkin has been shown to act as E3 ligase leading to K63 ubiquitination, p62-NBR1 recruitment and targeting of *M. tuberculosis* to autophagosomes. Finally, p62 is directly implicated in regulating inflammation. The PB1 domains of p62 homo and heterodimerize while interacting with MEKKK3. This complex further co-localizes to TRAF 6 oligomers, forming what is known as p62 speckles. This complex then phosphorylates and ubiquitinates IKK complex, inhibiting NFκB signaling. **(B)** NDP52/CALCOCO2: Upon mitochondrial damage the recruitment of PINK1/PARKIN E3 ligase help in ubiquitinating mitochondria and activating TBK1, which subsequently phosphorylate both NDP52 and p62 Ubiquitination of mitochondria and tagging with p62 and NDP52 helps in targeting mitochondria to autophagosome. During *Salmonella* infection, LUBAC complex and LSRAM1 E3 ligases ubiquitinate the bacteria. Phosphorylation of NDP52 by TBK1, help tagging of the bacteria with NDP52. Here cytosolic Galectin 8 also takes part in the process and interacts with NDP52. For recruitment of *M. tuberculosis* parkin mediates ubiquitination of *M. tuberculosis*. NDP52 is also recruited to *M. tuberculosis* and targets it to autophagosome. Rab35 and NDP52 also mediate targeting of *Streptoccocus* to autophagosomes. Bacterial PAMPS are recognized by TLR followed by recruitment of TLR adapters. The TLR adaptors get ubiquitinated and recognized by autophagy adapter NDP52. Along with the TLR adaptors, autophagy adaptors undergo degradation via autophagosome maturation called adaptophagy and controls inflammation. **(C)** OPTN: OPTN acts in mitophagy in a manner very similar to NDP52 where PINK1 and PARKIN E3 ligases are activated and recruited to mitochondria for ubiquitination. Ubiquitinated mitochondria are recognized by OPTN for targeting them to autophagosomes. In addition to mitophagy for degrading intracellular cargo OPTN performs aggrephagy as well. During bacterial infections, OPTN's role has been shown in the context of LUBAC complex mediated K63 and M1 polyubiquitination of *Salmonella*. Upon recruitment, OPTN targets the bacterium to autophagosomes for degradation. OPTN is also shown to inhibit IKK complex by interacting with RIPK. Interaction of OPTN with a deubiquitinating enzyme called CYLD, which deubiquitinates OPTN and RIPK allows RIPK mediated activation of IKK complex and inflammation. Similarly, PAMPs can activate TBK1, which gets autophosphorylated and subsequently binds to TBK binding domain of OPTN to alleviate inflammation. **(D)** TAX1BP1/CALCOCO3: Similar, to other autophagy adapters like NDP52 and OPTN TAX1BP1 also performs mitophagy where E3 ligases are not very well known, however, Parkin is the most likely candidate. In *Salmonella*, TAX1BP1 interacts with myosin motor VI and induces autophagosome and lysosome fusion subsequently helping in xenophagy of polyubiquitinated *Salmonella*. TAX1BP1 interacts with A20, an NFκB inhibitor to control inflammation via inhibiting IKK complex. Here, RNF 11 and Itch E3 ligases helps recruitment of TAX1BP1 to A20 for autophagic targeting. While we have used very selected examples to highlight the functional overlaps between different autophagy adaptors during selective autophagy, it must be noted that inhibition of selective autophagy like mitophagy also contributes to inflammation. This figure therefore showcases the complex regulatory events and points toward the existing lacunae in our understanding of selective autophagy, especially in the context of bacterial infection and inflammation.

redox homeostasis (Hashimoto et al., 2016). In addition to TAK-1, activated mTOR pathway and TLR-induced TBK-1 are also involved in inducing the interaction of p62 with Keap-1 (Ichimura et al., 2013). Although, p62 is involved in inflammation as well as in autophagy, its role in inflammation during intracellular infection is still elusive and needs further explorations.

NDP52

NDP52 is an important selective autophagy adaptor primarily performing mitophagy to maintain cellular homeostasis by removing damaged mitochondria from the cell (Heo et al., 2015). It is also found to play a significant role in xenophagy, as confirmed by several studies. It targets various bacteria including *Streptococcus pyogenes, Salmonella enterica* and *S. flexneri* to autophagy for their selective degradation (von Muhlinen et al., 2010; Mostowy et al., 2011; Minowa-Nozawa et al., 2017). Since, there is a substantial amount of functional redundancy found in autophagy adaptors, it results into the recruitment of two or more adaptors to the same bacterium. Additionally, few reports also highlight the fact that the same adaptor can induce targeting of different bacteria to the intrinsically different autophagosomes. For instance, p62 and NDP52 targets *Shigella* to autophagosomes in actin-septin dependent manner, whereas p62 and NDP52 are recruited independently to *Listeria* to target them into autophagosome via actin-septin independent pathways (Mostowy et al., 2011). This reinforces the view that different bacterial pathogens can evoke different pathways of selective autophagy and therefore it needs to be further investigated. Although xenophagy is aided by several cargo receptors, it is important to note that the recruitment of adaptors is independent of each other and they bind to different micro domains of bacteria (Cemma et al., 2011). NDP52 is able to interact with all the human ATG8 orthologs, but it selectively binds to LC3C via its non-canonical LIR (CLIR) domain to perform the antibacterial activities (von Muhlinen et al., 2012). Parkin, a well-known E3 ligase ubiquitinates NDP52 for mitophagy in a TBK1 phosphorylation dependent manner (Heo et al., 2015). Parkin is also involved in the recruitment of NDP52 to *M. tuberculosis* phagosomes upon infection, as the knockdown of *Park2* decreases the co-localization of *M. tuberculosis* with NDP52, p62 as well as NBR1 (Manzanillo et al., 2013). Besides ubiquitin, cytosolic Galectins also play an important role in cargo targeting to the autophagosome. NDP52 is shown to bind to Galectin 8 for removal of *Salmonella* via selective autophagy (Thurston et al., 2012). Besides, ubiquitination and phosphorylation there are few other proteins, which might show interaction with autophagy adaptors to activate them. Recently, Rab35 has been reported to control Group A *Streptococcus* (GAS) degradation by xenophagy via recruiting NDP52 (Minowa-Nozawa et al., 2017). However, Rab35 involvement in autophagy is not only restricted to xenophagy, but also in mitophagy, starvation-induced autophagy, both of which occur via recruiting NDP52 (Minowa-Nozawa et al., 2017).

NDP52 also take part in reducing inflammation via down-regulating the NF-κB signaling. In a sequencing study of CD patients versus healthy controls, whole exome sequencing of 42 CD patients revealed an interesting mechanism. A missense mutation Val248Ala in NDP52 failed to recognize polyubiquitinated adaptors resulting in high NF-κB activity, thereby causing more inflammation in CD patients. Importantly, the study also highlights the importance of NDP52/CALCOCO2 adaptophagy (Ellinghaus et al., 2013; Till et al., 2013), where

the turnover of autophagy adaptors plays an important role in regulating inflammation.

OPTN

Optineurin (OPTN) is a 67 KDa intracellular protein found in different tissues and has various domains like C-terminal zinc finger, leucine zipper domain, a LIR domain, and ubiquitin binding in ABIN and NEMO domain (UBAN) domain (Kim et al., 2016; Slowicka and van Loo, 2018). It also consists of coiled-coil motifs, which mediates its oligomerization. It is an important autophagy adaptor as demonstrated by its role in mitophagy, aggrephagy as well as xenophagy (Wild et al., 2011; Heo et al., 2015; Richter et al., 2016; Slowicka and van Loo, 2018). The role of OPTN in mitophagy was established in studies, which show that mutations in OPTN are associated with amyotrophic lateral sclerosis (ALS) and glaucoma due to mitochondrial dysfunction (Wong and Holzbaur, 2014). Studies have also shown that OPTN is required to restrict the growth of *S. enterica* upon infection (Wild et al., 2011). The key player in OPTN-mediated selective autophagy is TBK1, which phosphorylates it within LIR domain at Ser-177 and enhances its activity (Richter et al., 2016). Although, mitophagy and xenophagy are mediated by OPTN in ubiquitin-dependent manner, aggrephagy can be both dependent as well as independent of ubiquitin. It has been reported that OPTN recognizes targets like mutant SOD-1 and huntington protein (associated with ALS and Huntington's disease respectively) by an ubiquitin-independent pathway (Korac et al., 2013).

OPTN majorly inhibits inflammation by negatively regulating NF-κB signaling (Nagabhushana et al., 2011; Slowicka and van Loo, 2018). It is capable of competing with NEMO, to bind to RIPK1, a major factor for activating NF-κB, thereby resulting in down-regulation of NF-κB (Zhu et al., 2007). OPTN is also found to interact with a deubiquitinase enzyme CYLD, leading to deubiquitination of NEMO and RIPK1, thereby aiding in inhibition of TNF mediated NF-κB activation (Nagabhushana et al., 2011). However, role of OPTN *in vitro* is in contrast to the *in vivo* findings where OPTN knock out or knock in mice does not affect NF-κB signaling (Munitic et al., 2013; Slowicka et al., 2016). OPTN associated mitophagy have been extensively described, more recently it is shown to remove protein clusters associated with ER called aggrephagy (Tschurtschenthaler and Adolph, 2018). It might be further implicated to regulate inflammation since unused proteins are deleterious to the host cell. A decrease in the expression of OPTN has been correlated with the patients of CD. The reduced expression of OPTN was linked to a genetic variation due to SNP (rs12415716) in a subset of CD patients' cohort that was examined (Smith et al., 2015). In addition, OPTN also limits persistent ER-Stress. In intestinal cell, it targets IRE1-α degradation to combat the ER based inflammatory response (Tschurtschenthaler et al., 2017). On the contrary, OPTN mediated activation of IRF3 results into type 1 IFN production ultimately leading to bacterial clearance (Slowicka et al., 2016). As is evident here, the functional versatility of OPTN makes it an important autophagy adaptor.

TAX1BP1

TAX1BP1/CALCOCO3 is a close paralog of NDP52/CALCOCO2. Its role in xenophagy was first highlighted in the study that shows its involvement in the removal of *S. typhimurium* (Tumbarello et al., 2015). The clearance of the bacteria is dependent on the binding of TAX1BP1 to the myosin motor VI, which aids in the fusion of autophagosomes with the lysosomes (Tumbarello et al., 2015). Besides bacteria, the role of autophagy adaptors is also reported during viral infection. For instance, both TAX1BP1 and NDP52 can impact the replication of Measles Virus (MeV) in the cells by interacting with the MeV proteins and facilitating the maturation of autophagosomes (Petkova et al., 2017). These functions, are however, independent of their potential role in NFκB signaling. Surprisingly, knocking down OPTN using siRNAs does not have any effect on the MeV replication suggesting that there is specificity/selectivity among the adaptors to interact with different viral proteins (Petkova et al., 2017).

In an uninfected Streptozotocin (STZ)- induced diabetic mice model, TAX1BP1 overexpression in the heart attenuates inflammation, oxidative stress, and apoptosis that results in improved cardiac function (Xiao et al., 2018). It has been shown to interact with ubiquitin-editing enzyme A20, regulating NFκB and IRF3 signaling thereby controlling inflammation to increase the longevity of host cells (Shembade et al., 2007). The E3 ligase Itch and RNF11 recruitment to A20 is shown to be dependent on TAX1BP1 that restricts pro-inflammatory state in the cell (Shembade et al., 2009). Similarly, in RNA virus infection it controls RIG-1/MDA-5 mediated production of IFNβ (Parvatiyar et al., 2010; Choi et al., 2017). Few reports also manifest anti-inflammatory roles of TAX1BP1 during other viral infections. For example, dysregulation of TAX1BP1 in HTLV-1 infection can result in the induction of diverse forms of inflammatory disorders (Nakano et al., 2013). Recently, TAX1BP1 has been shown to play an important role in negatively regulating the VSV and Sendai virus associated apoptosis, as it gets degraded upon viral infection (Choi et al., 2017). Degradation of TAX1BP1 increases apoptosis and this could help in limiting the prolonged antiviral state of the cells. TAX1BP1 can also translocate to the mitochondria and interact with MAVS. This helps the recruitment of E3 ligase Itch to MAVS for its ubiquitination and degradation and thus restricting virus mediated apoptosis (Choi et al., 2017). Although, the anti-inflammatory roles of TAX1BP1 in uninfected and virus infected cells are well known, studies demonstrating its role in controlling bacterial associated inflammation remain unexplored.

UBIQUITIN LIGASES AND DEUBIQUITINASES IN INTRACELLULAR BACTERIAL INFECTIONS

One common theme across all the autophagy adaptors discussed above is ubiquitination of the cargos for subsequent adaptor binding. Whether the target is intracellular pathogens like *Salmonella*, *Lysteria* and *Mycobacteria* or cellular cargos like

damaged mitochondria, ERs, peroxisomes or selective proteins; all of them must get ubiquitinated before they are recognized by the autophagy machinery. Ubiquitination of the cargos requires subsequent action of three enzyme cascades- ubiquitin activating (E1), ubiquitin conjugating (E2) and ubiquitin ligase (E3). In humans, nearly 2 E1s, about 40 E2s and more than 600 E3 ligases are known. Since, E3 ligases are the most heterogeneous, they can mediate substrate specificity (Morreale and Walden, 2016) As far as proteins are concerned, ubiquitin chain length and ubiquitin linkage may impact the capacity of ubiquitinated proteins to get targeted for autophagy. Proteins which are coated with K-63 linked ubiquitin chains are mainly cleared through autophagy (Linares et al., 2013), whereas those which are decorated with K-48 or K-27 ubiquitin chains are targeted for proteasomal degradation (Grumati and Dikic, 2018). The UBA domain of p62 shows more affinity toward K-63 chains, even though it binds both K-63 as well as K-48 chains (Long et al., 2008). Similarly, M1-linked polyubiquitin chains on the bacteria attracts OPTN more (Noad et al., 2017). Several E3 Ligases are known which ubiquitinates cytosolic targets as well as bacteria and target them for selective autophagy. Parkin is one of the most studied E3 ligase and is mainly responsible for the ubiquitination of a plethora of mitochondrial membrane proteins and thus is involved in mitophagy (Heo et al., 2015). In addition to mitophagy, Parkin is also involved in ubiquitination of *M. tuberculosis* and targeting it to the autophagosomes via p62 and NDP52 receptor proteins (Manzanillo et al., 2013). Interestingly, Smurf-1, a newly identified ubiquitin ligase polyubiquitinates not only *M. tuberculosis* but also *L. monocytogenes* (Franco et al., 2017). Moreover, smurf-1 seems to work synergistically with Parkin, since BMDMs from Smurf-1$^{-/-}$, Parkin$^{-/-}$ double knockout animals supported enhanced replication of *M. tuberculosis* in comparison to single knockouts (Franco et al., 2017). Similar co-operativity was also observed during *in vivo M. tuberculosis* infection in mice. Additional E3 Ligases responsible for the pathogen ubiquitination are LRSAM1, ARIH, HOIPI and LUBAC complex. However, the ubiquitination pattern deployed by these enzymes may differ. LRSAM is known for forming K6 and K27 chains on the surface of *Salmonella,* whereas, ARIH forms K48 chains and HOIP1 is involved in linear ubiquitination (Huett et al., 2012; Noad et al., 2017; Polajnar et al., 2017). It has been reported that M1-linked polyubiquitination on the bacterial surface recruits OPTN via E3 ubiquitin ligase complex LUBAC. However, the recruitment of p62 and NDP52 occurs independently of LUBAC, demonstrating that the functions of few adaptors are not completely redundant (Noad et al., 2017; Slowicka and van Loo, 2018). During *Salmonella* infections, TRIM32, an E3 ligase, interacts with TRIF and adds a layer of complexity in its selective degradation in TAX1BP1 dependent manner. Here, deficiency of TAX1BP1 leads to inhibition of degradation of TRIF thereby turning off TLR3/4 mediated innate immune responses and inflammation (Yang et al., 2017). Other E3 ligases like RNF166 is found to be a key protein that controls the recruitment of ubiquitin as well as autophagy adaptors to *Salmonella*, by catalyzing K29- and K33-linked polyubiquitination of p62 (Heath et al., 2016). However, ubiquitination of bacteria by RNF166 is not studied. On the similar line, another protein UBQLN-1, consisting of ubiquitin like domain, an UBA domain and a STl1 motif, targets *M. tuberculosis* to autophagy after recruiting ubiquitin, p62 and LC3 (Sakowski et al., 2015). Many of the E3 ligases like LRSAM1 which are known to ubiquitinate other bacterial pathogens are unable to ubiquitinate *M. tuberculosis*. It is possible that while they may still get ubiquitinated, they could recruit certain deubiquitinase (DUBs) to strip themselves of ubiquitin tags and mask from the autophagy adaptors.

DUB's are the proteins, possessed mainly by the host cells, to execute deubiquitination. Besides, deubiquitinating intracellular cargos, these DUB's may also target intracellular pathogens for deubiquitination. DUB's are mainly present in all eukaryotic cells and almost more than 100 of them have been discovered in humans covering important regulatory functions of the cells. Since ubiquitination helps in the degradation process of pathogens including bacteria and viruses, many pathogens have evolved DUB's or DUB's like molecules so as to interfere with the host ubiquitination process. For example, in *C. trachomatis*, Chla1 and Chla2 have been reported to hydrolyse ubiquitinated and neddylated substrates. *C. pneumonia* has an Otubain like effector (OTU) called as ChlaOTU, having deubiquitinating activity which can cleave K63 and K48 linked polyubiquitin chains of the target/cargo (Sheedlo et al., 2015; Pruneda et al., 2016). It is also reported that NDP52 and ChlaOTU interacts at the bacterial entry site to reduce ubiquitin accumulation. Similarly, in *Shigella* and *Rickettsia*, ShiCE and RickCE function as deubiquitinating enzymes and prefer K63 linked targets (Pruneda et al., 2016). Structural analysis of DUB domain of Sde A in *L. pneumophila* revealed its molecular contacts with ubiquitin on bacteria containing phagophore. Importantly, unlike other eukaryotic counterparts, $SdeA_{Dub}$ recognizes Glutamine 40 patch of ubiquitin rather than Isoleucine 44 on bacterial phagosome (Sheedlo et al., 2015; Pruneda et al., 2016). Sid E effector family of *L. pneumophila* which remain involved in ubiquitination of the target substrates, therefore help bacteria to replicate in amoeba. This Sde E effector family also contain a DUB domain which help in reducing the ubiquitin level on LCV (Sheedlo et al., 2015). Besides bacteria, certain viruses like Herpes Simplex virus-1 (HSV-1) has UL-36, pertaining deubiquitinase activity responsible for deubiquitinating TRAF-3 (Wang et al., 2013). This activity is reported to remain conserved in Epstein Barr Virus and Cytomegalovirus. Interestingly, there are cases where in few pathogens the same enzyme performs two or more functions attributing to redundancy in enzyme functionality. *Yersenia* virulence factor Yop J, is an acetyl transferase and also contributes in deubiquitinating Iκβ and limit NFκβ induced inflammation (Rytkonen and Holden, 2007; Danelishvili et al., 2014). Intriguingly, most of the bacterial DUB's discovered so far have been shown to interfere with the host ubiqutination or deubiquitination pathways. Studies regarding their interaction with specific autophagy adapters which might lead to rescue of these pathogens from selective degradation remain unexplored. Interestingly, some pathogens may also modulate functions of host DUBs to help them evade xenophagic targeting. For example in a genome-wide siRNA knockdown study USP9Y, a deubiquitinase, was shown to help intracellular

M. tuberculosis survival (Kumar et al., 2010). In another recent study, OTULIN, a host DUB, specifically deubiquitinates M1 linked ubiquitin chains, thus maintaining balance of conjugation and deconjugation of ubiquitin chains on *Salmonella* (van Wijk et al., 2017). Knocking down OTULIN in the cells results in increased inflammation and *Salmonella* restriction (van Wijk et al., 2017)

FUTURE PERSPECTIVES

We emphasized in this review that autophagy is a cellular homeostatic function, which gets co-opted during various stress conditions including bacterial infections. Generation of pro-inflammatory state upon bacterial infections is a common antibacterial strategy adopted by the host. At the same time, activation of autophagy can also help to directly target the intracellular pathogens for degradation via xenophagy. Within an infected cell, where both inflammatory responses and anti-bacterial autophagy could occur simultaneously, what ensures the selectivity and balance between the two different arms of autophagy? This question is pertinent during pathogenic conditions since as discussed above, the cellular machinery involved in regulating autophagy for homeostasis, inflammation or xenophagy is more or less constant. Thus adaptors like p62, OPTN, TAX1BP1 and NDP52 could be recruited to different cargos including bacterial targets in varying proportions for subsequent targeting to autophagosomes. With the examples discussed in the above sections and many more which could not be discussed due to space constraints, it is plausible that while the adaptors are necessary for selective autophagy, they themselves are not solely responsible in ensuring selectivity of cellular cargos for degradation. This part is also depicted in the schematic shown in the figure summarizing these events (**Figure 3**). In the view of the above, the role of ubiquitin ligases becomes most critical in recognizing the autophagy cargo and subsequently tagging them for degradation. Questions like involvement of kind of ubiquitination like degree (mono or poly-ubiquitinatred) and linkage (K48, K63, K11, K27 etc.) in deciding the fate of cargo could also emerge in the future. The foundation for such selectivity is already available, given the distinction in fate of K48 versus K63 ubiquitin chains among many others. Similarly, several studies also point to distinctiveness originating due to the degree of ubiquitination like mono-, oligo- or poly-ubiquitination (Kwon and Ciechanover, 2017). To add further complexity, there are also reports suggesting role of autophagy independent of adaptors or ubiquitination. For example, *M. smegmatis*, a non-virulent strain of mycobacterium is degraded by LC3 targeting without involving membrane damage and ubiquitination. Here bacterial killing inside macrophages involves activation of autophagy via a TLR2-dependent mechanism in a p62 and NDP52 independent manner (Bah et al., 2016). Similarly, *M. marinum* ESX-1 secretion system is implicated in LC3 dependent phagocytosis but not ubiquitination (Bah et al., 2016). A better understanding of selective E3 ligase recruitment, activation and downstream recruitment of adaptors followed by activation across cargos like intracellular pathogens as well as cytosolic cargos during infection constitutes a major area of investigation in future. Such studies may yield clearer picture of how the observed selectivity and specificity in maintaining the equilibrium between homeostatic and defense arm of autophagy is ensured in the cells.

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Autophagy-Virus Interplay: From Cell Biology to Human Disease

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Autophagy is a highly conserved intracellular degradation process that targets protein aggregates and damaged organelles. Autophagy is also implicated in numerous viral infections, including human immunodeficiency virus-1 (HIV-1), influenza A (IAV) and herpes simplex virus-1 (HSV-1). Depending on the virus, autophagy can restrict or promote viral replication, and play key roles in modulating inflammation and cell survival. In this review, we consider examples of autophagy-virus interplay, highlighting the protective role of autophagy in human infections. We summarize recent discoveries and emerging themes illuminating autophagy's role in immunity and inflammation upon viral infection. Finally, we discuss future prospects and therapeutic implications, and potential caveats associated with using autophagy to control viral infections in humans.

Keywords: autophagy, inflammation, HIV-1, HSV-1, IAV, viral immunity

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INTRODUCTION

Autophagy captures cytoplasmic contents, such as excess or defective proteins and organelles, for degradation by the lysosome. It is initiated in response to various stimuli, including nutritional state of the cell and environmental stresses such as starvation and hypoxia. As such, autophagy is an important process of regulating cellular homeostasis and survival. It is a well-studied process that is orchestrated by over 35 autophagy-related (ATG) proteins and can be organized into multiple steps: phagophore initiation, membrane elongation, autophagosome formation and autophagosome fusion with hydrolytic lysosomes (**Figure 1A**; Mizushima et al., 2011). Autophagy can be selective in terms of cargo capture via the recruitment of selective autophagy receptors. Autophagy receptors can interact with ubiquitin tags that decorate the cargo [via its ubiquitin-binding domain (UBD)], and with LC3 proteins of nascent autophagosomes [via its LC3-interacting region (LIR) motif] (Stolz et al., 2014). Some autophagy receptors, particularly p62/SQSTM1 and optineurin, are regulated by Tank-binding kinase 1 (TBK1)-mediated phosphorylation, and are key players in the autophagic degradation of invasive pathogens (Wild et al., 2011; Pilli et al., 2012; Sparrer et al., 2017).

Autophagy plays a key role in cellular immunity to human infections (Randow et al., 2013; Paul and Münz, 2016). In the case of human viral infections, autophagy can be either proviral or antiviral (Levine et al., 2011; Jackson, 2015). Some viruses highjack the autophagy machinery for their intracellular survival, while others express specific proteins to evade autophagy and propagate in host cells. Antiviral autophagy can (1) selectively target pathogens for degradation, (2) promote pathogen recognition and inflammatory cytokine responses, (3) regulate inflammation, (4) control cell survival, (5) promote antigen presentation and/or (6) be regulated in a paracrine-mediated fashion, a departure from the classic cell autonomous route (**Figure 1B**; Levine, 2005;

Levine et al., 2011; Jackson, 2015; Paul and Münz, 2016). By focusing on these themes of antiviral autophagy, this review will highlight the protective nature of autophagy. We will draw from examples of human viral infections representing significant disease burden, whose interplay with autophagy has been supported by experimental and/or clinical evidence.

PATHOGEN OR VIRAL ANTIGEN CLEARANCE BY AUTOPHAGY

Selective autophagy has been reported to control several human viral infections *in vitro*, leading to the clearance of pathogens or viral antigens and host cell survival. One of the first viruses shown to engage the autophagy machinery was Sindbis virus (SV), a positive-stranded RNA alphavirus that typically causes mild disease in humans. Autophagy receptors p62 and SMURF1 were found to target SV capsid, in a ubiquitin-independent manner, to autophagosomes in human HeLa cells and mouse embryonic fibroblasts (MEFs) (Orvedahl et al., 2010, 2011). Depletion of p62 in HeLa cells or *Atg5* in mouse neurons *in vivo* led to an accumulation of toxic SV capsid and higher virus-induced mortality, without altering SV replication (Orvedahl et al., 2010). Whether SV can express proteins to counteract autophagy is not yet known.

Similar to SV, p62 is involved in targeting toxic Chikungunya virus (CHIKV) capsid to autophagosomes in HeLa cells, but in a ubiquitin-dependent manner (Judith et al., 2013). CHIKV, also a positive-stranded RNA alphavirus, is a mosquito-borne virus causing severe pathologies in humans that range from febrile arthralgia, rash to encephalopathy (Couderc and Lecuit, 2015). siRNA-mediated depletion of p62 led to an increase in both cell mortality and CHIKV replication, while p62 overexpression promoted cell viability in CHIKV-infected cells (Judith et al., 2013). In contrast, another autophagy receptor NDP52 was shown to have a proviral role although overall autophagy played a cytoprotective role in CHIKV infection (Judith et al., 2013).

Human immunodeficiency virus-1 (HIV-1), a lentivirus that is transmitted sexually through infected body fluids, is targeted by autophagy in human $CD4^+$ T cells *in vitro* (Sagnier et al., 2015). HIV-1 primarily infects $CD4^+$ T cells, consequently compromising the individual's immune defense and leading to acquired immunodeficiency syndrome (AIDS) (Maartens et al., 2014). HIV-1 integrates its DNA into the host's genome, and recruits its transactivator protein Tat to activate viral transcription. Tat protein is therefore essential for HIV-1 replication. However, Tat protein is targeted by p62 in HEK293T cells, which directs it to autophagosomes in a ubiquitin-independent manner (Sagnier et al., 2015). Consistent with this, depleting p62 in HEK293T cells resulted in accumulation of Tat protein, and enhancing autophagy in a chronically HIV-1-infected T cell line led to reduction of Tat protein levels (Sagnier et al., 2015). Furthermore, peripheral blood mononuclear cells (PBMCs) from HIV-1-infected non-progressor individuals showed higher number of HIV-1 particle-containing autophagic vesicles compared with HIV-1-infected normal progressors, suggesting a role for autophagy in limiting the pathogenesis of HIV-1 *in vivo* (Nardacci et al., 2014). However, HIV-1 can modulate autophagy and expresses multiple autophagy inhibitors, such as Vif, Nef, and Env, which operate in a cell type-specific manner (Liu et al., 2017). Hence, autophagy can be viewed to control HIV-1 replication by targeting viral components to degradation in specific cell types.

Herpes simplex virus-1 (HSV-1) is a ubiquitous, neurotropic α-herpesvirus with a global seroprevalence of 67% (Looker et al., 2015). It typically manifests as benign, self-limiting mucocutaneous ulcers, but in rare cases may cause life-threatening herpes simplex encephalitis (HSE) (Whitley and Roizman, 2001). Reports have identified HSV-1-encoded ICP34.5 and Us11 as autophagy inhibitors, which exert their effects by targeting Beclin-1 and protein kinase R (PKR), respectively (Orvedahl et al., 2007; Lussignol et al., 2013). Studies in primary MEFs and mice described increased autophagy following infection with ICP34.5-deficient HSV-1, as suggested by an increased number of autophagosomes and virions in neuronal autophagosomes, respectively (Tallóczy et al., 2006; Alexander et al., 2007). During HSV-1 infection, autophagy appears to be operating in a cell type-specific manner (Yordy et al., 2012). For example, in contrast to mitotic cells such as MEFs and mouse keratinocytes, post-mitotic mouse primary neuronal cells predominantly use autophagy over interferon (IFN) as a viral control mechanism (Yordy et al., 2012). In MEFs, HSV-1 was shown to be selectively targeted by p62 and SMURF1. HSV-1-infected *smurf1−/−* MEFs failed to target HSV-1 virions to autolysosomes, resulting in more HSV-1 virions in autophagosomes (Orvedahl et al., 2011). In mouse primary trigeminal neurons *in vitro*, HSV-1 infection triggered the formation of p62-mediated autophagosomes (Katzenell and Leib, 2016). Finally, infection of HEK293T cells with ICP34.5-deficient HSV-1 led to the recruitment of TBK1- and p62-mediated autophagy, and viral restriction (Sparrer et al., 2017). However, the exact viral target of p62 and SMURF1, and the role of ubiquitin in mediating receptor-virus interactions, is not yet known. Reports using human HFFF2 fibroblasts and mouse dendritic cells (DCs) have shown that autophagy is triggered by HSV-1 double-stranded DNA (dsDNA), and is independent of viral replication but dependent on STING (McFarlane et al., 2011; Rasmussen et al., 2011).

AUTOPHAGY AND CELLULAR IMMUNITY: PATHOGEN RECOGNITION AND CYTOKINE RESPONSES

Cellular immunity requires the detection of viral pathogen-associated molecular patterns (PAMPs) by their cognate receptors to produce antiviral cytokines, such as type-I IFNs (Iwasaki, 2012). Autophagy can deliver viral PAMPs to their receptors, and help amplify the production of inflammatory cytokines. The capacity of autophagy to facilitate viral recognition and modulate downstream cytokine production has been demonstrated in the case of HIV-1 infections. During HIV-1 infection in human primary plasmacytoid DCs, autophagy plays a key role in presenting the HIV-1 RNA genome to its cognate immune

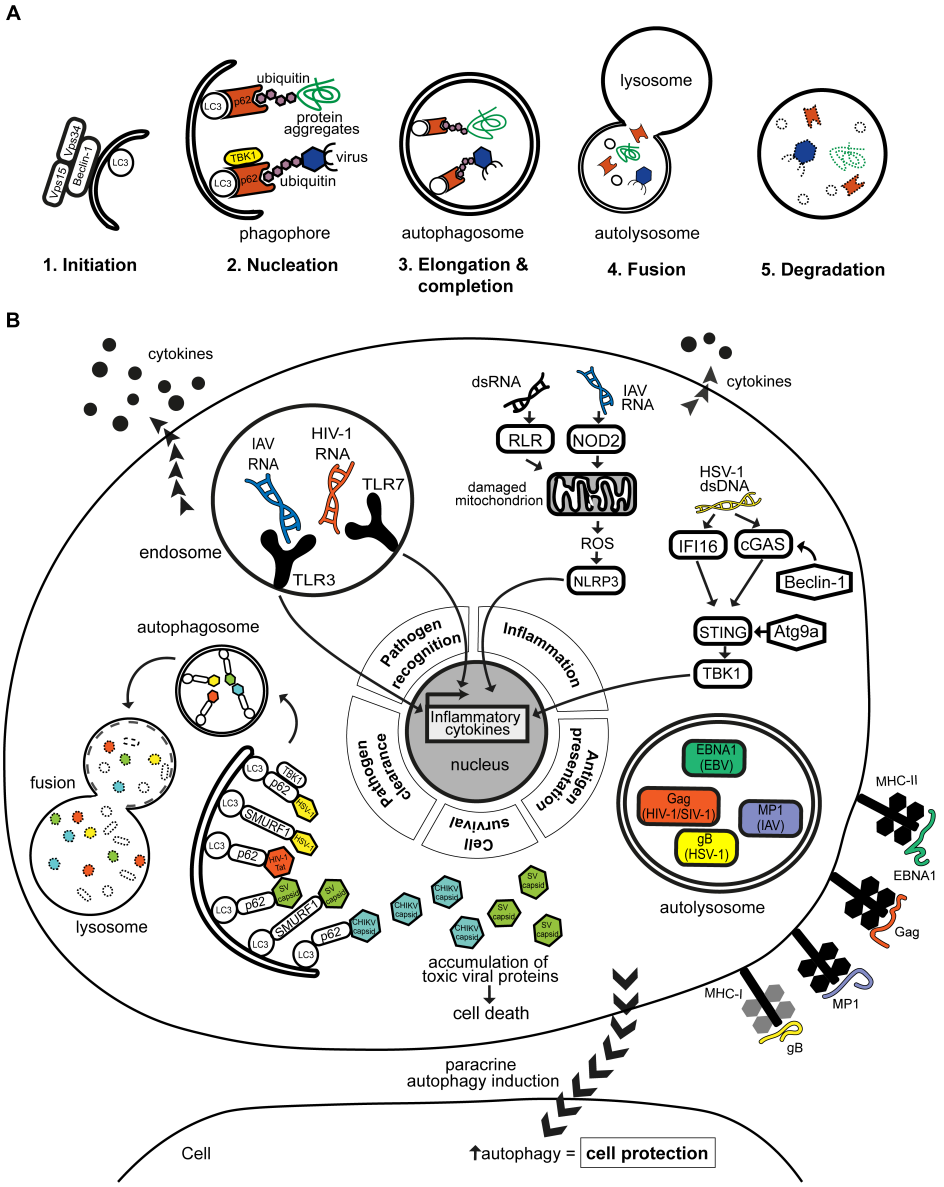


FIGURE 1 | (A) Autophagy is a regulated multi-step process that leads to cargo degradation. Autophagy can eliminate cargo such as virus and viral-derived antigens. It can be organized into 5 distinct steps beginning with (1) the initiation of phagophore formation which (2) nucleates around the intended cargo. The cargo can be selectively recruited by autophagy receptors such as p62, which can be regulated by TBK1. (3) The phagophore elongates and completes to form a structure termed autophagosome which then (4) fuses with nearby lysosomes carrying hydrolytic enzymes. This eventually leads to (5) the acidification and hence degradation of the contained cargo. **(B)** Autophagy plays an antiviral role in various human infections by modulating different aspects of the immune response. Autophagy facilitates viral clearance by recruiting selective autophagy receptors p62 and SMURF1 to target viral components to autophagosomes for lysosomal degradation. Reported targets include HSV-1, HIV-1 Tat protein, and the capsids of CHIKV and SV. Disruption of the targeting of viral proteins, such as CHIKV and SV capsids, may lead to their toxic accumulation and cause cell death. Autophagy also promotes pathogen recognition by aiding delivery of viral PAMPs, e.g., HIV-1 and IAV RNA genomes to cognate TLRs in endosomes, which results in enhanced production of antiviral cytokines. On the other hand, autophagy can prevent excessive inflammation by negatively regulating signaling pathways through Atg9a or Beclin-1, or by clearing mitochondria that are producing inflammatory-inducing signals such as reactive oxygen species (ROS). Autophagy supports amplification of inflammatory responses by regulating adaptive immune responses, through the processing and presentation of viral antigens, such as EBV EBNA1, IAV MP1, HIV-1/SIV gag and HSV-1 glycoprotein B, on MHC class I or II to T cells. Autophagy can also be induced in distant cells, i.e., in paracrine manner, which may confer protection to these cells as seen with multiple viral infections including CVB, HCV, and HSV-1.

receptor Toll-like receptor-7 (TLR7) in endosomes, leading to the induction of IFNα (Lee et al., 2007; Zhou et al., 2012). Silencing the expression of ATG7 in plasmacytoid DCs *in vitro* leads to a significant decrease in IFNα production following HIV-1 infection, highlighting a crucial role for autophagy in mediating TLR7-IFN signaling (Zhou et al., 2012).

Autophagy also plays an important role in mediating cytokine production during infection by influenza A virus (IAV). IAV, an RNA virus, is a pandemic threat and global health concern. It targets epithelial cells of the respiratory tract, and in severe cases may cause pneumonia or pulmonary damage (Paules and Subbarao, 2017). In the case of infection with highly virulent IAV strains H1N1 and H5N1, high morbidity has been attributed to excessive host-induced inflammatory cytokine production (Peiris et al., 2010). IAV infection was shown to induce autophagy in primary human blood macrophages, which regulates the production of CXCL10 and IFNα. When these cells were depleted of ATG5 or treated with the autophagy inhibitor 3-methyladenine (3-MA), they produced lower CXCL10 and/or IFNα levels (Law et al., 2010). The precise mechanism by which autophagy facilitates this aspect of IAV infection in human blood macrophages is unknown, but is thought to involve recognition of viral RNA by endosomal TLR3 (Law et al., 2010). In contrast, IAV-induced autophagy in MEFs can prevent IFNβ production, and enhance viral replication (Perot et al., 2018). The induction of autophagy during IAV infection is complex: it is initially cytoprotective but is later counteracted by the IAV matrix protein 2 (M2) which targets Beclin-1 to block lysosomal fusion with autophagosomes (Gannagé et al., 2009). Taken together, these data suggest an immunopathological role of autophagy in controlling cytokine production and IAV infection, in potentially a tissue-specific manner.

From the examples of HIV-1 and IAV, autophagy plays a fundamental role in modulating the primary antiviral response, by promoting viral recognition through TLR-dependent signaling and inflammatory cytokine production.

ANTI-INFLAMMATORY ACTIONS OF AUTOPHAGY

In addition to promoting inflammation, autophagy is crucial for preventing prolonged and excessive inflammation detrimental to the host. Components of the autophagy machinery, such as Beclin-1 and Atg9a, interact with the cytoplasmic type-I-IFN-inducing STING-TBK1 pathway. A study found that Beclin-1 interacts with dsDNA sensor cGAS to dampen IFNβ production in HEK293T cells stimulated with dsDNA or infected with HSV-1 (Liang et al., 2014). This interaction also leads to clearance of dsDNA through autophagy, limiting the otherwise persistent IFNβ-mediated inflammation (Liang et al., 2014). The depletion of Beclin-1 was shown to increase cGAS-mediated IFNβ production while reducing HSV-1 replication in RAW264.7 mouse macrophages (Liang et al., 2014). In MEFs stimulated with dsDNA, Atg9a had a similar role and negatively regulated the STING-TBK1-IFN pathway by binding to STING and preventing its assembly with TBK1 in LC3-positive structures (Saitoh et al., 2009). *Atg9a*-knockout mice revealed an increase in IRF3 phosphorylation and IFNβ production following dsDNA stimulation (Saitoh et al., 2009). However, whether HSV-1 infection (or infection of other dsDNA viruses) is subject to Atg9a-mediated regulation remains unknown.

Autophagy may also negatively regulate inflammation indirectly by clearing host DAMPs, such as reactive oxygen species (ROS) released by mitochondria. This has been observed in mice BMDCs where IAV genomic RNA is detected by the NOD2-RIPK2 pathway, which activates ULK1 to induce RIPK2-mediated autophagic clearance of damaged mitochondria (Lupfer et al., 2013). In RIPK2-deficient mice BMDCs infected with IAV, mitochondria accumulated in cells resulting in elevated production of superoxide. This led to the hyperactivation of the NLRP3 inflammasome and an increased secretion of the inflammatory cytokine interleukin (IL)-18 (Lupfer et al., 2013). A similar observation was made in *Atg5*-deficient MEFs and mouse primary macrophages stimulated with dsRNA analog poly(I:C), which led to excessive RIG-I-like-receptor signaling (Tal et al., 2009). Furthermore, ectopic P-granules autophagy protein 5 homolog (EPG5; a protein that regulates autolysosomal formation) has been shown to control pulmonary inflammation. Lung macrophages from *Epg5*−/− mice showed excessive production of inflammatory IL-1β and IL-13 cytokines, resulting in resistance to IAV infection (Lu et al., 2016).

These reports highlight the important role of autophagy in attenuating inflammation. The autophagy machinery can limit inflammation by regulating cytosolic NLR- and STING-mediated signaling pathways through disposal of their ligands, inactivation of their cognate receptors or interaction with their downstream effector molecules.

AUTOPHAGY AND CELL SURVIVAL

As shown from investigations of multiple human viral infections, autophagy plays a role in promoting cell survival and limiting pathogenesis. This has been demonstrated by the ability of mouse L cell mutant gro29 cells which have high basal autophagy to restrict HSV-1 replication (Le Sage and Banfield, 2012). In contrast, Atg16L^{HM} mice, which have reduced basal autophagy, showed high mortality following infection with CHIKV *in vivo* (Joubert et al., 2012). One mechanism by which autophagy may promote cell survival during viral infection is by degrading and preventing accumulation of toxic viral proteins, such as viral capsids, in the infected cells. This has been demonstrated in the case of CHIKV and SV infected cells, as discussed above (Orvedahl et al., 2010; Judith et al., 2013). Furthermore, reports have documented the cytoprotective effect of autophagy-enhancing drugs, such as vitamin D, MG132 and rapamycin, in viral infections. For example, primary human macrophages have shown benefit from vitamin D treatment, which limits HIV-1 replication *in vitro* (Campbell and Spector, 2012). Treating HSV-1-infected human corneal epithelial (HCE) cells with MG132 can reduce viral titres (Yakoub and Shukla, 2015). Moreover, pre-conditioning human fibroblasts *in vitro* with rapamycin has been shown to promote cell survival following HSV-1 infection (Ahmad et al., 2018). This cytoprotective role for autophagy that occurs early in HSV-1 infection appears to be a TBK1-dependent process (Ahmad et al., 2018). In agreement with this, TBK1

TABLE 1 | Opportunities for autophagy-modifying therapeutic intervention in human viral diseases.

Virus	Available treatment for acute disease	Vaccine	Post-infectious inflammation/ autoimmunity
CHIKV	None	No	Rheumatic inflammation
HIV-1	Antiretroviral therapy (ART)	No	HIV-associated neuroinflammation
HSV-1	Nucleoside analog (e.g., acyclovir)	No	Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis
IAV	Neuraminidase inhibitors (e.g., oseltamivir)	Yes	Acute infection-induced cytokine storm

deficiencies render human fibroblasts susceptible to HSV-1 infection and leads to increased cell mortality (Herman et al., 2012). These data support literature showing that TBK1 is a key player in protective autophagy against bacterial infections (Weidberg and Elazar, 2011), and extend its protective role to viral infection.

AUTOPHAGY AND ADAPTIVE IMMUNITY: ANTIGEN PRESENTATION

Through its degradative function, autophagy is particularly useful for generating endogenous peptide antigens for major histocompatibility complex (MHC)-II presentation (Dengjel et al., 2005; Paul and Münz, 2016). In viral infections, autophagy generates viral antigens loaded onto MHC-I and MHC-II for presentation to T cells (Münz, 2017). Epstein-Barr virus (EBV) is an oncogenic γ-herpesvirus causing a spectrum of human diseases ranging from mononucleosis to lymphomas and carcinomas (Taylor et al., 2015). Historically, EBV nuclear antigen 1 (EBNA1) was one of the first viral antigens shown to be processed by autophagy and loaded on MHC-II molecules of EBV-transformed B cell lines (Paludan et al., 2005). Inhibition of autophagy leads to accumulation of EBNA1 in autophagosomes of EBV-transformed lymphoblastoid cell lines, and a decrease in EBNA1-specific $CD4^+$ T cell recognition via MHC-II (Paludan et al., 2005).

As a result of reduced MHC-II antigen presentation, mice with *Atg5*-deficient DCs intradermally injected with HSV-1 showed significantly lower IFNγ production by $CD4^+$ T cells (Lee et al., 2010). In addition, autophagy can deliver viral antigens for MHC-I cross-presentation. Using a mouse BMA3.1A7 macrophage cell line for $CD8^+$ cell stimulation, HSV-1 glycoprotein B (gB) was presented on MHC-I in an autophagy-dependent manner (English et al., 2009; Radtke et al., 2013).

Autophagy is also vital for efficient stimulation of antiviral $CD4^+$ T cells in HIV-1/Simian immunodeficiency virus (SIV) and IAV infections. Knocking down LC3 protein or inhibiting autophagy using 3-MA in human DCs led to reduced antigen processing and MHC-II presentation, and a decrease in HIV-1-specific $CD4^+$ T cell response (Blanchet et al., 2010). On the other hand, enhancing autophagy in human DCs with rapamycin resulted in a more pronounced HIV-1-specific $CD4^+$ T cell response (Blanchet et al., 2010). Fusing SIV gag protein to LC3 in mice BMDCs was also shown to improve antigen-specific $CD4^+$ T cell responses *in vitro* (Jin et al., 2014). Similar results were obtained *in vivo* where immunizing mice with SIV gag-LC3 resulted in a stronger humoral immune response, with $CD4^+$ T cells producing higher levels of IFNγ, TNFα and IL-2 (Jin et al., 2014). Conjugating IAV matrix protein 1 (M1) to LC3 in HaCat human epithelial cells, B cells and DCs led to enhanced antigen-specific human $CD4^+$ T cell responses *in vitro*, as measured by IFNγ (Schmid et al., 2007).

Taken together, autophagy can perpetuate the initial response to viral infection by priming and mediating T cell responses of the adaptive immune system to ensure effective viral clearance.

BEYOND CELL AUTONOMOUS IMMUNITY: PARACRINE REGULATION OF AUTOPHAGY

Since its discovery, the primary focus of autophagy research has been to investigate its role on a cell autonomous level. Interestingly, two recent reports have demonstrated that autophagy can also be triggered at a cell population level (i.e., in a paracrine manner) to affect distant cells. A first report showed that autophagy could be triggered in distant and distinct cell types that can protect them from a variety of viral infections (Delorme-Axford et al., 2013). In this case, primary human placental trophoblasts can protect other cells from coxsackievirus B3 (CVB), hepatitis C virus (HCV), vesicular stomatitis virus (VSV) and vaccinia virus (VACV), by secreting signals that induce autophagy to resist infections. This concept is particularly relevant in the womb, allowing maternal trophoblasts to confer resistance to viral infections to the growing fetus.

A second report described the paracrine regulation of autophagy early in HSV-1 infection (Ahmad et al., 2018). In this case, HSV-1 infection of human fibroblasts was shown to induce autophagy in cells neighboring an infection site. Despite having functional basal autophagy, HSE patient-derived fibroblasts deficient in TBK1 specifically failed to mount paracrine-mediated autophagy during HSV-1 infection. The study further showed that autophagy induction early during infection may protect cells from death. The autophagic role of TBK1 has previously been associated with inflammation control in neurodegenerative amyotrophic lateral sclerosis (ALS) (Freischmidt et al., 2015). These observations highlight a potential involvement of TBK1 in controlling neuroinflammation through autophagy in HSE.

CONCLUSION

Many open questions remain concerning the precise role of autophagy in human viral infections. Studies looking at human responses *in vivo* are rare, due to difficulty of conducting these studies. However, a wealth of studies using animal

in vitro/ex vivo/in vivo and human *in vitro/ex vivo* models have given remarkable insights into the role of autophagy in disease manifestation.

In this review, we discuss human viruses modulated by autophagy that represent a significant clinical burden. We highlight how autophagy is protective and may be used to enhance current treatment options (**Table 1**; Whitley and Roizman, 2001; Moscona, 2005; Maartens et al., 2014; Couderc and Lecuit, 2015). Several reports have shown the protective role of p62-mediated selective autophagy in various human pathogens (e.g., CHIKV, HIV-1, and HSV-1), making p62 an attractive therapeutic target. Enhancement of autophagy through p62 may provide an important therapeutic avenue for treatment of human viral diseases (**Table 1**). However, p62 also participates in other biological processes, such as cell proliferation and ubiquitin-proteasomal degradation (Liu et al., 2016b), and research focusing on employing p62 for therapeutic benefit should be aware of potential pleiotropic effects. The ability of p62 to interact with viruses independent of its conventional ubiquitin-binding domain also warrants further investigation. As shown in the case of HSV-1 and HIV-1 infections, augmenting autophagy using stimulants (such as rapamycin and vitamin D) can be beneficial to restrict viral replication and/or promote cell survival. Enhancing autophagy in vaccine therapies has also been beneficial, taking advantage of the role of autophagy in antigen priming. Promising results were observed in the case of IAV and HIV-1/SIV-1 infections, whereby increasing the autophagic targeting of viral protein gave rise to a heightened adaptive immune response (Schmid et al., 2007; Jin et al., 2014). Moreover, autophagy is important for both MHC-I and/or -II antigen presentation in HSV-1 and HIV-1 infections, as well as for regulating inflammation by facilitating antiviral inflammatory cytokine production. Autophagy's role in fine-tuning inflammation is also important during IAV infection, where it promotes inflammatory cytokine production and prevents excessive inflammatory responses. In addition to the effects on acute disease outcome, modulating autophagy may have a promising role in the prevention or treatment of various viral post-infectious inflammation/autoimmune disorders for which there are limited treatment options (**Table 1**; Kovalevich and Langford, 2012; Armangue et al., 2014; Couderc and Lecuit, 2015; Liu et al., 2016a). Harnessing autophagy's inflammation-reducing capacity can potentially prevent the development of these states, or help to resolve the inflammatory symptoms.

Current research on autophagy is mostly focused on its role in cell autonomous immunity (Randow et al., 2013). However, recent studies have revealed a novel type of autophagy triggered in a paracrine manner in response to viral infections. Elucidating the role of paracrine-regulated autophagy may prove to be highly relevant in disease pathogenesis *in vivo*, and may be useful as a method of clinical intervention. It is tempting to speculate that similar mechanisms could also be extended to viral pathogenesis that disseminates to sensitive tissues, such as the central nervous system (CNS).

In conclusion, we have discussed here the protective nature of autophagy in light of important human viral infections, and highlighted potential therapeutic strategies that can be pursued through autophagy modulation. Most viral infections result in complex host-pathogen interplay, and therefore routes of intervention require careful consideration in terms of application. For example, certain studies of autophagy in HSV-1 infections have shown viral restriction whilst others have only demonstrated cytoprotective effects despite the presence of viral autophagy inhibitors, which may be partly due to cell type specificity. These studies reveal the intimate interactions of the virus and the host cell which will require further dissection if we wish to target the appropriate molecular pathways for antiviral therapies.

AUTHOR CONTRIBUTIONS

LA, SM, and VS-S conceptualized and wrote this review. Figure was prepared by LA. All authors approved the final version of this review and agreed to be accountable for the content of the work.

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Chemical Biology Strategies to Study Autophagy

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Growing amount of evidence in the last two decades highlight that macroautophagy (generally referred to as autophagy) is not only indispensable for survival in yeast but also equally important to maintain cellular quality control in higher eukaryotes as well. Importantly, dysfunctional autophagy has been explicitly shown to be involved in various physiological and pathological conditions such as cell death, cancer, neurodegenerative, and other diseases. Therefore, modulation and regulation of the autophagy pathway has emerged as an alternative strategy for the treatment of various disease conditions in the recent years. Several studies have shown genetic or pharmacological modulation of autophagy to be effective in treating cancer, clearing intracellular aggregates and pathogens. Understanding and controlling the autophagic flux, either through a genetic or pharmacological approach is therefore a highly promising approach and of great scientific interest as spatiotemporal and cell-tissue-organ level autophagy regulation is not clearly understood. Indeed, chemical biology approaches that identify small molecule effectors of autophagy have thus a dual benefit: the modulators act as tools to study and understand the process of autophagy, and may also have therapeutic potential. In this review, we discuss different strategies that have appeared to screen and identify potent small molecule modulators of autophagy.

Keywords: autophagy, high throughput, chemical biology, luciferase, small molecule screening, fluorescence microscopy

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INTRODUCTION

Macroautophagy (herein autophagy) is a major intracellular process that is critically crucial for maintaining cellular homeostasis. Autophagy has been reported in several organisms from different kingdoms ranging from yeast to humans suggesting that it is an evolutionarily conserved process. This process was first reported by Christian de Duve (Deter et al., 1967), when he observed organelles captured within the lysosomes with the help of electron microscopy (De Duve and Wattiaux, 1966). This entire phenomenon of cargo capture and ultimately its degradation in the lysosomes is called "autophagic flux" (Klionsky, 2007; Rabinowitz and White, 2010; Boya et al., 2013).

Basal levels of autophagy occur in all cells during nutrient rich conditions and help in housekeeping functions to maintain cellular quality control by clearance of damaged or surplus organelles and misfolded proteins, recycling and providing basic building blocks like amino acids for reuse (Mizushima and Klionsky, 2007; Musiwaro et al., 2013). However, the levels of autophagy are highly modulated in response to different stimulus, both intracellular and exogenous

such as starvation, pathogen invasion, organelle damage and protein aggregation in cytoplasm (Takeshige et al., 1992; Komatsu and Ichimura, 2010). Because autophagy is central to maintaining cellular homeostasis, defective autophagy has been attributed to a variety of disease conditions such as cardiovascular diseases, atherosclerosis, certain myopathies, innate and adaptive immune responses, neurodegeneration and cancer (Choy and Roy, 2013; Kroemer, 2015).

Dysfunction of autophagy leads to cell death, cancer, neurodegenerative, and other diseases. Therefore, studying the molecular aspects of autophagy is of current research interest for the treatment of various disease conditions. Genetic and pharmacological modulation of autophagy has been shown to be beneficial in many such situations (Rubinsztein et al., 2012). Modulation of autophagy has been shown to be beneficial in diseases such as diabetes, cancers, neurodegenerative disorders and some infectious diseases (Sarkar et al., 2007; Sarkar and Rubinsztein, 2008). Several studies in the recent years have discovered novel or repurposed drugs for restoring autophagic balance. For instance, Rapamycin, an autophagy inducer and its analogs were used by Ravikumar et al., to abrogate neurodegeneration in a *Drosophila* based model by enhancing the rates of autophagy (Ravikumar et al., 2004; Sarkar, 2013a). In some of these studies, distinct assays have been developed and used for a High Throughput Screening (HTS) to identify small molecules that modulate autophagy (**Table 1**). Several autophagy modulators have been discovered in the recent past but very few of them have led to potential candidate drug molecules. Many of these compounds are specific toward different targets in the autophagy pathway. For example, specific screens to identify novel candidate molecules such as ULK1 (Rosenberg et al., 2015), ATG4 (Ketteler and Seed, 2008), class III phosphatidylinositol 3-kinase (Farkas et al., 2011), and MTOR (Butcher et al., 2006), have been carried out. In addition, compounds with broad spectrum effects have also been identified as well (Sarkar, 2013b). The scope for the discovery of new autophagy modulators that can be later taken up to clinical trials is ever increasing. It has been postulated that deeper insights into autophagy through chemical modulation can lead to better understanding of various diseases. In addition, understanding of the mechanism of these molecules may provide deeper mechanistic insights and understanding of the finely regulated process of autophagy. Chemical biology approach to study autophagy can be compared to a genetic screen (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Harding et al., 1995; Titorenko et al., 1995), where further studies on the hits reveal more about the mechanism of autophagy. For example, just as the identification of a gene and its function, a manner in which a small molecule modulates autophagy can also shed some light regarding the regulation of autophagy (Seglen and Gordon, 1982; Kunz et al., 1993). In search of potential candidate drugs

TABLE 1 | Autophagy modulators identified through High Throughput Screening of Chemical compound libraries.

Compound name	Autophagy modulation	General/Selective autophagy modulator	Mechanism of autophagy modulation	Reference
ARP101	Inducer	General	Induction of autophagosome biogenesis	Jo et al., 2011
Bay 11	Inhibitor	General	Inhibition of autophagosome biogenesis	Mishra et al., 2017a
BRD5631	Inducer	Aggrephagy/Xenophagy	–	Kuo et al., 2015
Carbamazepine	Inducer	Xenophagy	By myo-inositol depletion and AMPK activation	Schiebler et al., 2015
Cardiac glycosides, e.g., Digoxin, Helveticoside	Inducer	General	Inhibition of Na^+K^+ATPases leading to increase in Ca^{2+} levels	Hundeshagen et al., 2011
KU55933 and Gö6976	Inhibitor	General	Inhibition of PI3K	Farkas et al., 2011
Loperamide	Inducer	Aggrephagy	Regulation of intracellular Ca^{2+} levels	Zhang et al., 2007
P29A03	Inducer	General	Increase in Beclin levels	Lee et al., 2013
P23C07	Inhibitor	General	Inhibition of autophagosomes fusion with lysosomes	Lee et al., 2013
Rottlerin	Inducer	General	Inhibition of mTOR through TSC2 pathway	Balgi et al., 2009
6-Bio	Inducer	Aggrephagy	GSK-3 beta inhibitor	Suresh et al., 2017
Fasudil	Inducer	General	–	Iorio et al., 2010
Flubendazole	Inducer	Xenophagy	Microtubules destabiliser	Chauhan et al., 2015
Minoxidil and clonidine	Inducer	Aggrephagy	Modulation of cAMP levels	Williams et al., 2008
Niclosamide	Inducer	General	Inhibition of mTOR	Balgi et al., 2009
Perhexiline	Inducer	General	Inhibition of mTOR	Balgi et al., 2009
SEN177	Inducer	Aggrephagy	Inhibition of glutaminyl cyclase	Jimenez-Sanchez et al., 2015
SMER10, SMER18, SMER28	Inducer	Aggrephagy	–	Sarkar et al., 2007
Trifluoperazine	Inducer	Aggrephagy/Xenophagy	Increase in FYVE containing vesicles	Zhang et al., 2007
Tamoxifen	Inducer	Xenophagy	Estrogen and G protein coupled receptor GPR30 antagonist shown to inhibit intracellular *Toxoplasma*	Dittmar et al., 2016
Valproic acid	Inducer	Xenophagy	By myo-inositol depletion and AMPK activation	Schiebler et al., 2015
XCT 790	Inducer	Aggrephagy/Xenophagy	ERR alpha inhibitor	Suresh et al., 2018
ZPCK	Inhibitor	General	Inhibition of cargo degradation within lysosomes	Mishra et al., 2017a

that moderate autophagy, identifying small molecule modulators of autophagy is the primary step. Small molecule study will further enhance the understanding of autophagy and related pathways. Thus, having a robust, sensitive assay to monitor autophagic flux that could be performed at a high throughput rate for the purpose of screening modulators of autophagy is of primary importance (**Figure 1**). In this review, we discuss some of the pharmacological strategies undertaken in the recent past to identify novel autophagy modulators (**Table 2**).

CONVENTIONAL AUTOPHAGY ASSAYS

The real time analysis of autophagy in cells tissues principally been performed via qualitative measures. These assays identify autophagosomes or measure the conversion of LC3I to LC3II (Atg8 in yeast) either through western blotting or microscopy (Klionsky et al., 2016). Owing to the conserved nature of autophagy (Mizushima et al., 1998; Kabeya et al., 2000; Meijer et al., 2007), the use of yeast as a model system to study autophagy is still widely recognized, even after the identification of homologous Atg sequences in mammalian cells. This is primarily because of the ease of handling and the vast array of biochemical and genetic tools available to carry out autophagy studies. Several different techniques to monitor autophagy are well established in yeast (Torggler et al., 2017). For example, Pho8Δ60 assay provides readout for bulk autophagy (Noda et al., 1995). Wild type alkaline phosphatase protein moves from ER (inactive) to vacuole where it gets activated. Deletion of first 60 amino acids from the N-terminal makes the mutated protein cytosolic which is taken up by the autophagosome machinery along with other cytosolic contents and delivered to vacuole for bulk degradation. The action of vacuolar proteases activates the Pho8Δ60, which can act on different substrates to dephosphorylate them. Depending on the substrate being used, the readout could be measured using either photometry or fluorimetry.

Other classical assays in yeast include monitoring the degradation of fluorescent tagged Atg8 (GFP-Atg8), either through microscopy or immunoblotting (Kirisako et al., 1999; Suzuki et al., 2001; Meiling-Wesse et al., 2002). Similarly, autophagic degradation of certain different cargoes like PGK1 or radiolabeled long-lived proteins and organelles like peroxisomes (discussed in later sections) and mitochondria can be chased (Tsukada and Ohsumi, 1993; Kissova et al., 2004; Sakai et al., 2006; Welter et al., 2010; Motley et al., 2012).

Although yeast studies provide a reliable and efficient way to study autophagy, considering the complexity in higher eukaryotes, the results cannot be always extrapolated. Keeping the role of autophagy in different physiological and pathological contexts in mind, several different autophagy assays have been developed in cell culture (Tooze et al., 2015; Orhon and Reggiori, 2017). Many of these assays rely on the status of LC3B protein, which is a mammalian homolog of yeast Atg8 protein and is involved in biogenesis and maturation of autophagosome (Kabeya et al., 2000; Mizushima and Yoshimori, 2007; Weidberg et al., 2010; Nguyen et al., 2016). LC3 gets conjugated to phosphatidyl ethanolamine (PE) on the autophagosome membrane and is the sole marker for autophagosomes right from the biogenesis to its degradation. The form of LC3B conjugated to PE is called LC3B II, while the cytosolic, unconjugated form is referred to as LC3B I. This led to development of various LC3 based assays for monitoring the autophagic flux. Other autophagy marker protein widely utilized for the purpose of autophagy assays is p62/SQSTM1, which is an adaptor protein that helps in cargo sequestration (Bjorkoy et al., 2005). Different fluorescent reporters are tagged to these markers (mRFP/GFP-LC3) to visualize them under the microscope (Kabeya et al., 2000). *In vivo* studies have also been conducted in the past using the fluorescently labeled LC3 marker. Mizushima et al., used a transgenic mice model expressing the GFP-LC3 protein to show that autophagy occurs in all the cell types. The basal levels of autophagy vary in different tissues and starvation stimulus induces autophagy over and above the basal levels in all the tissues (Mizushima et al., 2004). Tandem fluorescent tags on these proteins (mRFP/mCherry-GFP-LC3) provide an added benefit of visualizing different stages of autophagic flux (Kimura et al., 2007). This reporter is an indicator of conversion of autophagosomes into autolysosomes upon fusion with lysosomes, wherein the autophagosomes emit both mRFP and GFP signals ($mRFP^+$ GFP^+) whereas the autolysosomes emit only mRFP signal ($mRFP^+$ GFP^-) because GFP is acid-labile and is quenched in the acidic environment of the autolysosomes.

The cytoplasmic autophagic flux of proteins is too small to be chased over a time course using an assay (Welter et al., 2010). The turnover rate of cytosolic proteins through basal autophagy is less and does not provide a broad window or physiological range to carry out a screen using protein degradation as a measure. In turn, having an inducible cargo that is specifically degraded through autophagy provides a higher working range. The inadaptability of the conventional autophagy assays into a high throughput setting presents a major limitation and hence makes the small molecule screening a very cumbersome process (Cheong and Klionsky, 2008; Wang and Subramani, 2017).

HIGH THROUGHPUT ASSAYS TO MONITOR AUTOPHAGY

Multiple aspects and steps of the autophagy pathway have been exploited to establish several different HTS assay systems both in yeast and mammalian cells. These have also led to identification of potent novel autophagy modulators (**Figure 1**). Studies on these modulators have not only revealed their therapeutic potential but led to better understanding of the autophagy process.

Growth Based Autophagy Assays

MTOR is a nutrient sensor and hence is central to cells growth. MTOR also is a regulator of the autophagy pathway (Noda and Ohsumi, 1998; Loewith and Hall, 2011). Rapamycin, an inhibitor of MTOR, activates autophagy pathway (Abraham and Wiederrecht, 1996). This understanding has been widely utilized

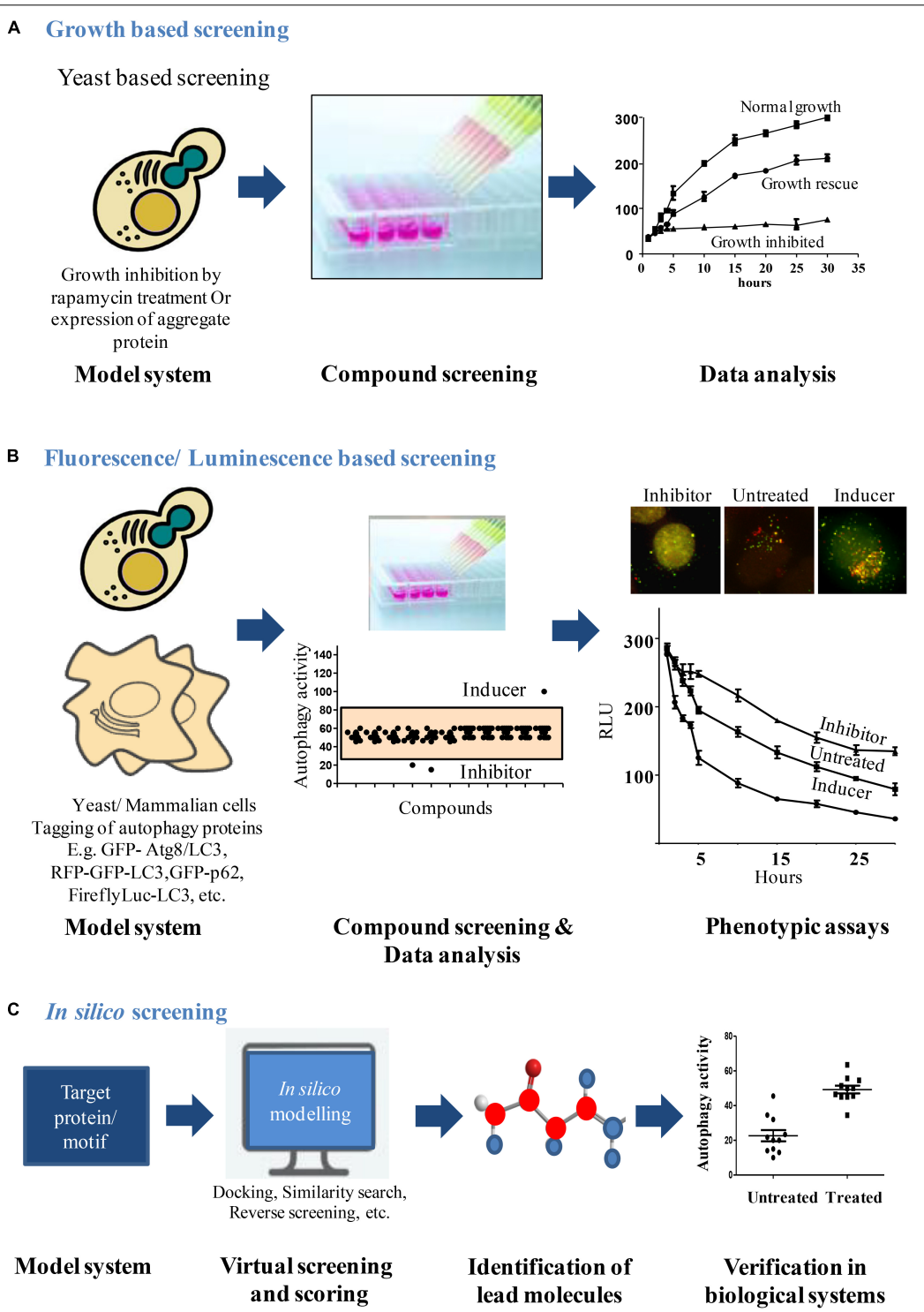


FIGURE 1 | Workflow for screening autophagy modulators. **(A)** Growth based screening: growth inhibition can be induced in yeast due to over expression of aggregate proteins or rapamycin treatment. This cytostatic effect exhibited by yeast can be used as a platform to screen compounds that rescue the growth lag through autophagy induction. After compound treatments, analysis of yeast growth curves identifies the compounds that rescued the growth lag. **(B)** Fluorescence/Luminescence based screening: fluorescent or luminescent reporters are tagged to autophagy proteins for transfection in yeast or mammalian model systems. Modulators of autophagy from chemical libraries are obtained by analyzing the fluorescent/luminescent signal intensities or by visualizing the autophagic vesicle formation by microscopy. **(C)** *In silico* screening: structures of autophagy proteins/motifs of interest can be obtained from data sources like Protein Data Bank and can be used as a model system to identify chemical molecules that bind using *in silico* modeling softwares. The selected lead molecules are then verified in biological system to validate its ability to modulate the process.

TABLE 2 | Summary of HTS assays for compound libraries.

Model system	Assay principle	Read out	Compound(s) identified	Autophagy modulation	Reference
Yeast	Rescue of rapamycin induced growth inhibition	Growth based assay	LY-83583	–	Butcher et al., 2006
	Rescue of rapamycin induced growth inhibition	Growth based assay	SMER 10, 18, and 28	Inducer	Sarkar et al., 2007
	Rescue of rapamycin induced growth inhibition	Growth based assay	SMIRs	Inhibitor	Sarkar et al., 2007
	Rescue of SNCA α-synuclein induced growth lag	Growth based assay	6-Bio	Inducer	Suresh et al., 2017
	Degradation of luciferase tagged peroxisomes	Luminescence	Bay11, ZPCK	Inhibitor	Mishra et al., 2017a
Mammalian cells	Increase in number of autophagosomes (GFP-LC3)	Fluorescence microscopy	ARP101	Inducer	Jo et al., 2011
	Increase in number of autophagosomes and autolysosomes (mCherry-GFP-LC3)	Flow cytometry	Cardiac glycosides	Inducer	Hundeshagen et al., 2011
	Degradation of autophagy adaptor proteins (GFP-p62, GFP-NBR1)	Flow cytometry	Lactacystin	Inhibitor	Larsen et al., 2010
	Reduction in intracellular *Mycobacterium tubercluosis*	Fluorescence microscopy	Valproic acid	Inducer	Schiebler et al., 2015
	Increase in autophagosomes and autolysosomes (mCherry-GFP-LC3)	High-content fluorescent microscopy	Flubendazole	Inducer	Chauhan et al., 2015
	Degradation of lipid droplets	Fluorescence microscopy	P23C07	Inhibitor	Lee et al., 2013
	Ratio of GFP-LC3 (autophagosomes) and cytosolic RFP-LC3ΔG (internal control) using the probe GFP-LC3-RFP-LC3ΔG	High-content fluorescent microscopy and flow cytometry	Deslanoside, Cladribine	Inducer	Kaizuka et al., 2016
	Ratio of GFP-LC3 (autophagosomes) and cytosolic RFP-LC3ΔG (internal control) using the probe GFP-LC3-RFP-LC3ΔG	High-content fluorescent microscopy and flow cytometry	Mebendazole Nelarabine	Inhibitor	Kaizuka et al., 2016
	Clearance of A30P α-synuclein	Fluorescence microscopy	Minoxidil and clonidine	Inducer	Williams et al., 2008
	Quantitation of Nuclear LC3	High-content fluorescent microscopy	NSC179818, NSC60785	–	Kolla et al., 2018
	Degradation of luciferase tagged adaptor protein (Luc2p-p62 and Luc2p-p62ΔU)	Luminescence	Temozolomide	Inducer	Min et al., 2018
	Renilla Luc tagged LC3 turnover	Luminescence	KU55933 and Gö6976	Inhibitor	Farkas et al., 2011
	Comparison of data expression pattern	*In silico* data mining	Fasudil	Inducer	Iorio et al., 2010

to develop assays to monitor autophagy via MTOR activity. Butcher *et al.,* developed an assay that monitored the growth of yeast cells each harboring a different plasmid from a pool of 3900 overexpression plasmids in the presence of rapamycin, which is an inhibitor of MTOR (Butcher et al., 2006). Yeast cells when cultured in the presence of rapamycin, undergo growth inhibition, because of block in TOR pathway. From the pool of overexpression plasmids, candidate gene products were identified that suppressed the cytostatic effect of rapamycin and were involved in the TOR pathway. They also characterized the mechanism of LY-83583. LY-83583 is a novel molecule that suppressed the rapamycin-induced growth inhibition and its several candidate targets were also implicated. Sarkar et al. (2007), used yeast to identify small molecule enhancers (SMERs) and inhibitors (SMIRs) of rapamycin using the same strategy. From the screening, 21 SMIRs and 12 SMERs were listed that were structurally non-redundant. They identified SMERs that could enhance autophagy independently of MTOR, and these SMERs (SMER 10, 18, and 28) when tested in mouse and Drosophila models decreased the toxicity associated with mutant Huntington protein, also reflecting on the therapeutic potential of these compounds (Sarkar et al., 2007). The HTS utilized a chemical genetic suppressor platform to rescue or elevate the growth inhibitory properties of rapamycin on wild type yeast cells (Huang et al., 2004). Therefore, because of the involvement of MTOR pathway in regulating autophagy, a simple screen based on the growth of yeast was able to give therapeutically potent small molecule hits.

A growth-based neurotoxicity assay in yeast was also utilized by Suresh et al., to identify novel autophagy enhancer 6-Bio that ameliorates α-synuclein toxicity. The compound 6-Bio effectively cleared toxic aggregates in an autophagy dependent manner in both yeast as well as mammalian cells. More importantly, the action of the compound was conserved and showed neuroprotection in a pre-clinical mouse model of Parkinson's disease (Suresh et al., 2017).

Fluorescence Based High Throughput Assays

Fluorescence based microscopy assays are the most commonly used techniques to monitor autophagic flux. Autophagy, being a multistep process involving several molecular players, presents with a number of markers that can be tagged with a fluorescent probe and the autophagy rates can be monitored. Interestingly, this has also been exploited to design several high content-based

imaging strategies to screen for novel autophagy modulators (**Figure 1**).

Clearance of toxic poly glutamine aggregates in cell culture was also demonstrated by using autophagy enhancers obtained from an image based HTS of GFP tagged LC3 puncta representing autophagosomes (Zhang et al., 2007). The number, size and intensity of the autophagosomes were analyzed and quantified using high throughput fluorescence microscopy. GFP-LC3 was used as a probe in an automated microscopy cell-based assay to identify chemical enhancers that rapidly led to an increase in autophagosome content (Balgi et al., 2009). The same reporter was also used by Jo et al. (2011), to identify ARP101, that inhibits matrix metalloproteinase-2 (MMP-2) selectively; as an inducer of autophagy- associated cell death in cancer cells. A high content, flow cytometry based screening approach was used to screen Prestwick Chemical Library containing FDA approved drugs by looking at autolysosome formation and degradation and also endolysosomal activities under basal and stimulated autophagy conditions (Hundeshagen et al., 2011). This study used three different probes to investigate different stages of autophagic flux (GFP-LC3 for autophagosome, mCherry-GFP-LC3 for autophagosome and autolysosome) and endocytic activity (GFP-Rab7). From the screening, cardiac glycosides were validated as potent enhancers of autophagic flux. The same GFP-LC3 probe has also been used by Kuo et al. (2015), to screen 59,541 small molecules prepared by stereoselective diversity-oriented chemical synthesis and identification of enhancers of autophagy.

Larsen et al. (2010), followed the degradation of three fluorescent tagged autophagy markers: GFP-p62, GFP-NBR1, or GFP-LC3 by flow cytometry of live cells after their promoter has been turned off. Relative degradation rates of these three promoters was analyzed under basal autophagy conditions. Through single cell analysis, GFP-LC3B was found to be the most stable protein whereas GFP-NBR1 was the reporter that was most effectively degraded. The degradation of GFP-p62 was observed to show the strongest response to nutrient limitation condition and was reported to be the best reporter out of the three. Chemical screening strategies have also been used to identify novel target processes that activate autophagy (Chauhan et al., 2015). In this study, LC3B puncta in HeLa cells stably expressing mRFP-GFP-LC3B were analyzed using high-content (HC) image analysis of and revealed a novel role of microtubules, which when altered resulted in autophagy induction.

Autophagy dependent degradation of lipid droplets (LDs) was also used for the development of a high content screening platform to discover novel autophagy modulators (Lee et al., 2013). In this study, an indolizine-based fluorescent skeleton called Seoul-Fluor (SF) (Kim et al., 2011) that stains the hydrophobic LDs was used and its subsequent degradation via autophagy was followed.

Two anticonvulsants were discovered as mTOR independent autophagy enhancers, from a functional cell-based screening of FDA-approved drugs that were further shown to clear intracellular population of *Mycobacterium tuberculosis* (Schiebler et al., 2015). In this screen, a library of 214 compounds was screened for its ability to kill intracellular luminescent strain of *M. bovis* BCG (bacille-Calmette-Guerin, live attenuated strain of *M. bovis*) within macrophages. These hits were further validated both in primary macrophages and autophagy null cells and also for their effect on autophagy in an mTOR independent manner. The probe GFP-LC3-RFP-LC3ΔG developed by Kaizuka et al. (2016), serves as a cumulative index for autophagy activity. The probe utilizes the protease activity of the ATG4 family of proteins. Upon cleavage of the fusion protein by ATG4, GFP-LC3 gets associated with the autophagosomes and then degraded upon subsequent fusion to lysosomes. RFP-LC3ΔG on the other hand is cytosolic due to the deletion of glycine at the C-terminal of LC3. This probe can be utilized in different settings like high throughput microscopy, flow cytometry and microplate readers and is also amenable to screening small molecule modulators of autophagy by comparing the ratio of GFP/RFP.

A high content screening in HeLa cells using EGFP-LC3 reporter identified several autophagy inhibitors. These compounds were then further analyzed using an array of phenotypic cell-based assays. The screening strategy identified several hitherto unknown target proteins amongst the well defined targets like Vps34 and ULK1 (Peppard et al., 2014). In a first of a kind, a high content screening using the fluorescent LC3 reporter, a library of 1539 chemical compounds was aimed to identify modulators that affected the nuclear localization of LC3. Potent modulators were identified that may help in the understanding of LC3 nuclear-cytoplasmic localization (Kolla et al., 2018).

Parkinson's disease associated protein A30P α-synuclein is a substrate for autophagy and has been used to study aggregate clearance by autophagy in the past. One such study used A30P α-synuclein clearance by autophagy as a primary screen to identify novel autophagy enhancers. Using this screen L-type Ca^{2+} channel antagonists, the K^{+}_{ATP} channel opener minoxidil, and the G_i signaling activator clonidine were identified as autophagy inducers that work independent of MTOR. This important discovery revealed that MTOR is dispensable for autophagy induction. The authors showed that cAMP can modulate autophagy by controlling IP3 activity (Williams et al., 2008). As MTOR is central to several other pathways as well, identification of an alternative pathway opened the scope of controlling autophagy independent of MTOR.

Luminescence Based High Throughput Assays

Luciferase being a sensitive reporter protein comes in handy when an assay has to be scaled to a high throughput format (**Figure 1**). Availability of different luciferase variants further helps in the design of an assay according to the needs. These luciferase variants have different degrees of sensitivity (Nanoluc is more sensitive to Firefly luciferase), different sizes and spectra (*Renilla* luciferase is smaller in size to Firefly luciferase) or different properties (Gaussian luciferase is secretory in nature while Renilla luciferase is cytosolic and Firefly luciferase naturally has a peroxisomal targeting signal). Depending on the need of the assay and the process to be studied, an appropriate luciferase

variant may be used in the study. A *Gaussian* luciferase reporter based assay that quantitatively measures the autophagy rate by monitoring proteolytic activity of ATG4B, can be done at a large scale and is quantifiable (Ketteler and Seed, 2008). This luciferase release assay is well suited for upstream signaling events that either increase or decrease the rates of autophagy. A luciferase-based assay that exploited the property of long lived proteins to be solely degraded via autophagy pathway provided a direct relevance of the autophagy modulation in aggregate prone cells. This assay demonstrated autophagic clearance of an expanded polyglutamine *in vitro* and *in vivo* conditions (Ju et al., 2009). This assay takes into account the selective degradation of autophagy cargo using a sensitive luciferase-based reporter. Dynamic and sensitive assay could be achieved by following the cargo that is selective for degradation through autophagy. Peroxisomes provide highly inducible cargo with high turnover rates which are specifically degraded through autophagy machinery under starvation conditions (Sakai et al., 2006). This high turnover of peroxisomes when combined to the sensitivity of luciferase reporter, provides a very sensitive assay to monitor autophagic flux which is also amenable to high throughput setting. Based on this principle, Mishra et al., designed a screening strategy that allows measurement of autophagic cargo (facultative organelle, peroxisomes) clearance rather than ATG8 based changes in autophagosome number. The principle of the assay is based on detection of the levels of firefly and *Renilla* luciferase activities to monitor the flux of selective and general autophagy, respectively, in *S. cerevisiae* (Mishra et al., 2017b). Reporter strains were constructed that expressed Renilla luciferase and firefly luciferase with a peroxisome targeting signal (PTS1) under a fatty acid responsive promoter. These cells when grown in the presence of fatty acid or glycerol containing media, leads to the expression of peroxisomal firefly luciferase and *Renilla* luciferase which is cytosolic. These cells are then subjected to starvation to induce autophagy. Induction of autophagy leads to selective autophagic degradation of peroxisomes (pexophagy) and also non-selective bulk degradation of cytoplasm. The rate of decay in firefly luciferase activity depicts pexophagy whereas *Renilla* levels depict general autophagy. The dual luciferase assay provides the added advantage of monitoring autophagy in real time, is more sensitive and gives kinetic assessment of two different types of autophagy processes simultaneously. Interestingly, the action of the autophagy modulators identified from the screen was conserved across higher eukaryotes (Mishra et al., 2017a). The autophagy inhibitor Bay11 identified from the screen acted at the autophagosome biogenesis step and ZPCK inhibited the degradation of cargo inside the vacuole/lysosome. These inhibitors had a conserved mode of action across yeast, animals and plants.

Luciferase based HTS autophagy assay has been reported for mammalian cells as well. In a study by Min et al. (2018), a luciferase variant Luc2p was fused with the wild type p62/SQSTM1 or a deletion version of p62 (p62 lacking the ubiquitin binding domain) and transfected into glioma cells. The lysates from the two populations (wild type and mutant p62) were compared to monitor the autophagic flux. The performance of this probe was reported to be comparable to GFP-LC3-RFP-LC3ΔG probe described earlier in the review (Min et al., 2018).

In vitro and *in silico* Assays

In recent years, many groups have also carried out a target driven autophagy screen using purified proteins and substrates. To identify substrates for ULK1 that might be involved in the process of autophagy, Egan et al. (2015), screened degenerate peptide libraries to identify a consensus motif for ULK1 mediated phosphorylation. After identifying novel phosphorylation sites, multiple targets for ULK1 were discovered. These substrates were then used to screen for potent inhibitors of ULK1 phosphorylation.

Renilla luciferase based turnover of LC3 was used to screen two kinase inhibitor libraries for identifying inhibitors of autophagic flux (Farkas et al., 2011). This study identified specific and more potent inhibitors of the upstream signaling component; class III phosphatidylinositol 3-kinase. Inhibitors specific to Ulk1 kinase activity, an upstream protein involved in autophagy initiation were obtained from a screen that utilized purified stress-activated Ulk1 and then looked at the phosphorylation of its substrate, Atg13 at Serine 318 position (Rosenberg et al., 2015).

Iorio et al. (2010) used the large dataset of drug expression pattern integrated into "drug network" and identified the previously hitherto unknown functions of several well characterized drugs. This is a dataset of expression profiles constructed while comparing the transcriptional responses induced by different small molecules in human cell lines. Through data mining, they identified fasudil as a novel autophagy enhancer taking the help of the same drug network (Iorio et al., 2010).

DISCUSSION

Although the core autophagy machinery and the proteins involved in disease conditions might be known, but the exact mechanism of action and how the autophagic flux is regulated is not completely understood which leads to many unanswered questions. Understanding and controlling the autophagic flux either through a genetic or pharmacological approach is a highly promising approach and of great scientific interest. Studies with genetic modulations of autophagic flux have been carried out in the past with immense success. Yoshinori Ohsumi, a pioneer in autophagy field was awarded the Nobel Prize in 2016 for his contribution to the study of autophagic flux. However, chemical modulation has an advantage over genetic manipulations that the phenotype could be observed just on the addition of the compound and the action could be reversed on its withdrawal. The method is less laborious, and the putative modulators could be used as leads for pharmacological purposes in certain disease conditions. However, there are limitations associated with the chemical approach because of the bioavailability issues, toxicity and the secondary or off-target effects associated with the chemical compound. Also, tissue specific effects are difficult to monitor.

To identify novel small molecule modulators of autophagy having a robust and sensitive screening system is the primary step. Therefore, HTS assays for autophagy are of utmost importance as they enable us to screen several small molecules in a small space of time with the inclusion of all possible biological and technical replicates. The data obtained from these assays should be amenable for direct comparison between the control and test groups and statistical analysis. Several high throughput assays have been developed in the recent past to identify small molecule modulators of autophagy. But some limitations associated with these assays must be overcome for a highly potent and effective HTS assay system. Many of these assays have issues with sensitivity and range. They do not directly look at the cargo or possess a higher physiological working range to detect smaller changes in autophagic flux. Although these assays are quantitative but may lack in one of the many parameters required to attain an ideal autophagy assay. An ideal assay would incorporate all these properties such as cargo build up, high sensitivity, ease of experimentation, broader physiological range, and live cell readout in a single high throughput format. Dynamic, sensitive and highly effective assay could be achieved by following the cargo that is inducible and selective for degradation through autophagy.

AUTHOR CONTRIBUTIONS

PM and RM conceived the idea and wrote the manuscript. VA conceptualized and contributed to the figure.

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REVIEW
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Chemical Screening Approaches Enabling Drug Discovery of Autophagy Modulators for Biomedical Applications in Human Diseases

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Autophagy is an intracellular degradation pathway for malfunctioning aggregation-prone proteins, damaged organelles, unwanted macromolecules and invading pathogens. This process is essential for maintaining cellular and tissue homeostasis that contribute to organismal survival. Autophagy dysfunction has been implicated in the pathogenesis of diverse human diseases, and therefore, therapeutic exploitation of autophagy is of potential biomedical relevance. A number of chemical screening approaches have been established for the drug discovery of autophagy modulators based on the perturbations of autophagy reporters or the clearance of autophagy substrates. These readouts can be detected by fluorescence and high-content microscopy, flow cytometry, microplate reader and immunoblotting, and the assays have evolved to enable high-throughput screening and measurement of autophagic flux. Several pharmacological modulators of autophagy have been identified that act either via the classical mechanistic target of rapamycin (mTOR) pathway or independently of mTOR. Many of these autophagy modulators have been demonstrated to exert beneficial effects in transgenic models of neurodegenerative disorders, cancer, infectious diseases, liver diseases, myopathies as well as in lifespan extension. This review describes the commonly used chemical screening approaches in mammalian cells and the key autophagy modulators identified through these methods, and highlights the therapeutic benefits of these compounds in specific disease contexts.

Keywords: autophagy, autophagy reporter, autophagy substrate, autophagy modulator, screening method, neurodegenerative diseases, cancer, lifespan extension

INTRODUCTION

Macroautophagy, herein referred to as autophagy, is an intracellular degradation process essential for ensuring cellular homeostasis. This well-conserved catabolic process mediates the targeted degradation of unwanted or excess cytoplasmic materials, such as aggregation-prone proteins, pathogens and damaged organelles like mitochondria, amongst others (Ravikumar et al., 2010). This process is also involved in the bulk degradation of cytoplasmic macromolecules and recycling of the breakdown products especially during nutrient deprivation to provide energy homeostasis, thereby forming a crucial connection between anabolism and catabolism (Boya et al., 2013; Kaur and Debnath, 2015). Due to its vital function as a homeostatic regulator, impairment of the autophagy is implicated in several human pathologies including certain cancer, metabolic syndromes, infectious diseases, liver diseases, myopathies, aging and neurodegenerative disorders (Mizushima et al., 2008). Therefore, therapeutic modulation of autophagy holds great potential in the development of treatment strategies for these diseases (Rubinsztein et al., 2012).

Autophagy is evolutionarily-conserved from yeast to humans. The *de novo* formation of phagophores, the double-membrane structures that expand to form double-membrane vesicles called autophagosomes, require multiple autophagy-related (*Atg*) genes in the autophagic machinery, such as the Atg5-Atg12-Atg16 complex and the phosphatidylethanolamine-conjugated microtubule-associated protein 1 light chain 3 (LC3-II) (Kabeya et al., 2000; Mizushima et al., 2011; Ktistakis and Tooze, 2016). Maturation of autophagosomes into the degradative autolysosomes occurs either via the multi-step route involving the fusion of autophagosomes with late endosomes to form amphisomes which subsequently fuse with the lysosomes, or via the direct route involving the fusion between autophagosomes and the lysosomes (Nakamura and Yoshimori, 2017). The autophagic cargo engulfed by the autophagosomes are ultimately degraded in the acidic autolysosomes by the lysosomal hydrolases, which are only active at the low pH maintained by the vacuolar-type H^+-ATPase (V-ATPase) on the lysosomal membrane (Saftig and Klumperman, 2009). Finally, the breakdown products are recycled and utilized as inputs to cellular metabolism for energy generation (Rabinowitz and White, 2010). The rate at which this dynamic turnover of cellular contents occurs through the process of autophagy is referred to as autophagic flux. Autophagic flux encompasses all stages of autophagy which includes autophagosome formation, fusion with the lysosomes and cargo degradation in the autolysosomes (**Figure 1**).

Key upstream modulators of autophagy include the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which promotes cellular biosynthesis and inhibits autophagy (Saxton and Sabatini, 2017). Regulation of autophagosome formation by mTORC1 is mediated via the ULK1–Atg13–FIP200 complex; mTORC1 suppresses autophagy under nutrient-rich conditions by phosphorylation-dependent inactivation of ULK1 and Atg13 (Mizushima, 2010; Zachari and Ganley, 2017). Various signals such as growth factors and nutrients impinge on mTORC1 to negatively influence autophagy (Kim and Guan, 2015). Conversely, during nutrient starvation, autophagy is promoted by inhibition of the mTORC1 activity (Carroll et al., 2014; Russell et al., 2014). Furthermore, ULK1 can be directly phosphorylated and activated by the energy sensor AMPK to stimulate autophagy (Egan et al., 2011; Kim et al., 2011). In addition, several mTORC1-independent pathways have been described where autophagy is negatively regulated by the elevation in intracellular inositol, Ca^{2+} and nitric oxide levels, amongst others (Sarkar, 2013b). Molecular mediators of the late stage of autophagy involving autophagosome maturation include Rab7, SNAREs (N-ethylmaleimide-sensitive factor-attachment protein receptors), GABARAPs, BRUCE and Beclin1-interacting partners such as Atg14L, UVRAG and Ambra1 (He and Levine, 2010; Nguyen et al., 2016; Wang et al., 2016; Reggiori and Ungermann, 2017; Ebner et al., 2018). At a transcriptional level, autophagy is governed by the transcription factor EB (TFEB) (Settembre et al., 2011), which in itself is activated by lysosomal Ca^{2+} (Medina et al., 2015).

Chemical modulation of autophagy by targeting the mTOR-dependent and mTOR-independent pathways has proven to be of potential biomedical relevance due to therapeutic advantages, especially in neurodegenerative disorders as well as in diverse human pathological conditions such as in certain liver diseases, myopathies, infectious diseases, metabolic diseases, cancer and aging (Rubinsztein et al., 2012; Sarkar, 2013b; Levine et al., 2015). Hence, the discovery of potent small molecules regulating autophagy is of great interest. Here we review the chemical screening strategies for autophagy drug discovery, and highlight the potential benefits of autophagy modulators in human diseases.

CHEMICAL SCREENING STRATEGIES FOR IDENTIFYING AUTOPHAGY MODULATORS

A number of *in vitro* screening methods have been designed for identifying compounds (Sarkar, 2013a; Joachim et al., 2015; Seranova et al., 2019). The assays are primarily based on the perturbations of autophagy reporters or autophagy cargoes as readouts (**Figure 1**), which can be measured via fluorescence or high-content imaging, immunoblotting, flow cytometry and microplate reader (Mizushima et al., 2010; Klionsky et al., 2016; **Figure 2** and **Table 1**). Some of these screening methods can be subjected to high-throughput applications. Below are descriptions of the common screening approaches in mammalian cells, and the identification and therapeutic benefits of key autophagy modulators.

CHEMICAL SCREENING METHODS BASED ON AUTOPHAGY REPORTERS

Screening methods based on autophagy reporters are the most commonly used approaches to detect changes in the numbers of autophagosomes and autolysosomes (**Table 1**). The protein

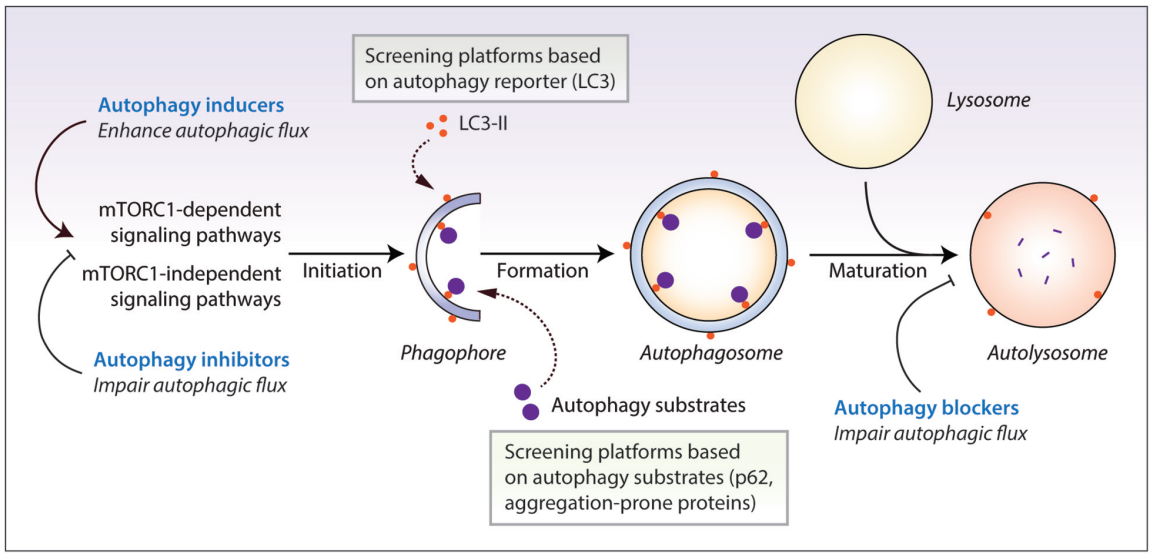


FIGURE 1 | Autophagy reporter and substrate based screening strategies and the impact of autophagy modulators at different stages of the autophagy process. Autophagy is regulated by the mechanistic target of rapamycin complex 1 (mTORC1) or mTORC1-independent pathways. This process initiates by the formation of phagophores that expand and engulf autophagy substrates to form autophagosomes, which then fuse with the lysosomes to form autolysosomes where the autophagic cargo is degraded. Autophagy inducers and inhibitors increase or decrease autophagosome formation, respectively, at the early stages of autophagy, whereas autophagy blockers prevent lysosomal degradation and/or autophagosome maturation at late stages of autophagy. Autophagic flux is thus enhanced by autophagy inducers but is retarded by autophagy inhibitors and blockers. Chemical screening methods for identifying autophagy modulators are commonly based on the readouts of perturbations in autophagy reporters such as LC3-II, or autophagy substrate clearance such as aggregation-prone proteins or p62/SQSTM1.

TABLE 1 | Chemical screening methods for identifying autophagy modulators in mammalian cells.

Autophagy screening assays	Detection methods	Strengths	Limitations
GFP-LC3	Fluorescence or high-content microscopy	(1) Simple readout easy to detect	(1) Can not distinguish between autophagy inducer and blocker
		(2) High-throughput application	(2) Can not assess overall autophagic flux
mRFP-EGFP-LC3	Fluorescence or high-content microscopy	(1) Can distinguish between autophagy inducer, inhibitor and blocker	(1) Assay depends on proper acidification of the lysosomes that can be affected by lysosomotrophic agents
		(2) Measures autophagosome flux	(2) Can not precisely assess overall autophagic flux as it does not measure cargo clearance.
		(3) High-throughput application	
GFP-LC3-RFP-LC3ΔG	Fluorescence or high-content microscopy, Flow cytometry, Microplate reader	(1) Measures overall autophagic flux	(1) Can not distinguish between autophagy inhibitor and blocker
		(2) Versatile detection methods	(2) Homologous recombination of two LC3 sequences could result in non-degradable GFP-LC3ΔG
		(3) High-throughput application	
Inducible p62-fLuc or GFP-p62	Microplate reader, Flow cytometry	(1) Measures clearance of autophagic cargo indicating overall autophagic flux	(1) Can not distinguish between autophagy inhibitor and blocker
		(2) Possible high-throughput application	(2) Transcriptional changes in leaky p62 transgene could affect readout
Inducible EGFP-HDQ74 or HA-α-syn$^{(A53T)}$	Immunoblotting	(1) Measures clearance of autophagic cargo indicating overall autophagic flux	(1) Can not distinguish between autophagy inhibitor and blocker
			(2) High-throughput analysis not possible

The detection methods, strengths and limitations of the autophagy reporter and substrate based screening assays are highlighted.

reporter that is widely used to study autophagy is microtubule-associated protein 1 (MAP1) light chain 3 (LC3). The nascent LC3 is cleaved at its C-terminal arginine residue by Atg4 to form the cytoplasmic LC3-I, which is then post-translationally conjugated with phosphatidylethanolamine at its C-terminal glycine residue by Atg7 to form the autophagosome-associated LC3-II (Kabeya et al., 2000). The lipidated LC3-II remains associated to the autophagosomes throughout their lifespan, and is present on

both the outer and inner membranes. Following the maturation of autophagosomes with lysosomes to form autolysosomes, the LC3-II on the inner surface is degraded whereas the LC3-II on the outer surface is delipidated and removed by Atg4B for recycling (Tanida et al., 2004). A number of fluorescent-tagged reporters of LC3, such as GFP-LC3 (Kabeya et al., 2000), mRFP-GFP-LC3 (Kimura et al., 2007) and GFP-LC3-RFP-LC3ΔG (Kaizuka et al., 2016), have been used to study autophagy and undertake chemical screening.

Identification of Autophagy Modulators by GFP-LC3 Screening Method

The most common LC3-based reporter that has been used in several studies is GFP-LC3, which labels autophagosomes, autolysosomes as well as phagophores (Kabeya et al., 2000). For the GFP-LC3 screening method, image-based analysis is done by quantifying the GFP^+ puncta per cell to measure perturbations in autophagosome number. In general, an autophagy inducer as well as an autophagy blocker will increase GFP-LC3 puncta whereas an autophagy inhibitor will decrease GFP-LC3 puncta (**Figure 2**). A number of high-throughput and small-scale screens have been undertaken with this strategy that has been also utilized to assess the key hits arising from other screening methods; and some of the primary chemical screens utilizing GFP-LC3 readout are highlighted below.

Using GFP-LC3 as the primary screening method in a stable human glioblastoma H4 cell line, an image-based chemical screen with 480 bioactive compounds was performed wherein the number, size and intensity of GFP-LC3 spots were taken into consideration while selecting potent autophagy modulators (Zhang et al., 2007). Compounds were treated at 3–12 μM concentrations for 24 h. This screen identified 8 autophagy inducers, which included a number of FDA-approved drugs such as fluspirilene, trifluoperazine, pimozide (antipsychotic drugs), niguldipine, nicardipine, amiodarone (drugs used for cardiovascular conditions) and loperamide (used in diarrhea). While fluspirilene, trifluoperazine are dopamine antagonists, the other drugs are Ca^{2+} channel antagonists that lower intracellular Ca^{2+}; all of which induced

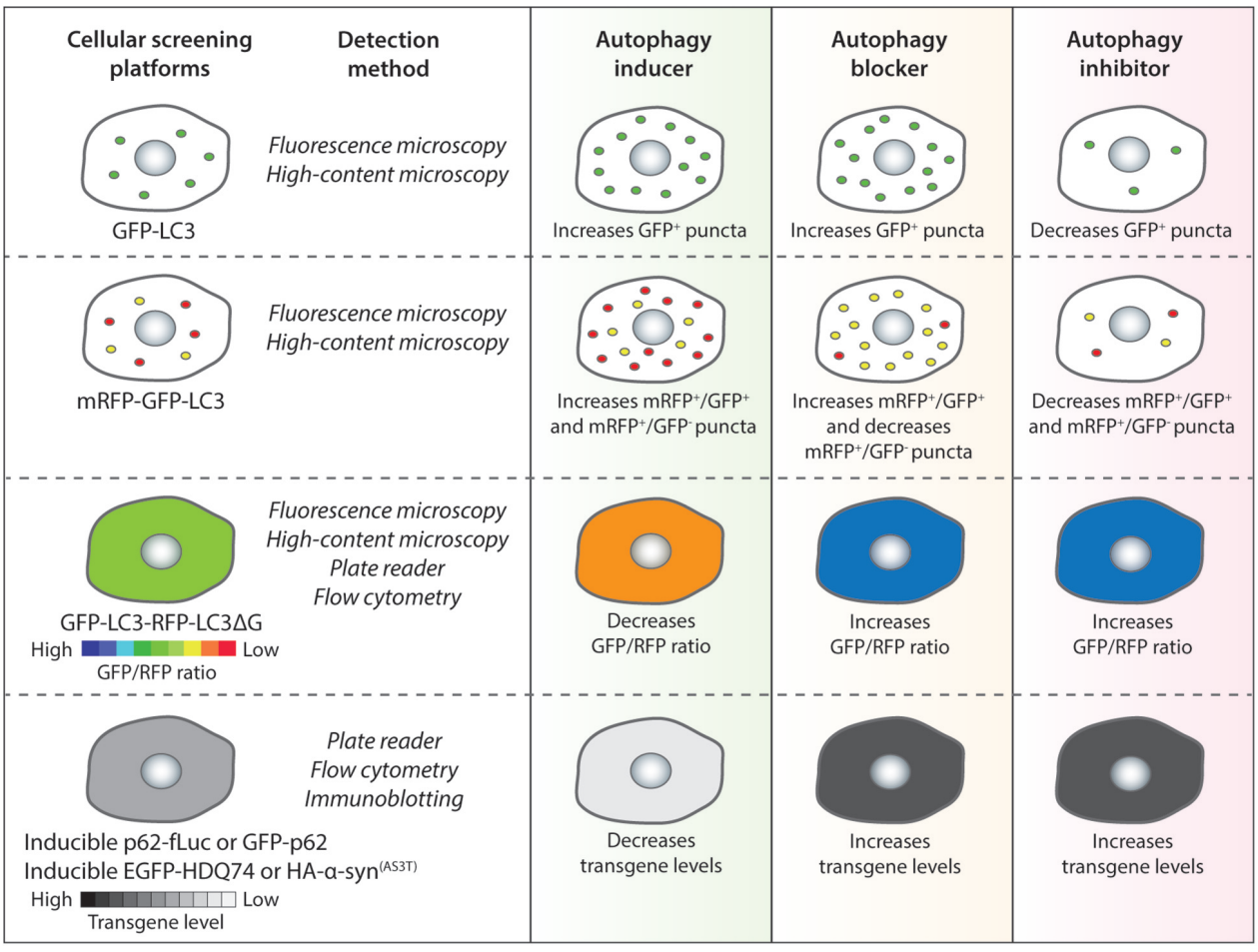


FIGURE 2 | Autophagy chemical screening strategies in mammalian cells. Chemical screening methods that are commonly used for identifying autophagy modulators are based on autophagy reporters (LC3) or autophagy substrates (p62 or aggregation-prone proteins). The detection methods for the respective assays and the expected readouts for autophagy inducers, blockers or inhibitors are indicated as a general guidance.

autophagy independently of mTOR (Zhang et al., 2007). Another image-based chemical screen was performed with a library of 3584 pharmacologically active compounds in human breast cancer MCF-7 cells stably expressing GFP-LC3 (Balgi et al., 2009). Treatment of compounds was done at ∼15 μM concentration for 4 h. This screen identified 3 FDA-approved drugs such as perhexilene, niclosamide and amiodarone, as well as rottlerin, as autophagy inducers; all of which were shown to inhibit mTORC1 (Balgi et al., 2009). However, other screens have reported amiodarone (Ca^{2+} channel antagonist) to act independently of mTORC1 for inducing autophagy at a much lower dose than what is required to inhibit mTORC1 (Williams et al., 2008); and likewise, perhexilene is a Ca^{2+} channel blocker that could be also mTOR-independent. Furthermore, one of the largest chemical screens for identifying autophagy modulators was undertaken in HeLa cells stably expressing GFP-LC3 with 59541 stereochemically and skeletally diverse compounds derived from diversity-oriented synthesis (Kuo et al., 2015). Compounds were treated for 4 h in 8-point dose with a maximal concentration of 10 μM. Several hits were subjected to a secondary screen at 10 μM concentration from which BRD5631 was identified as the potent autophagy inducer along with other hits like BRD2716 and BRD34009; all of which did not affect mTOR activity. Interestingly, the hit rate in the primary screen for compounds having an alkyl amine was higher than that for all of the compounds. This effect was augmented by the additional presence of a single lipophilic group, such as diphenyl alkyne, biphenyl, cyclohexane or naphthalene (Kuo et al., 2015). While the above screens were undertaken in immortalized human cell lines, another chemical screen was done with 1280 pharmacologically active compounds in mouse embryonic fibroblasts (MEFs) stably expressing GFP-LC3 (Li et al., 2016). Compounds were treated at 0.02–46 μM concentrations for 16 h in the presence or absence of chloroquine (autophagy blocker) to determine their effects on autophagic flux. Out of the 27 autophagy inducers identified, few were characterized further. These include anti-psychotic drugs such as indatraline hydrochloride (dopamine inhibitor), chlorpromazine hydrochloride and fluphenazine dihydrochloride (dopamine receptor antagonists). Fluphenazine was found to inhibit mTORC1 whereas indatraline and chlorpromazine were mTOR-independent (Li et al., 2016).

Although GFP-LC3 is a straightforward, widely-used screening assay, its inability to distinguish between autophagosomes and autolysosomes is a major inadequacy of this reporter. Accumulation of autophagosomes can occur either due to induction of autophagosome formation (by autophagy inducers) or due to block in autophagosome maturation (by autophagy blockers) in the early and late stages of autophagy, respectively (Rubinsztein et al., 2009). Since autophagy is a dynamic, multi-step process, it is imperative to measure autophagosome flux in order to assess the status of autophagy. Therefore, the hits from the primary GFP-LC3 screen are subjected to rigorous secondary assays (such as autophagosome formation and maturation, and autophagic substrate clearance, amongst others) (Mizushima et al., 2010; Klionsky et al., 2012) for characterizing autophagy modulators.

Identification of Autophagy Modulators by mRFP-GFP-LC3 Screening Method

In order to overcome the problem of the GFP-LC3 reporter, a tandem fluorescent-tagged mRFP-GFP-LC3 reporter can be employed to determine autophagosome maturation for distinguishing between the autophagosomes and the autolysosomes. This mRFP-GFP-LC3 reporter is pH-sensitive. When overexpressed in cells, the autophagosomes exhibit both mRFP and GFP signals, whereas the autolysosomes emit only mRFP signal because the acid-labile GFP signal is quenched in the acidic environment (Kimura et al., 2007). For the mRFP-GFP-LC3 screening method, image-based analysis is done by quantifying the $mRFP^+$ and GFP^+ puncta per cell to measure perturbations in the number of autophagosomes ($mRFP^+/GFP^+$) and autolysosomes ($mRFP^+/GFP^-$). In general, an autophagy inducer (acting at early stage) will increase autophagosomes and autolysosomes, an autophagy inhibitor (acting at early stage) will decrease both these compartments, whereas an autophagy blocker (acting at late stage) will increase autophagosomes and decrease autolysosomes (**Figure 2**). Alternative versions of the mRFP-GFP-LC3 reporter have been described that may provide better readouts. These include replacing mRFP with mCherry that has superior photostability over mRFP (Pankiv et al., 2007), and substituting GFP with mWasabi that is more acid-sensitive than GFP (Zhou et al., 2012).

This pH-sensitive reporter has been primarily utilized as a secondary screening strategy following primary screens utilizing the more simpler GFP-LC3 method. In a high-throughput screen with 59541 compounds in GFP-LC3 platform, 400 screen hits were subjected to additional screening in stable HeLa cells expressing mCherry-GFP-LC3 (Kuo et al., 2015). These compounds were treated at 10 μM concentration for 24 h, after which 250 compounds increased (putative inducers) and 80 compounds decreased (putative inhibitors/blockers) the number of $mCherry^+/GFP^-$ autolysosomes. Following further characterization, potent mTOR-independent autophagy inducers identified were BRD5631, BRD2716, and BRD34009 (Kuo et al., 2015). In another study, HeLa cells stably expressing mRFP-GFP-LC3 was subjected to three drug libraries such as the Prestwick Chemical Library, Microsource Spectrum 2000 library and Johns Hopkins Library that encompass 3791 compounds including FDA-approved drugs and bioactive molecules (Chauhan et al., 2015). Compounds were treated at 10 μM concentration for 4 h. However, high-content image analysis was done based only on GFP-LC3 puncta and total integrated area per cell, but not together with mRFP-LC3 that was utilized later during secondary characterization. 80 compounds were identified, out of which 55 were novel and 25 were previously reported as autophagy modulators. Further characterization of the hits including the mRFP-GFP-LC3 analysis identified flubendazole as a novel autophagy inducer that is also an antihelminthic drug. Flubendazole was shown to impact on dynamic and

acetylated microtubules to inhibit mTOR and disrupt Bcl2-Beclin 1 complex for inducing autophagy (Chauhan et al., 2015). More recently, a primary screen with mRFP-GFP-LC3 has been performed in U343 glioma cell spheroids (3D tumor spheroids) by dynamic live-cell imaging (Pampaloni et al., 2017). A subset of the Enzo Life Sciences Screen-Well Natural Compounds library comprising of 94 compounds were used at 1, 12.5, and 50 μM concentrations, followed by long-term time-lapse fluorescence imaging over 24 h at an interval of 1 h. Instead of measuring puncta formation, this study quantified the readout based on the ratio of mRFP and GFP emission intensities over time. Apart from validating this approach with the Enzo Life Sciences Screen-Well Autophagy library consisting of known autophagy modulators, the screen with selected natural compounds identified six potent autophagy inducers and four inhibitors. The autophagy-inducing natural compounds include PI-103, nonactin, valinomycin, quercetin, ivermectin, and harmine (Pampaloni et al., 2017).

The mRFP-GFP-LC3 reporter or its alternative versions can be subjected to high-throughput image-based screens to analyse autophagosome flux. This assay requires proper acidification of the lysosomes that could be affected by lysosomotrophic agents. However, autophagic substrate clearance along with other secondary assays should be assessed following the primary screen in order to assess the overall autophagic flux.

Identification of Autophagy Modulators by GFP-LC3-RFP-LC3ΔG Screening Method

A novel autophagy probe, GFP-LC3-RFP-LC3ΔG, has been recently developed for evaluating autophagic flux that can be used for high-throughput screening approaches (Kaizuka et al., 2016). When overexpressed in cells, the Atg4 family proteases can cleave this reporter into equimolar amounts of GFP-LC3 and RFP-LC3ΔG. While GFP-LC3 on the autophagosomes is degraded or recycled after fusion with the lysosomes, RFP-LC3ΔG cannot be lipidated due to a deletion in its C-terminal glycine and thus remains in the cytosol serving as an internal control. This GFP-LC3-RFP-LC3ΔG reporter can be subjected to both qualitative (by ratiometric imaging via fluorescence microscopy) and quantitative (via microplate reader or flow cytometry) analyses by measuring the fluorescence of GFP-LC3 and RFP-LC3ΔG, and then calculating the GFP/RFP ratio (Kaizuka et al., 2016). Autophagy inducers are expected to decrease GFP/RFP ratio by enhancing autophagic flux, whereas autophagy inhibitors or blockers will increase GFP/RFP ratio by reducing autophagic flux (**Figure 2**).

Two chemical screens employing the GFP-LC3-RFP-LC3ΔG screening method have been undertaken using a selected library of 34 known autophagy-regulating compounds and 1054 approved drugs under basal or starvation conditions in HeLa cells stably expressing this reporter (Kaizuka et al., 2016). The GFP/RFP ratio was calculated from fluorescence measurement via a microplate reader. For the first screen with known autophagy-regulating compounds, cells were treated for 6, 12 or 24 h with concentrations previously shown to modulate autophagy. A number of known autophagy modulators, but not all, acted as expected primarily after 12 or 24 h treatment. Specifically, autophagy inducers such as rapamycin (Blommaart et al., 1995) and Torin 1 (Thoreen et al., 2009) decreased GFP/RFP ratio whereas autophagy blockers like bafilomycin A1 (Yamamoto et al., 1998) and chloroquine (Seglen et al., 1979) increased GFP/RFP ratio (Kaizuka et al., 2016). For the second screen with approved drug library, cells were treated for 24 h at 10 μM concentration with few exceptions at 5 μM. The screen hits included 47 autophagy-inducing drugs (comprising of certain anti-cancer drugs, antibiotics and cardiotonic drugs) and 43 autophagy inhibitory drugs. Although many of these hits were previously reported, 13 inducers and 18 inhibitors/blockers were identified as novel autophagy modulators, of which some of the novel autophagy inducers were adefovir pivoxil, methyltestosterone, norethisterone, oxaprozin, and zidovudine (Kaizuka et al., 2016). This GFP-LC3-RFP-LC3ΔG probe has been demonstrated to be capable of measuring basal and induced autophagic flux in Zebrafish and in tissues of transgenic mice (Kaizuka et al., 2016), and is thus valuable for monitoring autophagic flux *in vivo*.

Although this reporter can be used for high-throughput applications and *in vivo* studies to measure the overall autophagic flux, it is not ideal for investigating the distinct stages of autophagy such as autophagosome formation and maturation. Importantly, the two LC3 sequences of GFP-LC3-RFP-LC3ΔG in retrovirally transfected cells can undergo homologous recombination, which will generate GFP-LC3ΔG that is incapable of being degraded by autophagy. In addition, the expression levels of this reporter define the accuracy of the readout, and hence analysis in different cell lines or tissues will require comparable expression (Kaizuka et al., 2016; Geng and Klionsky, 2017).

CHEMICAL SCREENING METHODS BASED ON AUTOPHAGY SUBSTRATES

In addition to the screening approaches based on LC3 reporters, autophagy substrate clearance has also been utilized as a primary screening assay for identifying autophagy modulators (**Table 1**). This method measures the autophagic cargo flux, which together with LC3-based secondary assays for autophagosome flux can indicate the overall autophagic flux.

Identification of Autophagy Modulators by Clearance of Aggregation-Prone Proteins

A number of neurodegeneration-associated aggregation-prone proteins are predominantly degraded by autophagy (Menzies et al., 2017), and hence screening methods can be based on their clearance as readouts (Sarkar, 2013a). The well-established substrates undergoing autophagic degradation include mutant huntingtin (with expanded polyglutamine repeats) and mutant α-synuclein (A53T or A30P mutants) associated with Huntington's and Parkinson's disease,

respectively (Webb et al., 2003; Ravikumar et al., 2004). Since the steady-state level of proteins is not ideal for accurately reflecting any impact on their degradation, stable inducible cell lines are required for analyzing autophagic substrate clearance where the transgene product is temporally synthesized by doxycycline followed by treatment with compounds after the expression is turned off (Wyttenbach et al., 2001; Webb et al., 2003; Sarkar et al., 2009). In general, autophagy inducers will enhance the clearance of aggregation-prone proteins, whereas autophagy inhibitors or blockers will retard their clearance (**Figure 2**).

Independent studies using a stable inducible PC12 cell line expressing EGFP-tagged mutant huntingtin (EGFP-HDQ74) identified mTOR-independent autophagy inducers such as trehalose (Sarkar et al., 2007a) as well as inositol-lowering agents (lithium, carbamazepine, valproic acid, L-690330) (Sarkar et al., 2005) and nitric oxide synthase inhibitors (L-NAME) (Sarkar et al., 2011). These studies also identified autophagy inhibitory compounds such as agents increasing inositol or inositol 1,4,5-trisphosphate (IP_3) levels (myo-inositol, prolyl endopeptidase inhibitor 2) (Sarkar et al., 2005) and nitric oxide donors (DEA NONOate, DETA NONOate) (Sarkar et al., 2011). Utilizing stable inducible PC12 cell line expressing hemagglutinin (HA)-tagged A53T α-synuclein (HA-α-syn$^{(A53T)}$) as the primary screening method, a chemical screen was undertaken with 72 hits arising from an yeast screen involving 50729 compounds (Sarkar et al., 2007b). Cells were treated with compounds at 2 mg mL^{-1} concentration for 24 h after the initial doxycycline-induced synthesis of the transgene product (A53T α-synuclein), followed by immunoblotting analysis to measure its clearance. A number of novel autophagy modulators were identified which enhanced the autophagy substrate clearance. These include 4 small molecule enhancers of rapamycin (SMERs) and 13 small molecule inhibitors of rapamycin (SMIRs), of which SMER10, SMER18, and SMER28 were characterized to be autophagy inducers acting independently of mTOR. Further screening of the chemical analogs of these SMERs identified 18 additional autophagy inducers, such as 1 SMER10, 7 SMER18 and 10 SMER28 analogs that are capable of enhancing substrate clearance; although not substantially better than the respective parent compounds (Sarkar et al., 2007b). Another screen also utilizing a stable inducible PC12 cell line expressing HA-tagged A30P α-synuclein (HA-α-syn$^{(A30P)}$) was undertaken with a library of 253 compounds including FDA-approved drugs and pharmacological probes (Williams et al., 2008). Drug treatment was done at 1 μM for 24 h after the synthesis of the transgene product, followed by immunoblotting analysis. This study elucidated a cyclic mTOR-independent autophagy pathway with multiple drug targets, in which cAMP regulates IP_3 levels that impact on calpain activity, which in turn activates $G_{s\alpha}$ that regulates cAMP levels. Some of the autophagy-inducing compounds identified include L-type Ca^{2+} channel blockers (verapamil, loperamide, amiodarone), calpain inhibitors (calpastatin), ATP-sensitive K^+ channel agonist (minoxidil), cAMP reducing agents (rilmenidine, clonidine) and inositol lowering agents (valproic acid), whereas Ca^{2+} channel openers [(±)-Bay K8644] and agents elevating cAMP (dibutyryl cAMP, forskolin) and cytosolic Ca^{2+} (thapsigargin) levels were autophagy inhibitory (Williams et al., 2008). In addition to these immunoblotting based methods, the effects of autophagy modulators on autophagy-dependent clearance of EGFP-tagged mutant huntingtin aggregates can be validated by fluorescence microscopy in wild-type (*Atg5*$^{+/+}$) and autophagy-deficient (*Atg5*$^{-/-}$) mouse embryonic fibroblasts (MEFs) (Kuma et al., 2004; Sarkar et al., 2009).

Although autophagic clearance of aggregation-prone proteins is informative for autophagic flux, only low-throughput approaches are possible that creates a major hurdle for high-throughput applications. Nonetheless, this method could be used as a secondary assay for characterization of selected hits arising from screens with LC3-based reporters.

Identification of Autophagy Modulators by p62/SQSTM1 Clearance

An alternative approach to the clearance of aggregation-prone proteins is to monitor the autophagic degradation of a known autophagy substrate, p62/SQSTM1, which also functions as an adaptor protein during selective autophagy for recruiting specific autophagic cargo to the autophagosomes (Bjorkoy et al., 2005; Pankiv et al., 2007). Similarly, to the method involving aggregation-prone proteins, screening approaches based on p62 clearance would ideally require a stable inducible cell line where the transgene product is temporally expressed before the treatment with compounds. The p62 reporters, such as GFP-p62 (Larsen et al., 2010) or luciferase-tagged p62 (Brown et al., 2016; Min et al., 2018), could be utilized for medium- to high-throughput screens by flow cytometry or microplate reader (for analyzing p62 levels) or by fluorescence imaging (for analyzing p62 aggregates). Genetic screens have been undertaken with p62-based reporters (Pietrocola et al., 2015; Strohecker et al., 2015; DeJesus et al., 2016; Hale et al., 2016), and therefore, similar chemical screening approaches are also possible. In addition, analyzing the steady-state levels of endogenous p62 by immunoblotting is often used as a secondary assay for characterization of autophagy modulators (Klionsky et al., 2012). It is expected that an autophagy inducer will decrease p62 levels or aggregates, whereas an autophagy inhibitor or blocker will cause its accumulation (**Figure 2**). Recently, an assay based on LC3B-II and p62 time-resolved fluorescence resonance energy transfer (TR-FRET) has been described to monitor autophagy independent of any exogenous labels. This method is based on the proximity of the donor and the acceptor antibodies of LC3-II and p62, in which autophagy inducers increase LC3-II signal and decrease p62 signal, autophagy inhibitors do not display any turnover of either signals, whereas autophagy blockers will increase LC3-II signal without any turnover of p62 signal (Bresciani et al., 2018).

Although p62 is a specific autophagy substrate in most mammalian cell lines (Klionsky et al., 2012), its autophagic degradation should be confirmed in the cell-type and the time-points to be used in the screens. Moreover, transcriptional upregulation of p62 has been reported during some instances of autophagy activation, such as under prolonged starvation or with certain pharmacological inducers (Klionsky et al., 2012;

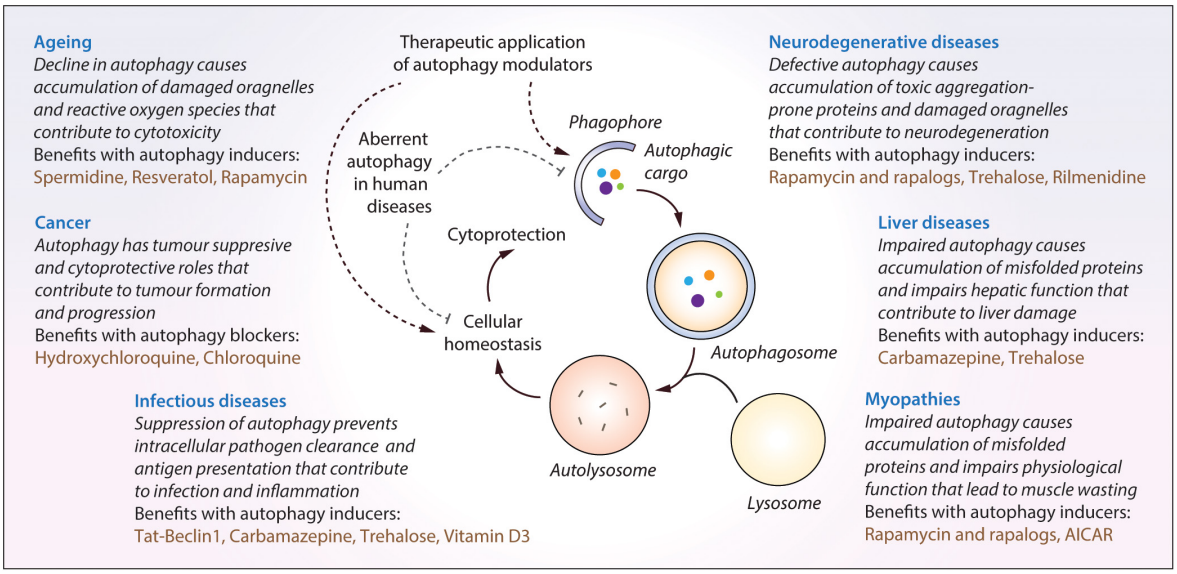


FIGURE 3 | The impact of malfunctioning autophagy and the therapeutic benefits of autophagy modulators in diverse human diseases. Autophagy is implicated in diverse human diseases due to its vital role in maintaining cellular homeostasis. Defective or aberrant autophagy contributes to the cytotoxicity underlying many pathological conditions whereas pharmacological upregulation of autophagy is beneficial in various transgenic models. Key autophagy modulators exerting therapeutic benefits in neurodegenerative disorders, cancer, infectious diseases, liver diseases, myopathies and lifespan extension, as well as the impact of malfunctioning autophagy in these contexts, are highlighted.

Sahani et al., 2014; Kuo et al., 2015), and therefore, any perturbation in p62 protein levels needs to be accompanied by qPCR assessment of its mRNA levels.

BIOMEDICAL APPLICATIONS OF AUTOPHAGY MODULATORS IN HUMAN DISEASES

Autophagy plays an essential role for tissue homeostasis and cellular survival by removing unwanted materials like malfunctioning aggregated proteins and damaged organelles from the cells; however, deregulation of this process could contribute to cytotoxicity (Mizushima et al., 2008). Autophagy dysfunction has been implicated in the pathogenesis of diverse human diseases (Levine and Kroemer, 2008; Jiang and Mizushima, 2014), and therefore, therapeutic exploitation of autophagy is of potential biomedical relevance (**Figure 3**). A number of independent studies and chemical screens have identified several autophagy modulators, which have been shown to impart beneficial effects in various transgenic disease models (**Table 2**; Rubinsztein et al., 2012; Sarkar, 2013b; Levine et al., 2015). Some of the key studies in specific disease contexts are highlighted below.

AUTOPHAGY MODULATORS IN NEURODEGENERATIVE DISEASES

Basal autophagy in the brain is critical for maintaining cellular homeostasis in post-mitotic cells like neurons, which is evident from the genetic studies in mice where brain-specific deletion of essential autophagy genes resulted in neurodegenerative phenotypes (Hara et al., 2006; Komatsu et al., 2006). Particularly, autophagy is the primary degradation pathway for several aggregation-prone proteins associated with neurodegeneration (Rubinsztein, 2006; Nixon, 2013). However, defective autophagy has been reported in several neurodegenerative diseases, including neurodegenerative lysosomal storage disorders, and is considered a major causative factor for neurodegeneration (Nixon, 2013; Sarkar, 2013b; Menzies et al., 2017; Seranova et al., 2017). Therefore, induction of autophagy for enhancing the clearance of mutant aggregation-prone proteins is considered a potential treatment strategy. The therapeutic benefits of autophagy inducers have been robustly demonstrated in the context of neurodegeneration where upregulation of autophagy was protective in several *in vitro* and *in vivo* transgenic models of neurodegenerative diseases (Rubinsztein et al., 2012; Sarkar, 2013b; Levine et al., 2015; Seranova et al., 2017). Stimulating autophagy with mTOR inhibitors like rapamycin or its analogs had beneficial effects in fly and mouse models of Huntington's disease, Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), spinocerebellar ataxia type 3 (SCA3) and prion disease (Ravikumar et al., 2004; Berger et al., 2006; Sarkar et al., 2008; Menzies et al., 2010; Spilman et al., 2010; Cortes et al., 2012; Wang et al., 2012; Ozcelik et al., 2013; Jiang et al., 2014). Likewise, several mTOR-independent autophagy inducers such as, but not limited to, lithium, carbamazepine (inositol lowering agents), rilmenidine (cAMP reducing agent), trehalose (AMPK activator), SMERs and BRD5631 have been shown to be protective in fly, Zebrafish, mouse or induced pluripotent stem cell (iPSC) models of AD,

FTD, HD, amyotrophic lateral sclerosis (ALS) and Niemann-Pick type C1 (NPC1) disease (Sarkar et al., 2005, 2007a,b; Fornai et al., 2008; Williams et al., 2008; Rose et al., 2010; Zhang et al., 2011, 2018; Shimada et al., 2012; Wang et al., 2012; Li et al., 2013; Maetzel et al., 2014; Kuo et al., 2015). The most widely used mTOR-independent autophagy inducer *in vivo* is trehalose (Sarkar et al., 2007a), a disaccharide that stimulates autophagy by inhibiting SLC2A family of glucose transporters and activating AMPK (DeBosch et al., 2016), which in turn can directly influence the phosphorylation of the autophagy-initiating kinase ULK1 (Egan et al., 2011; Kim et al., 2011). Remarkably, trehalose had beneficial effects in mouse models of AD, PD, HD, FTD, SCA17, ALS, as well as cellular and iPSC-derived neuronal models of prion and NPC1 disease, respectively (Tanaka et al., 2004; Aguib et al., 2009; Rodriguez-Navarro et al., 2010; Schaeffer et al., 2012; Castillo et al., 2013; Du et al., 2013; Zhang et al., 2014; Chen et al., 2015; Tanji et al., 2015). Additional autophagy-inducing agents reported to be cytoprotective in neurodegenerative models such as HD, PD, ALS, FTD and Lafora disease include Tat-Beclin 1 peptide, calpastatin, verapamil, metformin, AUTEN-67, AUTEN-99, 6-Bio and fluphenazine (Ma et al., 2007; Williams et al., 2008; Shoji-Kawata et al., 2013; Barmada et al., 2014; Berthier et al., 2016; Billes et al., 2016; Papp et al., 2016; Kovacs et al., 2017; Suresh et al., 2017). A combinatorial approach in enhancing autophagy has been shown with rapamycin and mTOR-independent autophagy inducers such as lithium, trehalose or SMERs. Higher efficacy was achieved via the additive effects of dual treatment on autophagy induction and cytoprotection in cell and fly models of HD than the effects of single compounds (Sarkar et al., 2007a,b, 2008).

AUTOPHAGY MODULATORS IN CANCER

The ability of autophagy in the maintenance of metabolic homeostasis has drawn considerable attention as a potential target for cancer therapy via its pro-survival and pro-death mechanisms (Rabinowitz and White, 2010; Levy et al., 2017). Autophagy plays tumor suppressive role by mitigating oxidative stress, removing superfluous mitochondria and preventing DNA damage and genome instability; and on the other hand, shows pro-tumor activity by preventing the induction of tumor suppressors, increasing resistance to apoptosis and maintaining tumor metabolism through recycling of nutrients (Mathew et al., 2007; Galluzzi et al., 2015; Kimmelman and White, 2017). Depending on the cancer context and the opposing effects of autophagy, either inhibitors or inducers of autophagy could be exploited for cancer therapy (Galluzzi et al., 2017; Levy et al., 2017). Since autophagy promotes tumorigenesis in most contexts, inhibition of autophagy has gathered considerable interest for cancer therapy. Accumulating evidence demonstrate that autophagy inhibitors/blockers exerted therapeutic benefits in cancer models. The clinically- approved autophagy inhibitors chloroquine or hydroxychloroquine (HCQ), which impair lysosomal acidification and block autophagic flux (Murakami et al., 1998; Boya et al., 2005), caused tumor shrinkage in preclinical studies; and thus HCQ being more potent with lesser side-effects is used in ongoing clinical trials either alone or in combination with other treatments (Briceno et al., 2003; Amaravadi et al., 2007; Cook et al., 2014; Chude and Amaravadi, 2017; Levy et al., 2017; Onorati et al., 2018). Autophagy inhibitory compounds, such as Lys05 and ROC-325, which exhibited anti-tumor activity in mice have been suggested to be more potent than HCQ (McAfee et al., 2012; Carew et al., 2017). In addition, autophagy inhibitors preventing autophagosome formation such as ATG4B antagonists (compounds NSC185058 and UAMC-2526), Vps34 (vacuolar protein sorting protein 34) inhibitor (compound SAR405), ULK1 (Unc-51-like kinase 1) inhibitor (compound SBI-0206965), USP10/USP13 (ubiquitin-specific peptidases) inhibitor (Spautin-1) and agents causing transcriptional inhibition of autophagy genes (pyrvinium pamoate), also exerted anti-proliferative and anti-tumor effects in cellular and *in vivo* models of cancer (Liu et al., 2011; Deng et al., 2013; Akin et al., 2014; Ronan et al., 2014; Shao et al., 2014; Egan et al., 2015; Kurdi et al., 2017). On the contrary, various chemical agents or natural products exerting antiproliferative or anti-tumor activity either alone or in combination with chemotherapeutic agents could induce autophagy or autophagic cell death, which include Torin 1, AC-73, MC-4, metformin, silibinin, Abrus agglutinin, curcumin, liensinine, spermidine, vitamin D3, and imatinib (Buzzai et al., 2007; Ertmer et al., 2007; Wang et al., 2008; Thoreen et al., 2009; Qian et al., 2011; Francipane and Lagasse, 2013; Law et al., 2014; Jiang et al., 2016; Pietrocola et al., 2016; Panda et al., 2017; Son et al., 2018; Spinello et al., 2018).

AUTOPHAGY MODULATORS IN INFECTIOUS DISEASES

Autophagy plays an important role in innate defense mechanism by removing intracellular pathogens; a process termed xenophagy (Levine et al., 2011; Deretic et al., 2013). The role of autophagy in regulating intracellular infections initially emerged through studies on *Mycobacterium tuberculosis* (*Mtb*) (Gutierrez et al., 2004; Singh et al., 2006). Subsequently, several other bacterial pathogens like *Salmonella* and *Listeria*, and viral pathogens like HIV and Dengue were shown to utilize host autophagy pathways for their own advantage (Jia et al., 2009; Kyei et al., 2009; Yoshikawa et al., 2009; Heaton and Randall, 2010). A genome-wide siRNA screen to identify host factors required for intracellular *Mtb* survival within macrophages revealed that a large number of host factors acted via regulation of autophagy to help the bacteria (Kumar et al., 2010). Induction of autophagy with rapamycin, carbamazepine, SMER28, and vitamin D3 were shown to prevent bacterial survival or HIV replication in macrophages (Gutierrez et al., 2004; Floto et al., 2007; Yuk et al., 2009; Kumar et al., 2010; Campbell and Spector, 2011, 2012; Schiebler et al., 2015). Notably, carbamazepine reduced bacterial burden, improved lung pathology and stimulated adaptive immunity in mice infected with multidrug-resistant *Mtb* (Schiebler et al., 2015). Rapamycin also controlled viral and

TABLE 2 | Therapeutic benefits of autophagy modulators in diverse human diseases.

Diseases	Selected autophagy modulators	Mechanisms of autophagy modulation	Therapeutic benefits in animal and iPSC models
Neurodegenerative diseases	Rapamycin, CCI-779 (Inducers)	Inhibition of mTORC1 (Blommaart et al., 1995; Ravikumar et al., 2004)	HD flies (Ravikumar et al., 2004; Sarkar et al., 2008), FTD flies (Berger et al., 2006), HD mice (Ravikumar et al., 2004), AD mice (Spilman et al., 2010), FTD mice (Wang et al., 2012; Ozcelik et al., 2013; Jiang et al., 2014), SCA3 mice (Menzies et al., 2010), Prion disease mice (Cortes et al., 2012)
	Lithium (Inducer)	Reduction of inositol and IP_3; mTORC1-independent (Sarkar et al., 2005)	HD flies (Sarkar et al., 2008), AD mice (Zhang et al., 2011), FTD mice (Shimada et al., 2012), ALS mice (Fornai et al., 2008)
	Carbamazepine (Inducer)	Reduction of inositol and IP_3; mTORC1-independent (Sarkar et al., 2005)	AD mice (Li et al., 2013), FTD mice (Wang et al., 2012), ALS mice (Zhang et al., 2018), NPC1 patient iPSC-derived neurons (Maetzel et al., 2014)
	Trehalose (Inducer)	mTORC1-independent (Sarkar et al., 2007a); Inhibition of SLC2A and activation of AMPK (DeBosch et al., 2016)	HD mice (Tanaka et al., 2004), AD mice (Du et al., 2013), PD mice (Tanji et al., 2015), FTD mice (Rodriguez-Navarro et al., 2010; Schaeffer et al., 2012), SCA17 mice (Chen et al., 2015), ALS mice (Castillo et al., 2013; Zhang et al., 2014), NPC1 patient iPSC-derived neurons (Maetzel et al., 2014)
	Rilmenidine, Clonidine (Inducers)	Reduction of cAMP; mTORC1 independent (Williams et al., 2008)	HD mice (Rose et al., 2010), HD zebrafish (Williams et al., 2008), HD flies (Williams et al., 2008)
	Verapamil (Inducer)	Reduction of Ca^{2+}; mTORC1 independent (Williams et al., 2008)	HD zebrafish (Williams et al., 2008), HD flies (Williams et al., 2008), NPC1 patient iPSC-derived neurons (Maetzel et al., 2014)
	SMER28 (Inducer)	Mechanism unknown; mTORC1 independent (Sarkar et al., 2007b)	HD flies (Sarkar et al., 2007b)
	BRD5631 (Inducer)	Mechanism unknown; mTORC1 independent (Kuo et al., 2015)	NPC1 patient iPSC-derived neurons (Kuo et al., 2015)
	Metformin (Inducer)	Activation of AMPK (Buzzai et al., 2007)	HD mice (Ma et al., 2007), LD mice (Berthier et al., 2016)
	6-Bio (Inducer)	Inhibition of mTORC1 signaling (Suresh et al., 2017)	PD mice (Suresh et al., 2017)
	AUTEN-67, AUTEN-99 (Inducers)	Inhibition of MTMR14 (Papp et al., 2016; Kovacs et al., 2017)	HD flies (Billes et al., 2016; Papp et al., 2016; Kovacs et al., 2017), PD flies (Kovacs et al., 2017)
Cancer	Chloroquine, Hydroxychloroquine (Blockers)	Mechanism unknown; Impairment of lysosomal acidification and autophagosome-lysosome fusion (Murakami et al., 1998; Boya et al., 2005)	*Myc/p53ER*^{TAM} induced lymphoma mice (Amaravadi et al., 2007), mice bearing MCF7-RR and LCC9 ER+ breast cancer xenografts (Cook et al., 2014)
	Lys05, ROC-325 (Blockers)	Mechanism unknown; Impairment of lysosomal acidification and autophagosome-lysosome fusion (McAfee et al., 2012; Carew et al., 2017)	Mice bearing c8161 melanoma, 1205Lu melanoma and HT-29 colon cancer xenografts (McAfee et al., 2012), mice bearing 786-0 RCC xenografts (Carew et al., 2017)
	NSC185058, UAMC-2526 (Inhibitors)	Inhibition of ATG4B (Akin et al., 2014; Kurdi et al., 2017)	Mice bearing Saos-2 osteosarcoma xenograft (Akin et al., 2014), Mice bearing HT29 colorectal tumor xenograft (Kurdi et al., 2017)
	Pyrvinium pamoate (Inhibitor)	Mechanism unknown; Reduction in *Atg* gene expression; mTORC1 independent (Deng et al., 2013)	Mice bearing 4TI mammary carcinoma xenograft (Deng et al., 2013)
	Torin 1 (Inducer)	ATP-competitive inhibition of mTORC1 (Thoreen et al., 2009)	Mice bearing Tu12 and Tu22 colon cancer xenografts (Francipane and Lagasse, 2013)
Infectious diseases	Tat-Beclin 1 (Inducer)	Interaction with the negative autophagy regulator GAPR-1 (Shoji-Kawata et al., 2013)	Mice infected with chikungunya or West Nile virus (Shoji-Kawata et al., 2013), murine or human macrophages infected with *L. monocytogenes* bacteria and HIV (Shoji-Kawata et al., 2013)
	Vitamin D3 (Inducer)	Increase in Beclin 1 (Wang et al., 2008); Increase in *Atg* gene expression (Yuk et al., 2009)	Human macrophages infected with *M. tuberculosis bacteria* or HIV or coinfection (Yuk et al., 2009; Campbell and Spector, 2011, 2012)

(Continued)

TABLE 2 | Continued

Diseases	Selected autophagy modulators	Mechanisms of autophagy modulation	Therapeutic benefits in animal and iPSC models
	Carbamazepine (Inducer)	Reduction of inositol and IP_3; mTORC1-independent (Sarkar et al., 2005)	Human macrophages infected with *M. tuberculosis bacteria* or coinfection with HIV (Schiebler et al., 2015), mice infected with multidrug-resistant *M. tuberculosis bacteria* (Schiebler et al., 2015)
	Trehalose (Inducer)	mTORC1-independent (Sarkar et al., 2007a); $PI(3,5)P_2$ agonist, activation of TRPML1 Ca^{2+} channel (Sharma et al., 2017)	Human macrophages infected with *M. tuberculosis bacteria* or coinfection with HIV (Sharma et al., 2017), PBMCs from HIV patients (Sharma et al., 2017)
	Flubendazole (Inducer)	mTORC1 inactivation; nuclear translocation of TFEB (Chauhan et al., 2015)	Human dendritic cells infected with *HIV,* and HeLa cells infected with *E. coli bacteria* (Chauhan et al., 2015)
	Nitazoxanide (Inducer)	Inhibition of mTORC1 signaling (Lam et al., 2012)	Human acute monocytic leukemia cells or PBMCs infected with *M. tuberculosis bacteria* (Lam et al., 2012)
	Nortriptyline (Inducer)	Mechanism unknown	Human macrophages infected with *M. tuberculosis bacteria* (Sundaramurthy et al., 2013)
Liver Disease	Carbamazepine (Inducer)	Reduction of inositol and IP_3; mTORC1-independent (Sarkar et al., 2005)	AATD mice (Hidvegi et al., 2010), NAFLD and AFLD mice (Lin et al., 2013), FSD patients (Puls et al., 2013), AATD patient iPSC-derived hepatic cells (Choi et al., 2013), NPC1 patient iPSC-derived hepatic cells (Maetzel et al., 2014)
	Lithium, Valproic acid (Inducers)	Reduction of inositol and IP_3; mTORC1-independent (Sarkar et al., 2005)	AATD patient iPSC-derived hepatic cells (Choi et al., 2013)
	Trehalose (Inducer)	mTORC1-independent (Sarkar et al., 2007a); Inhibition of SLC2A and activation of AMPK (DeBosch et al., 2016)	NAFLD mice (DeBosch et al., 2016)
	Rapamycin (Inducer)	Inhibition of mTORC1 (Blommaart et al., 1995)	NAFLD mice (Lin et al., 2013), NPC1 patient iPSC-derived hepatic cells (Maetzel et al., 2014)
Myopathies	Rapamycin, CCI-779 (Inducers)	Inhibition of mTORC1 (Blommaart et al., 1995; Ravikumar et al., 2004)	Collagen type VI muscular dystrophy mice (Grumati et al., 2010), *LMNA* cardiomyopathy mice (Choi et al., 2012; Ramos et al., 2012)
	AICAR (Inducer)	Activation of AMPK (Buzzai et al., 2007)	DMD mice (Pauly et al., 2012)
	Simvastatin (Inducer)	Inhibition of Rac1-mTOR pathway (Wei et al., 2013)	DMD mice (Whitehead et al., 2015)
Lifespan extension	Spermidine (Inducer)	Inhibition of histone acetyltransferase and increase in *Atg* gene expression (Eisenberg et al., 2009)	Flies (Eisenberg et al., 2009), worms (Eisenberg et al., 2009), mice (Eisenberg et al., 2016)
	Resveratrol (Inducer)	Activation of SIRT1 (Morselli et al., 2010)	Flies (Wood et al., 2004), worms (Wood et al., 2004; Morselli et al., 2010), mice (Baur et al., 2006)
	Rapamycin (Inducer)	Inhibition of mTORC1 (Blommaart et al., 1995)	Flies (Bjedov et al., 2010), mice (Harrison et al., 2009)

Autophagy modulators have shown beneficial effects in a number of transgenic disease models, such as but not limited to, neurodegenerative disorders, cancer, infectious diseases, liver diseases and myopathies as well as in lifespan extension. Selected examples of autophagy modulators are highlighted in specific pathological contexts. AATD, α1 antitrypsin deficiency; AD, Alzheimer's disease; AFLD, Alcoholic fatty liver disease; ALS, Amyotrophic lateral sclerosis; AMPK, 5′ adenosine monophosphate-activated protein kinase; Atg, Autophagy-related genes; cAMP, 3′,5′-cyclic adenosine monophosphate; DMD, Duchenne muscular dystrophy; FSD, Fibrinogen storage disease, FTD, Frontotemporal dementia; GAPR-1, Golgi-associated plant pathogenesis-related protein 1; HD, Huntington's disease; HIV, Human immunodeficiency virus; IP_3, Inositol 1,4,5-trisphosphate; iPSC, Induced pluripotent stem cells; LD, Lafora disease; LMNA, Lamin A/C gene; MTMR14, Myotubularin related protein 14; mTORC1, Mechanistic target of rapamycin complex 1; NAFLD, Non-alcoholic fatty liver disease; NPC1, Niemann-Pick type C1 disease; PBMC, Peripheral blood mononuclear cells; PD, Parkinson's disease; $PI(3,5)P_2$, Phosphatidylinositol 3,5-bisphosphate; RCC, Renal cell carcinoma; SCA, Spinocerebellar ataxia; SIRT1 Sirtuin 1; SLC2A, Solute carrier 2A; TRPML1, Transient receptor potential cation channel mucolipin subfamily member 1.

bacterial pathogens both *in vitro* and *in vivo* (Donia et al., 2010). In an integrated chemical and RNAi screening for modulators of intracellular mycobacteria, one of the top three compounds was nortriptyline which significantly suppressed *Mtb* survival within macrophages and induced autophagy (Sundaramurthy et al., 2013). Other compounds limiting bacterial or HIV infections through activation of autophagic flux were nitazoxanide (anti-protozoan drug) and flubendazole (antihelminthic drug) (Lam et al., 2012; Chauhan et al., 2015). Similarly, the naturally occurring disaccharide trehalose, a potent mTOR-independent enhancer of autophagy in diverse cell-types (Sarkar et al., 2007a), can also induce autophagy and xenophagy in *Mtb*-infected

macrophages that resulted in the killing of bacteria (Sharma et al., 2017). In this study, trehalose was found to act as a PI(3,5)P_2 (phosphatidylinositol *3,5*-bisphosphate) agonist for activating the lysosomal Ca^{2+} channel TRPML1 (Sharma et al., 2017), which in turn released lysosomal Ca^{2+} that caused nuclear translocation of TFEB to induce autophagy (Medina et al., 2015). Excitingly, trehalose also seemed to be effective during HIV-*Mtb* co-infection and limits *Mtb* survival by reversing the HIV-mediated block in autophagy flux (Sharma et al., 2017). Similarly, vitamin D3 could also kill *Mtb* during HIV co-infection by inducing autophagy (Campbell and Spector, 2012). Several host factors currently being tested for anti-*Mtb* therapeutics function by regulating host autophagy and xenophagy. For example, inhibition of host Src kinase by the compound AZD0530 induced autophagy and lysosomal maturation to clear *Mtb* (Chandra et al., 2016). A pioneering anti-infective, autophagy-inducing agent is Tat-Beclin 1, which is a peptide representing a region of the autophagy regulator Beclin 1 that interacts with the HIV-1 accessory protein NEF, and this domain is linked with the HIV-1 Tat transduction domain to make it cell permeable (Shoji-Kawata et al., 2013). Tat-Beclin 1 prevented the replication of a number of viral and bacterial pathogens *in vitro* in autophagy-dependent manner, as well as induced autophagy and anti-viral activity in mice infected with chikungunya or West Nile virus (Shoji-Kawata et al., 2013). Thus, it is evident that regulators of autophagy and xenophagy have tremendous potential for novel therapeutics against various infectious diseases. It is now clear that within an infected host cell, there is a possibility of uncoupling between homeostatic autophagy and anti-bacterial xenophagy (Chandra et al., 2015; Sharma et al., 2018). Therefore, it is desirable to perform chemical screening pertaining to infection-specific xenophagy flux for identifying novel regulators of bacterial/viral survival within the host cells through the autophagy pathway.

AUTOPHAGY MODULATORS IN LIVER DISEASES

Liver autophagy is essential for various hepatic functions and is implicated in various liver conditions including α1-antitrypsin (AAT) deficiency, non-alcoholic fatty liver disease (NAFLD), hepatocellular carcinoma and viral hepatitis (Rautou et al., 2010; Ueno and Komatsu, 2017). Chemical modulation of autophagy has been shown to have beneficial effects in some of these diseases. Carbamazepine, an mTOR independent autophagy inducer acting by reducing inositol levels (Sarkar et al., 2005), reduced hepatic load of mutant α1-antitrypsin Z and hepatic fibrosis in a mouse model of AAT deficiency (Hidvegi et al., 2010), as well as decreased hepatocellular aggregate-related toxicity in patients suffering from fibrinogen storage disease (Puls et al., 2013). A high-throughput drug screen in hepatocyte-like cells derived from iPSC lines of patients with AAT deficiency also revealed inositol-lowering autophagy-inducing agents, such as carbamazepine, lithium, and valproic acid, in facilitating the clearance mutant AAT (Choi et al., 2013). Carbamazepine as well as the mTOR inhibitor rapamycin also rescued dysfunctional autophagic flux and improved cell viability in hepatic-like cells differentiated from patient-derived iPSC lines of Niemann-Pick type C1 (NPC1) disease (Maetzel et al., 2014). In addition, autophagy induction with trehalose, carbamazepine, rapamycin or hydrogen sulfide reduced steatosis, lipid accumulation and liver injury in high-fat diet-induced NAFLD in mice (Lin et al., 2013; Sun et al., 2015; DeBosch et al., 2016). Furthermore, the anti-diabetic drug metformin, which indirectly inhibits mTOR, induced SIRT1-mediated autophagy in primary hepatocytes and ameliorated hepatic steatosis *in vivo* (Song et al., 2015). Overall, these studies indicate that activation of autophagy via inhibition of mTOR, lowering inositol levels or with trehalose are effective modes of inducing autophagy in the liver.

AUTOPHAGY MODULATORS IN MYOPATHIES

Basal autophagy is required for maintaining muscle mass and myofiber integrity (Masiero et al., 2009), and thus deregulation of muscle autophagy is implicated in myopathies and muscular dystrophies (Sandri et al., 2013). Sustained activation of mTORC1 in skeletal muscle of TSC1-deficient mice could cause late-onset myopathy related to suppression of autophagy (Castets et al., 2013). Upregulation of autophagy, primarily by inhibiting the mTORC1 pathway, has been reported to have beneficial effects in certain transgenic disease models. Autophagy induction by rapamycin or low-protein diet increased myofiber survival and attenuated dystrophic phenotype in a mouse model of collagen type VI muscular dystrophy (Grumati et al., 2010). Likewise, activation of autophagy by dietary changes or with the AMP-activated protein kinase (AMPK) agonist, AICAR (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside), improved dystrophic phenotypes in mouse models of Duchenne muscular dystrophy (DMD) (De Palma et al., 2012; Pauly et al., 2012). A potential role of simvastatin, which has been reported to induce autophagy by inhibiting the Rac1-mTOR pathway (Wei et al., 2013), has been suggested in improving the physiological function of skeletal muscle in DMD transgenic mice (Whitehead et al., 2015). In addition, rapamycin or its analog, temsirolimus, ameliorated cardiomyopathy and improved skeletal and cardiac muscle function in mouse models of *LMNA* (lamin A/C gene) cardiomyopathy that recapitulate Emery-Dreifuss muscular dystrophy (EDMD) (Choi et al., 2012; Ramos et al., 2012).

AUTOPHAGY MODULATORS IN LIFESPAN EXTENSION

The functionality of autophagy declines with aging (Rubinsztein et al., 2011), and thus restoring adequate autophagy is considered a possible anti-aging strategy for lifespan extension. There are a number of lifespan expanding strategies, and in many of such approaches, autophagy acts as a common denominator

for promoting longevity (Madeo et al., 2010; Hansen et al., 2018). Pharmacological treatment with autophagy inducers has been linked to increasing longevity in transgenic *in vivo* models (Madeo et al., 2015). Lifespan extension via induction of autophagy with naturally- occurring polyamines such as spermidine, which is an acetyltransferase inhibitor, was shown in yeast, flies, worms and mice (Eisenberg et al., 2009, 2016); and likewise also reported with the natural phenol resveratrol, which is a deacetylase activator, in yeast, flies, worms as well as in mice on high-fat diet (Howitz et al., 2003; Wood et al., 2004; Baur et al., 2006; Morselli et al., 2010). Although both spermidine and resveratrol impacts on the acetylproteome, stimulation of autophagy by resveratrol requires the nicotinamide adenine dinucleotide-dependent deacetylase sirtuin 1 (SIRT1) whereas the effect of spermidine was SIRT1 independent (Morselli et al., 2010, 2011). Inhibition of mTOR by rapamycin also extended lifespan in yeast, flies and mice (Alvers et al., 2009; Harrison et al., 2009; Bjedov et al., 2010; Lamming et al., 2013). In addition, lifespan extension in multiple organisms including mice and apes could be achieved by caloric restriction, which is a physiological inducer of autophagy via AMPK activation, mTORC1 inhibition and SIRT1 activation (Mair and Dillin, 2008; Colman et al., 2009; Mercken et al., 2014; Mattison et al., 2017). In some of these studies reporting lifespan extension by autophagy activation, the role of autophagy has been specifically determined by abolishing the anti-aging effects via knockdown of essential autophagy genes (Madeo et al., 2015; Nakamura and Yoshimori, 2018).

CONCLUSION

The methodologies for measuring autophagy have evolved over the past decade and it is now feasible to undertake high-throughput chemical screens for identifying modulators of autophagic flux. A number of pharmacological modulators of autophagy have been identified via screening approaches or individual studies; some of which have been demonstrated to exert therapeutic benefits in diverse human diseases. Most of the key autophagy modulators have been identified either by the GFP-LC3 screening method in HeLa cells or via assessing the clearance of aggregation-prone proteins in inducible PC12 cell lines. While analysis of changes in autophagosome number with GFP-LC3 reporter requires shorter treatment period (such as 8–24 h), analysis of clearance of aggregation-prone proteins requires longer treatment duration (such as 24–72 h) depending on the nature of the transgene product. Following the primary screen, it is pertinent to characterize the high-confidence screen hits with secondary autophagy assays because there are no single assays to determine autophagic flux. These normally include analysis of autophagosome formation with bafilomycin A_1 via immunoblotting with anti-LC3 antibody, analysis of autophagosome maturation with mRFP-GFP-LC3 reporter, and analysis of autophagy substrate (p62) clearance via immunoblotting with anti-p62 antibody (Mizushima et al., 2010; Klionsky et al., 2016).

Although the methods described in this review are those that have been generally used in the field, alternative autophagy assays could also be employed for chemical screening. One potential approach is the use of Keima, a fluorescent acid-stable protein that exhibits bimodal excitation spectra in neutral and acidic pH, such as in autophagosomes and autolysosomes, respectively (Katayama et al., 2011). The cumulative fluorescence readout can be used to measure bulk autophagic flux. This protein can also be utilized for selective autophagic flux, such as with mitochondria-targeted Keima to measure mitophagy (Katayama et al., 2011; Sun et al., 2017). However, Keima-based assays solely depend upon the lysosomal acidity and thus cannot be performed in fixed cells where the pH gradient across lysosomal membranes is lost. In addition, other screening approaches could be based on fluorescent-tagged early markers of autophagy initiation, such as with WIPI-1 (Proikas-Cezanne and Pfisterer, 2009) and DFCP1 (Axe et al., 2008); however, these methods will not capture the late events of autophagy pathway involving autophagosome maturation and cargo degradation.

For the therapeutic exploitation of autophagy modulators, mTOR-independent autophagy inducers are generally favorable and considered to have lesser side-effects than the mTOR inhibitors like rapamycin. This is because mTOR controls vital cellular functions like cell growth and translation and thus its inhibition can lead to undesirable side-effects unrelated to autophagy induction. For clinical translation to patients, it is important to determine the efficacy and penetrance of the autophagy modulators in the target organs. Future directions could include identifying specific inducers of autophagy acting at the level of autophagic machinery rather than the upstream signaling pathways.

AUTHOR CONTRIBUTIONS

PP, AF, SV, DK, and SS wrote the manuscript. ES and SS made the figures. PP and SS made the tables. PP, AF, SV, ES, VS, MC, PD, JT, TR, DK, and SS reviewed the manuscript.

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METHODS
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Generation and Characterization of Germline-Specific Autophagy and Mitochondrial Reactive Oxygen Species Reporters in *Drosophila*

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Oogenesis is a fundamental process that forms the egg and, is crucial for the transmission of genetic information to the next generation. *Drosophila* oogenesis has been used extensively as a genetically tractable model to study organogenesis, niche-germline stem cell communication, and more recently reproductive aging including germline stem cell (GSC) aging. Autophagy, a lysosome-mediated degradation process, is implicated in gametogenesis and aging. However, there is a lack of genetic tools to study autophagy in the context of gametogenesis and GSC aging. Here we describe the generation of three transgenic lines mcherry-Atg8a, GFP-Ref(2)P and mito-roGFP2-Orp1 that are specifically expressed in the germline compartment including GSCs during *Drosophila* oogenesis. These transgenes are expressed from the *nanos* promoter and present a better alternative to UASp mediated overexpression of transgenes. These fluorescent reporters can be used to monitor and quantify autophagy, and the production of reactive oxygen species during oogenesis. These reporters provide a valuable tool that can be utilized in designing genetic screens to identify novel regulators of autophagy and redox homeostasis during oogenesis.

Keywords: GFP-Ref(2)P, mCherry-Atg8a, mito-roGFP2-Orp1, autophagic flux, redox, germline stem cell, autophagy, Atg8a

INTRODUCTION

In multicellular organisms, the production of functional gametes depends on the activity of specialized stem cells called "germline stem cells" (GSCs) located in the gonads (reviewed in Fuller and Spradling, 2007; Dansereau and Lasko, 2008). Like most other types of stem cells, GSCs are also subject to cellular damage that causes premature aging and render them inactive leading to their depletion within the gonads (López-Otín et al., 2013; Signer and Morrison, 2013; Oh et al., 2014). Cellular damage caused by genotoxic agents and reactive oxygen species can damage organelles, proteins, and DNA within the stem cells. Thus, it is crucial to maintaining the integrity and quality of GSCs, as cellular and genetic defects may be passed onto the next

generation which can be detrimental to the survival of the species. The prolonged sustenance of GSCs, therefore, require efficient homeostasis mechanisms to be operational constitutively within these cells. Such homeostasis mechanism(s) must function to significantly reduce cellular damage. Macro-autophagy (autophagy) is the cellular mechanism that is involved in removing toxic protein aggregates and damaged organelles such as mitochondria from within the cytoplasm (Takeshige et al., 1992; Mizushima, 2007; Yu et al., 2018). Autophagy is necessary during animal development and impaired autophagy has been implicated in several diseases including cancer, neurodegenerative diseases, infectious diseases, cardiopathy and autoimmunity (Jiang and Mizushima, 2014; Schneider and Cuervo, 2014). However, the involvement of autophagy during gametogenesis, reproduction and germline aging is not extensively studied.

Autophagy proceeds with the sequestration of cytoplasmic contents within a double-membraned vesicle termed as autophagosome which fuses with the lysosome to form an autolysosome. The cargo within the autolysosome is degraded by the lysosomal enzymes. The degradation products are exported from the autolysosome to the cytoplasm where they can be reused in several metabolic processes (He and Klionsky, 2009; Boya et al., 2013; Bento et al., 2016). The complex process of autophagosome formation and its subsequent fusion to the lysosome is highly regulated by Autophagy-related (Atg) proteins (Mizushima et al., 1998, 2011; Klionsky, 2012; Mulakkal et al., 2014).

In metazoans, 16 Atg proteins have been shown to be essential for the autophagy. The process is initiated at the pre-autophagosome structure/phagophore assembly site (PAS) where Atg1 kinase complex consisting of Atg1, FIP200, Atg13, and Atg101 assembles (reviewed in Scott et al., 2004; Nagy et al., 2014b; Hurley and Young, 2017). PAS formation can occur at the ER and is termed as omegasome in mammals (Axe et al., 2008). The activity of Atg1 kinase is required for recruitment of Vps34 complex comprising of Beclin-1 (Atg6 in *Drosophila*), Vps34, Vps15 and Atg14 to the phagophore where it catalyzes the formation of PI3P which is essential for vesicle nucleation (Kihara et al., 2001; Itakura et al., 2008; Juhász et al., 2008). Several membrane sources can contribute to the formation of the phagophore/isolation membrane (Chan and Tang, 2013). The formation of Phosphatidylinositol 3-phosphate (PI3P) at the phagophore site is crucial for the recruitment of WIPI family proteins (Burman and Ktistakis, 2010). WIPI2 (Atg18 in *Drosophila*) family proteins interact with Atg16L1 (Atg16 in *Drosophila*) that subsequently allow for recruitment of Atg12-Atg5-Atg6L1 complex at the PAS (Romanov et al., 2012; Walczak and Martens, 2013). The formation of the Atg12-Atg5-Atg16 complex is catalyzed by several enzymes. Atg12 is a ubiquitin-like protein that is activated by Atg7 E1-like enzyme and subsequently transferred to E2 like enzyme Atg10 allowing for the formation of Atg12-Atg5 complex. The Atg12-Atg5 complex can form a ternary complex with Atg16L1 (Atg16 in *Drosophila*), finally catalyzing lipidation of Atg8 on the autophagosome membrane (Mizushima et al., 1998; Kim et al., 2016; Nagy et al., 2017). Atg8/MAP1LC3, a ubiquitin-like molecule (microtubule associated protein 1 light-chain 3, LC3, in mammals) is a component of conjugation system that is also essential for elongation and completion of the autophagosome (Ichimura et al., 2000; Kabeya et al., 2000). C-terminal amino acid residues of Atg8 are removed by ATG4 protease to expose a C-terminal glycine that is covalently bound to phosphatidylethanolamine (PE) by series of enzymes Atg7 (E1-like enzyme), Atg3 (E2-like enzyme), and the Atg12 complex (consisting of Atg12-Atg5-Atg16). The Atg8-PE (Atg8-II) is localized to the autophagosome membrane and this lipidation event has been exploited to study autophagy. Typically, Atg8 is fused with fluorescent proteins such as GFP or RFP/mCherry at its amino terminus and are driven by tissue-specific promoters, endogenous Atg8a promoters, by UAS and UASp sequences (Scott et al., 2004; Denton et al., 2009, 2012; Nelson et al., 2014; Hegedus et al., 2016; Jacomin and Nezis, 2016; Bali and Shravage, 2017). However, a few of these transgenes do not express in the germline or are expressed at very high levels in the germline complicating autophagy assays. An alternative to monitoring autophagy is to assay for lipid conjugation of Atg8 proteins by immunoblotting (Barth et al., 2011; Mauvezin et al., 2014; Nagy et al., 2015; Lörincz et al., 2017). The lipidated protein Atg8-II migrates at a faster rate on polyacrylamide gels than its unprocessed form, Atg8-I. However, immunoblotting techniques typically use protein extracts prepared from the entire tissue or group of cells which eliminate individual differences in autophagy status in individual cell types within the tissue (Klionsky et al., 2016).

p62/SQSTM1 is an autophagy adaptor first described in mammals and subsequently shown to function during autophagy in *Drosophila* (Ref(2)P in *Drosophila*) (Bartlett et al., 2011). The ability of p62/Ref(2)P to interact with ubiquitin or polyubiquitin chains in different proteins via the UBA domain enables it to deliver cargo to autophagosome via the LIR domain (which is also known as the Atg8 interacting domain in *Drosophila*). Interestingly, p62 is capable of both homo-oligomerization and hetero-oligomerization via PB1 domains thus allowing the formation of complex protein aggregates that can be recognized by the autophagy machinery and targeted for degradation (Mulakkal et al., 2014; Nagy et al., 2015; Lörincz et al., 2017). Ref(2)P has been used to measure autophagic activity in *Drosophila* fat body cells where an increase in the size of Ref(2)P punctae under stress conditions was observed followed by a reduction in number due to the clearance of protein aggregates by autophagy (Pircs et al., 2012). The increased levels of Ref(2)P which marks the protein aggregates during the inhibition of autophagy serves as an *in vivo* measure of long term autophagic activity. Thus, it can be used as a tool to detect the protein inclusion formation and changes in autophagic activity under various physiological conditions (Bartlett et al., 2011; Pircs et al., 2012; Devorkin and Gorski, 2014; Mauvezin et al., 2014). In *Drosophila,* there are no transgenic lines that can detect Ref(2)P in GSCs and any assays performed in the germline are based on the use of anti-Ref(2)P antibody which becomes a limiting factor for designing forward genetic screens.

The elongation of phagophore is catalyzed by Atg9/Atg9L1 complex in addition to the Atg1 and Vps34 complexes, and Atg12 and Atg8 conjugation systems. The Atg9 complex consists of Atg9, a transmembrane protein, Atg18, and Atg2 (Nagy

et al., 2014a). Atg9 containing vesicles shuttle between the PAS/phagophore and membrane source (Golgi/endosomes). Membrane scission occurs at the end of the elongation process resulting in the generation of a closed autophagosome laden with cargo. Following this event, most Atg proteins are removed or disassembled from the autophagosome membrane catalyzed by PI3-P phosphatases which aid in the elimination of PI3-P from the membrane. In most metazoans, autophagosomes fuse with endosomes to form amphisomes before being fused to lysosomes by kiss-and-run like process. Several proteins which are involved in vesicle fusion including SNAP29, Syntaxin17, Rab7, Atg14, HOPS complex and PLEKHM1 are shown to be required for this process (Takáts et al., 2013; Guo et al., 2014; Takats et al., 2014; Liu et al., 2015; McEwan et al., 2015; Hegedus et al., 2016). The outer membrane of the autophagosome fuses to the lysosome to form the autolysosome. The cargo along with the inner membrane of the autophagosome is degraded in the autolysosome by the lysosomal hydrolases. The degradation products are exported from the autolysosome back into the cytoplasm where they are used for anabolic processes (Mizushima, 2007; Bento et al., 2016; Nakamura and Yoshimori, 2017; Yu et al., 2018).

Autophagy is also triggered in response to reactive oxygen species (Scherz-Shouval and Elazar, 2011; Filomeni et al., 2015). Hydrogen peroxide (H_2O_2), one of the metabolites of the intracellular redox reaction, is an important signaling molecule, is required for peroxisomal catabolism and used by cells in a controlled manner to oxidize substrates. However, it is also the main source of peroxide ions which oxidize and damage proteins, lipids, and DNA (Sies, 2017). One of the main sources of H_2O_2 is the mitochondrial electron transport chain (Dröge, 2002; Sena and Chandel, 2012; Shadel and Horvath, 2015). Superoxide ions O_2^- are produced by electron transport chain complex I and III present in different compartments of the mitochondria. O_2^- ions are reduced by superoxide dismutase (SOD) enzymes. Mn-dependent SOD2 in the mitochondrial matrix is responsible for the conversion of O_2^- to H_2O_2. H_2O_2 is detoxified to water by catalase and glutathione peroxidase (Dröge, 2002). However, H_2O_2 can be damaging when it reacts with the thiol group (−SH) within the proteins to form sulphenic acid (−SOH) altering their activity or rendering them inactive (Finkel, 2012). Such cellular damage within mitochondria leads to depolarization of mitochondria which results in a decrease of ATP synthesis and metabolic activities. Multiple proteins within the cells have been demonstrated to sense production of H_2O_2 and react with it (Ma et al., 2007; Gutscher et al., 2008).

Oxidant receptor peroxidase 1 (Orp1), a component of redox relay in yeast *Saccharomyces cerevisiae*, is homologous to glutathione peroxidase and senses H_2O_2 (Ma et al., 2007). Orp1 is shown to be able to successfully oxidize roGFP2 based on H_2O_2 sensing *in vitro*. This was first demonstrated by Gutscher et al. (2008), by making an Orp1-roGFP2 fusion protein. This construct was then modified by adding a mitochondrial localization signal (mito-roGFP2-Orp1) by Albrecht and colleagues enabling measurement of H_2O_2 production within the mitochondria (Albrecht et al., 2011, 2014). The probe senses the redox state of Orp1 through roGFP2. Thiol groups in Orp1 are oxidized by H_2O_2. This alters the redox state of Orp1 that causes reduction or oxidation of the roGFP2 which results in the switch in excitation of fluorescence from 488 to 405 nm. The shift in excitation of roGFP2 is a reliable measure of the H_2O_2 production.

Here we describe generation and characterization of transgenic lines that express mCherry-Atg8a, GFP-Ref(2)P and mito-ro-GFP2-Orp1 under the *nanos* gene promoter. Our data show that these transgenes could be used reliably to monitor autophagy and H_2O_2 production within the germ cells in *Drosophila* during gametogenesis. These transgenes lack the problems associated with UASp driven transgenes which can overexpress the Atg proteins complicating and affecting the interpretation of autophagy. We believe that these transgenes will aid in conducting screens designed to identify genes affecting autophagy and redox status within the germ cells in *Drosophila*.

MATERIALS AND METHODS

Fly Maintenance

All the transgenic fly stocks were maintained at 25°C on standard cornmeal sucrose malt agar. The stocks were maintained homozygous for all transgenes. All insertions were tested for expression of the transgene using confocal microscopy. Insertion with the highest expression (strong fluorescence) was chosen for characterization. mCherry-Atg8a and GFP-Ref(2)P were mapped on to the chromosome, balanced and crossed to get the following combination; ywhsFLP1; mCherry-Atg8a/CyO; GFP-Ref(2)P/TM6b. The following flies were used; yw; UASp.mCherry.Atg8a; Dr/ TM3, Ser (BL37750, RRID:BDSC_37750), w; +; nosGal4VP16 (BL4937, RRID:BDSC_4937), y sc v; +; Atg8a-RNAi (BL34340, RRID:BDSC_34340), y sc v; +; Atg5-RNAi (BL 34899, RRID:BDSC_34899), y $Atg8a^{KG07569}$/FM7c; +; + (BL14639, RRID:BDSC_14639).

Feeding, Starvation and Pharmacological Treatments

For each treatment, 24–96 h old flies in a group of 15 females and 10 males were housed in a vial.

Feeding

Flies were transferred every day into a fresh vial containing cornmeal sucrose malt agar supplemented with yeast paste/pellets.

Starvation

Flies were starved on vials containing 20% sucrose in 2.5% agar.

Chloroquine Treatment

Flies were transferred every day into a vial containing sucrose agar with chloroquine to a final concentration of 3 mg/ml.

Rapamycin Treatment

Rapamycin was added into food just before pouring food (60°C) to a final concentration of 200 μM.

To determine the optimal time of starvation where most number of mCherry-Atg8a punctae would occur

(**Supplementary Figure S3D**), nosP-mCherry-Atg8a line was subjected to starvation for 1, 2, 3, and 4 days by transferring them daily into a vials containing 20% sucrose in 2.5% agar. nosP-GFP-Ref(2)P flies after being exposed to pharmacological treatments were dissected in Grace's medium and imaged immediately on Nikon SMZ 1270 microscope fitted with Nikon DS-Fi2 camera. All images were acquired at the same magnification (**Supplementary Figure S1C**).

Cloning of pC4 nosP-nos 3′UTR

nanos3′UTR was PCR amplified from CantonS genome using primers nos3′UTRF25′-tctagaagagggcgaatccagctctggagcaga and nos3′UTRR 5′-tctagaccattttgggagacgccttgaacctaagtg and digested with XbaI and PstI. The resulting fragment of 1236 bp was cloned in XbaI, PstI digested pCasper4 to obtain pC4-nos3′UTR. Nanos promoter was amplified using primers nosPF 5′-aagcttcgaccgttttaacctcgaaatatg and newnosPR 5′-tggcgaaaatccgggtcgaaagttacg to obtained 935 bp fragment. This fragment was cloned in pGEMt-Easy to obtain pGEMt-nosP. pGEMt-nosP was digested using EcoRI and the resulting 963bp fragment was cloned in EcoRI digested pC4-nos3′UTR to obtain pC4-nosP-nos3′UTR.

nosP-GFP-Ref(2)P-nos3′UTR

2537 bp NotI-XbaI fragment from pUASt-GFP-Ref(2)P (Chang and Neufeld, 2009) was cloned in NotI-XbaI digested pC4-nosP-nos3'UTR to generate pC4-nosP-GFP-Ref(2)P-nos3'UTR.

Chang and Neufeld, 2009 have referred pUASt-GFP-Ref(2)P as GFP-Ref(2)P in their published article. However, GFP-Ref(2)P is generated using EGFP sequence from pEGFP (Clonetech, United States; Scott et al., 2004).

nosP-mCherry-Atg8a-nos3′UTR

1162 bp XbaI fragment from pmCherry-Atga (Bali and Shravage, 2017) was cloned in XbaI digested pC4-nosP-nos3′UTR to generate pC4-nosP-mCherry-Atg8a-nos3′UTR.

nosP-mito-roGFP2-Orp1-nos3′UTR

1241 bp NotI-XbaI fragment from pUASt-mito-roGFP2-Orp1 (Albrecht et al., 2011, 2014) was cloned in NotI-XbaI digested pC4-nosP-nos3′UTR to generate pC4-nosP-mito-roGFP2-Orp1-nos3′UTR.

All constructs were sequenced (1st Base, Malaysia), the sequences analyzed for accuracy and were verified to match the published sequence. Transgenic lines were generated at C-CAMP, Bangalore, India, using standard microinjection techniques.

Fat Body Dissection

Third instar larvae were floated using 20% sucrose and transferred to a Petri dish. The larvae were dissected in 1xPBS, the cuticle was torn along the length of the larvae using forceps to expose all the internal organs including the fat body.

Immunostaining

Third instar larvae were dissected in 1xPBS and fixed with 4% paraformaldehyde (PFA) overnight at 4°C. Fixed larval carcasses were washed with 0.1% PBTx (1xPBS + 0.1% Triton X-100) four times for 5 min each and blocked in PBTxGS (0.1% PBTx + 5% Normal Goat Serum) for 2–4 h at RT. After blocking, carcasses were incubated in primary antibody solution overnight at 4°C. The next day samples were washed with 0.1% PBTx four times for 20 min each and blocked in PBTxGS for 1–2 h at RT. Carcasses were incubated in secondary antibody solution for 2–3 h at RT (protected from light) followed by four washes with 0.1% PBTx for 20 min each. One μg/ml DAPI solution prepared in 0.1% PBTx was added to the sample and incubated for 10 min followed by washing with 0.1% PBTx three times for 10 min each. In the final step, the fat body along with the larval gonads was separated from the carcasses and mounted in Prolong Gold anti-fade reagent (Invitrogen Inc, United States).

Dissected adult ovaries were fixed in 4% PFA for 15 min, washed three times with 0.1% PBTx for 5 min each and blocked with 1% PBTx containing 0.5% BSA for 1 h at room temperature. The ovaries were incubated with primary antibody in 0.3% PBTx containing 0.5% BSA overnight at 4°C. The next day primary antibody was removed and the sample was washed with 0.1% PBTx for 15 min then blocked with 10% NGS in 0.1% PBTx for 2 h at room temperature. Ovaries were then stained with secondary antibody in 0.1% PBTx containing 10% NGS for 2 h at room temperature protected from light. Excess antibody was removed by three washes with 0.1%PBTX for 15 min each. One μg/ml DAPI solution in 0.1% PBTx was added to the samples and incubated at room temperature for 10 min followed by washing with 0.1% PBTx solution 3 times for 5 min each. Ovarioles were mounted in Prolong Gold.

The following antibodies and dilutions were used; anti-Cathepsin L (Abcam Cat# ab58991, RRID:AB_940826) 1:400 for larval tissues and 1:300 for adult ovaries; anti-ATP5α 1:100 (Thermo Fisher Scientific Cat# 43-9800, RRID:AB_2533548), anti-GABARAP 1:100 (Cell Signaling Technology Cat# 13733), anti-Ref(2)p 1:1000 (Abcam Cat# ab178440), anti-GFP 1:10 (DSHB Cat# DSHB-GFP-12A6, RRID:AB_2617417) anti-α spectrin 1:20 (DSHB Cat# 3A9 (323 or M10-2), RRID:AB_528473). Secondary antibodies used were Alexa fluor 555 goat anti-rabbit 1:250 (Molecular Probes Cat# A-21429, RRID:AB_141761) and Alexa fluor 488 goat anti-rabbit 1:250 (Molecular Probes Cat# A-11070, RRID:AB_142134).

Redox Chemical Treatment

Ovaries of mito-roGFP2-Orp1 flies were dissected in Grace's medium and washed in 1xPBS for 2 min. The ovaries were incubated in 4 mM diamide (Sigma Cat # D3648) in 1xPBS in order to oxidize or 40 mM DTT (SRL Cat# 17315) in 1xPBS in order to reduce for 10 min at room temperature with gentle shaking. They were washed once in 1xPBS for 2 min before being treated with the redox conservative reagent; 20 mM *N*-ethyl maleimide (Sigma Cat# E3876) in 1xPBS for 10 min at room temperature. The untreated set and pharmacologically treated samples were proceeded directly for redox conservation. They were washed once in 1xPBS before being fixed in 4% PFA for 15 min at room temperature. Following fixation, two washes of 1xPBS for 5 min each were given. The ovaries were mounted in 80% glycerol and imaged the same day. The redox

treatment procedure is modified from Albrecht et al., 2011; (Nilangekar and Shravage, 2018).

Imaging and Analysis

All imaging was performed on Leica SP8 Confocal microscope using 63x oil objective. Images acquired were 8 bit, 1024 × 1024 pixel resolution at 100 Hz scanning. Frame accumulation was performed with 6 frames for mCherry-Atg8a and GFP-Ref(2)P. Images were analyzed using ImageJ. For mCherry-Atg8a mean intensity measurements, an ROI was drawn around the GSCs, identified by the location and size of their nuclei. mCherry-Atg8a and GFP-Ref(2)P punctae were counted manually and the area of germarium were measured using ImageJ. For colocalization analysis, JACoP plugin was used (Bolte and Cordelières, 2006). Both the channels were thresholded in the plugin and Pearson's coefficient were recorded. Microsoft Excel was used for statistical analysis. Student's *T*-Test of two samples assuming unequal variance was performed for all comparisons. Graphs were plotted in GraphPad Prism 7.

Imaging and Image Analysis of Mito-roGFP2-Orp1

roGFP2 was excited at 405 and 488 nm "line by line" and its emission from both of these excitations was collected between 500 to 530 nm. Frame accumulation was performed with 6 frames for both the channels.

The ratiometric analysis was performed using ImageJ by the following steps; the background was subtracted (roll ball = 50 pixels), images were converted to 32 bit, the 488 channel was thresholded and the background pixels were set as "NaN," the intensity in the GSCs from the same region in both channels was measured. To generate the ratio image, "Ratio Plus" plugin was used and the resultant image was displayed in the "Fire" lookup table.

Western Blotting

For lysate preparation, 25–30 flies were dissected in a buffer containing 1 M NaCl, 50 mM Tris and protease inhibitors (Merck Cat # 11697498001). The ovaries were transferred to 1.5 ml tube and the buffer was replaced with RIPA buffer (1 M NaCl, 50 mM Tris, 1% Nonidet-P 40, 5% Sodium deoxycholate, 1% SDS) containing protease inhibitors. The tissue was homogenized on ice using plastic pestles. The homogenate was centrifuged at 20,000 rcf for 3 min at 4°C, the supernatant was transferred to a fresh tube and this step was repeated two more times. This lysate was quantified using BCA (Thermo Fisher Cat # 23227) and final concentration with addition to 2x Laemmli buffer (4% SDS, 5% 2-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 125 mM Tris) was adjusted so that equal quantities of protein could be loaded for the sets.

SDS-PAGE and Western Blotting

Total of 66, 50, and 55 μg/well protein sample was loaded for 12, 24, and 48 h treated samples respectively. Protein samples were run in 4–20% gradient SDS polyacrylamide gel (Bio-Rad Cat # 4561096) and transferred onto a PVDF membrane (Bio-Rad Cat # 1620177) by the wet transfer method. The membranes were blocked in 5% non-fat milk in TBST (50 mMTris, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, washed thrice with TBST for 5 min each. The membranes were then incubated with primary antibody solution at 4°C with gentle shaking overnight. The membranes were washed thrice with TBST for 5 min each. HRP linked secondary antibody binding was performed at room temperature for 1 h or 4°C overnight. The membranes were then washed thrice with TBST for 5 min each. Detection was done using ECL kit (Bio-Rad Cat # 1705062) and chemiluminescence was detected on the Bio-Rad ChemiDoc XRS+ system. The following antibodies and dilutions were used; anti-actin 1:100 (DSHB Cat# JLA20, RRID:AB_528068), anti-mCherry 1:10 (DSHB Cat# DSHB-mCherry-3A11, RRID:AB_2617430), anti-GFP (Novus Cat# NB 600-308, RRID:AB_341929), HRP-Goat anti mouse 1:4000 (Cloud-Clone Corp. Cat # SAA544Mu19), HRP-Goat anti rabbit 1:4000 (Cloud-Clone Corp. Cat # SAA544Rb19), anti-mCherry 1:1000 (Thermo Fisher Scientific Cat# PA5-34974, RRID:AB_2552323), HRP-Goat anti rat 1:4000 (Cloud-Clone Corp. Cat # SAA544Ra09).

RESULTS

Expression Analysis of GFP-Ref(2)P

Ref(2)P (SQSTM1/p62 in mammals) is one of the cargo receptors that binds polyubiquitylated substrates and aids in their recruitment into autophagosomes that are marked for degradation. In *Drosophila*, Ref(2)P was first characterized for its role in sigma rhabdovirus multiplication (Dezelee et al., 1989). Recently, it was shown that Ref(2)P is the *Drosophila* ortholog of mammalian p62, and the conserved PB1 and UBA domains are necessary for protein aggregate formation (Nezis et al., 2008). Genetic and pharmacological experiments in *Drosophila* demonstrate that reduction of autophagy activity leads to an accumulation of Ref(2)P-positive protein aggregates, suggesting that it can be used as a marker of autophagic activity (Nezis et al., 2008; Bartlett et al., 2011; Devorkin and Gorski, 2014).

We created a pCasper4 based plasmid that possesses 935 bp *nanos* promoter and a 1236 bp 3′ UTR of *nanos* that stabilizes transcripts in the germline to generate GFP-Ref(2)P (Doren et al., 1998; Rørth, 1998; Nilangekar and Shravage, 2018; **Figure 1**). Nanos is a maternally expressed and is required for the process of oogenesis and egg production. Nanos expression is detected very early in primordial germ cells of the embryo which later become the germline cells of larval gonads and of the adult ovaries (Wang et al., 1994; Rørth, 1998; Dansereau and Lasko, 2008). We tested larval and adult ovaries for GFP-Ref(2)P expression and its subcellular localization. GFP-Ref(2)P puncta could be readily detected in the GSCs of both larval ovaries and adult ovaries (**Figure 2** and **Supplementary Figures S1A,B**). Larval ovaries from these transgenic lines showed high levels of GFP-Ref(2)P in the developing GSCs. GFP-Ref(2)P expression was weaker and diffused in the support cells and in the region where

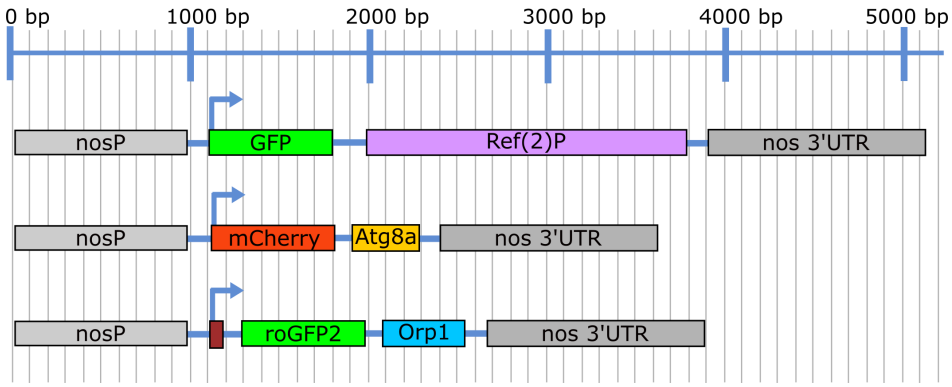


FIGURE 1 | Illustration showing the expression cassettes for the three nosP transgenic constructs. Blue line depicts the size in base pairs (bp).

niche cells would develop (**Supplementary Figure S1A**). Several of the germaria and late egg chambers possessed rod-shaped distribution of GFP-Ref(2)P which was described previously (Nezis, 2012; **Supplementary Figure S1D**). The distribution of GFP-Ref(2)P puncta within the germarium exhibited differences. Interestingly, majority of GFP-Ref(2)P puncta were found to be localized in the region 2 (2a and 2b) of germarium while region 1 appeared to have very few or no punctate GFP structures. In our analyses upto 30% germaria did not possess GFP-Ref(2)P punctae (**Supplementary Figure S2D**). Antibodies against GFP and p62 were used to validate the expression of GFP-Ref(2)P during oogenesis. Significant overlap between anti-GFP and GFP-Ref(2)P punctate structures was observed and further supported by high Pearson's coefficient. Approximately 60% of the GFP-Ref(2)P puncta were positive for anti-p62 as indicated by Pearson's correlation coefficient (**Supplementary Figures S2A–C**).

The transgenic line was subjected to nutrient stress and pharmacological treatments to validate their utility in various autophagy assays. Females expressing GFP-Ref(2)P were grown on sucrose only diet (nitrogen starvation), rapamycin (autophagy inducer) and 4-hydroxy-chloroquine (CQ) (autophagosome-lysosome fusion inhibitor; for details please refer to methods sections) for varying periods of time (Klionsky et al., 2016). The treatments were carried out for 6, 12, 24, 48, and 96 h. The ovary morphology changes in response to these stress stimuli are shown for each treatment in **Supplementary Figure S1C**. The number of GFP-Ref(2)P puncta decreased significantly in germaria obtained from starved females as compared to females reared on nutrient-rich food indicating induction of autophagy. CQ neutralizes lysosomal pH which subsequently prevents autophagosome-lysosome fusion (Ahlberg et al., 1985; Yoon et al., 2010). Upon CQ treatment, the number of GFP-Ref(2)P puncta significantly increased, as compared to germaria obtained from starved females indicating a disruption autophagic degradation of Ref2P (**Figure 2A**). Rapamycin induces autophagy by inhibiting mTOR kinase (Noda and Ohsumi, 1998; Klionsky et al., 2016). Females grown on food containing rapamycin exhibited a significant reduction of GFP-Ref(2)P puncta suggesting a robust induction of autophagy in germaria (**Figures 2A,B**).

Autophagy dependent cleavage of GFP-Ref(2)P is one of the ways to monitor its degradation. GFP is comparatively resistant to lysosomal hydrolases due to its compact globular structure. The liberation of free GFP from GFP-Ref(2)P following its delivery to the lysosome and can be reliably assayed using an immunoblot assay (Pircs et al., 2012; Devorkin and Gorski, 2014; Klionsky et al., 2016). We subjected GFP-Ref(2)P transgenic females to starvation induced autophagy and CQ treatment, extracted protein from the ovaries and tested if free GFP is liberated (**Figure 2C** and **Supplementary Figure S8**). As seen from **Figure 2C**, the anti-GFP antibody could detect changes in the levels of GFP-Ref(2)P fusion protein (∼130 kDa) in fed, starved and CQ treated conditions. For instance, high molecular weight bands > 130 kDa [GFP-Ref(2)P aggregates] were detected in starved and CQ treated conditions. Interestingly, a number of bands ranging from ∼ 60kDa–25kDa, intermediate degradation products of GFP-Ref(2)P, could be detected with the anti-GFP antibody. In particular, the predominant GFP-Ref(2)P intermediate degradation product was detected at ∼60 kDa. This GFP-Ref(2)P intermediate degradation product was seen to be enriched in starved condition and its abundance was found to be decreased upon CQ treatment. Free GFP (∼27 kDa) was not detected in any of the conditions tested and possible reasons are discussed in later section (Pircs et al., 2012; **Figure 2C** and **Supplementary Figures S8A,B**). Taken together, our data suggest that the GFP-Ref(2)P transgenic lines could be used as a reporter of autophagy in the female germline of *Drosophila*.

Expression Analysis of mCherry-Atg8a

Due to its ubiquitous expression in most tissues, Atg8a has been routinely used to monitor autophagy using various techniques including western blotting and immunofluorescence microscopy. Atg8a has been shown to be induced in germline cells as well as follicle cells in response to starvation during *Drosophila* oogenesis (Nezis et al., 2010a; Barth et al., 2011; Hegedus et al., 2016; Bali and Shravage, 2017).

Transgenic lines expressing *mCherry-Atg8a* under the *nanos* promoter were generated as described in materials and methods. mCherry-Atga8a expression was monitored in larval ovaries. mCherry-Atg8a was found to be diffused in larval GSCs but

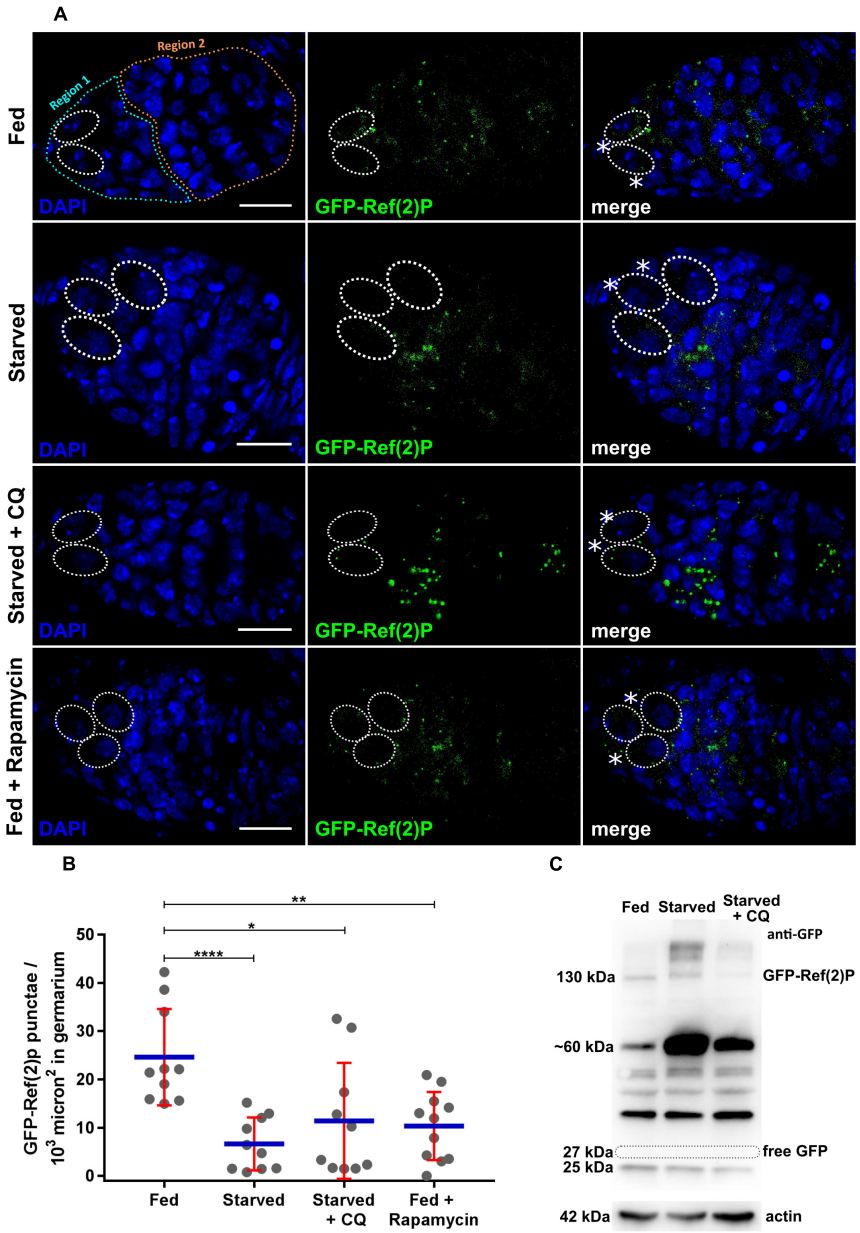


FIGURE 2 | Expression of nosP-GFP-Ref(2)P nos 3′UTR. **(A)** GFP-Ref(2)P punctae in germarium of flies subjected to fed (for 6 days), fed + starved (2 days fed + 4 days), starved + Chloroquine (2 days fed + 4 days), fed + rapamycin (2 days fed + 4 days). Dotted ovals mark the GSCs and the asterisks mark the cap cells. Scale bar 10 μm. **(B)** Interleaved scatter graph showing distribution of GFP-Ref(2)P punctae in germaria as a function of various treatments carried out for 12 h. Error bars represent SD in red and the mean is blue. $n = 10$ for fed, starved, starved and chloroquine and 11 for fed with rapamycin respectively. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. **(C)** Western blot analysis using anti-GFP antibodies of ovarian extracts expressing GFP-Ref(2)P. 130 kDa GFP-Ref(2)P band could be detected in fed, starved and starved + CQ treated ovaries for 48 h. Loading control actin is shown below for the same samples.

was punctate in the differentiated cells of larval ovaries. Unlike in GFP-Ref(2)P transgenic lines, no signal was detected in the fat body cells surrounding the larval ovaries (**Supplementary Figure S3A**). mCherry-Atg8a puncta could be detected in germaria and late egg chambers in adult ovaries (**Figure 3A** and **Supplementary Figures S3B**, **4A**).

The most dramatic change in localization of Atg8a appears when autophagy is induced, where cytoplasmic Atg8a in fed conditions localizes to autophagosomes and autolysosomes and is observed as punctate structures in fluorescence microscopy. Ovaries from adult females reared on 20% sucrose (nitrogen deprivation for 1–4 days) and fed flies (yeast) were dissected and assayed for mCherry-Atg8a expression (**Supplementary Figure S3C**). Our analyses suggested upregulation of autophagy by 24 h of starvation and a significant upregulation of autophagy by 48 h of starvation (**Supplementary**

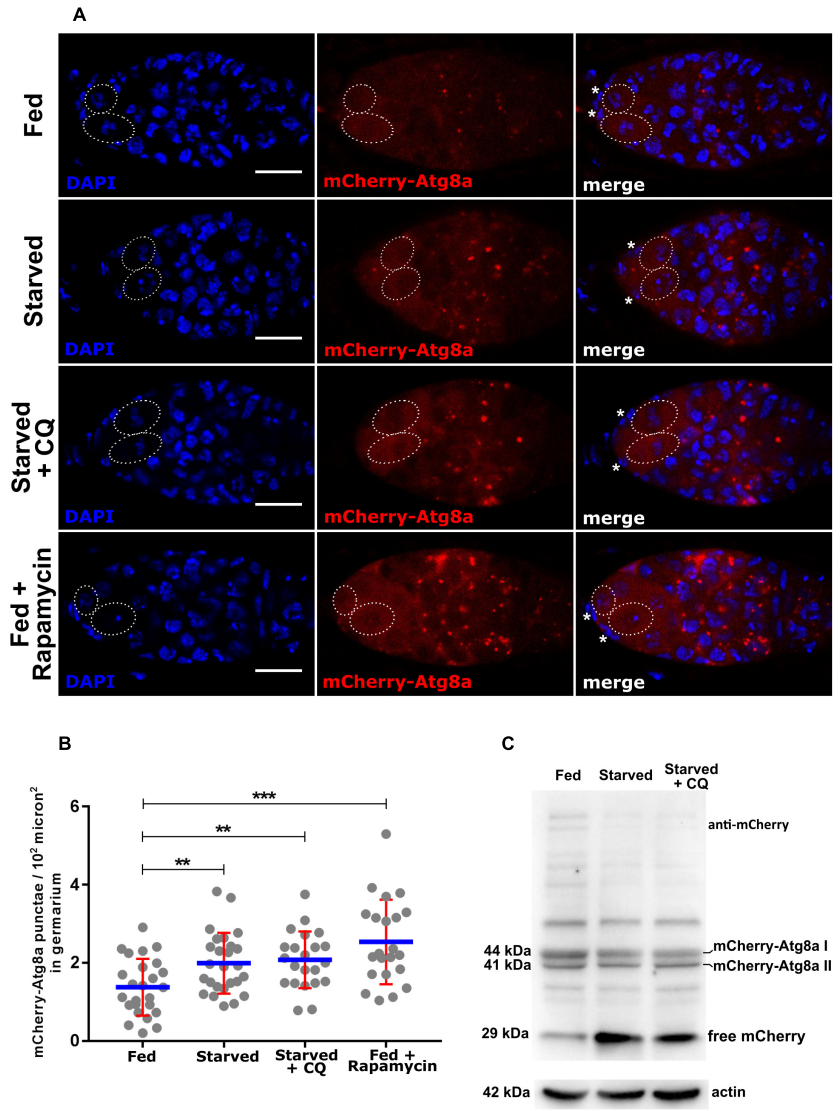


FIGURE 3 | Expression of nosP mCherry-Atg8a nos 3′UTR. **(A)** mCherry-Atg8a punctae in germarium of flies subjected to fed, starved, starved and chloroquine or fed with rapamycin treatment. Dotted ovals mark the GSCs and the asterisks mark the cap cells. Scale bar 10 μm. **(B)** Interleaved scatter graph showing distribution of mCherry-Atg8a punctae in germarium of flies subjected to fed (for 6 days), fed + starved (2 days fed + 4 days), starved + Chloroquine (2 days fed + 4 days), fed + rapamycin (2 days fed + 4 days). Error bars represent SD in red and the mean is blue. *n* = 25, 26, 22 and 22 for fed, starved, starved and chloroquine and fed with rapamycin respectively. $^{**}p < 0.01$, $^{***}p < 0.001$. **(C)** Western blot analysis using anti-mCherry antibodies of ovarian extracts expressing mCherry-Atg8a. 44, 41, and 29 kDa bands corresponding to mCherry-Atg8a-I, mCherry-Atg8a-II, and free mCherry respectively could be detected in fed, starved and starved + CQ treated ovaries for 48 h. Loading control actin is shown below for the same samples.

Figure S3D). However, we could detect an increase in total mCherry-(Atg8a) intensity in the GSCs in starved vs. fed GSCs (**Supplementary Figure S4E**). A significant increase in the number of mCherry-Atg8a punctae was detected in the entire germaria of ovaries dissected from fed vs. starved females (**Figures 3A,B**). The increase in mCherry-Atg8a punctae was concentrated in region 2 of germarium (**Figure 3A**). We next tested if disrupting autophagosome-lysosome fusion led to the accumulation of mCherry-Atg8a puncta. As expected, mCherry-Atg8a positive punctate structures accumulated within the germaria isolated from females grown on food containing CQ. In addition, germaria from rapamycin-treated transgenic females exhibited significantly higher number of mCherry-Atg8a puncta (autophagosomes and autolysosomes) as compared to transgenic females reared on nutrient rich food (**Figures 3A,B**). These data suggest that nosP-mCherry-Atg8a transgenic line could be used to monitor autophagy in the germarium.

Immunoblot analyses of mCherry-Atg8a (Atg8a) is recommended as it provides independent confirmation of autophagy induction (Mauvezin et al., 2014; Mulakkal et al., 2014; Nagy et al., 2015; Klionsky et al., 2016; Lörincz et al., 2017).

The degradation of mCherry-Atg8a II fusion protein within the lysosome is differential. The Atg8a-II part of the fusion protein is degraded rapidly while the mCherry part is relatively resistant to destruction by the lysosomal enzymes. The liberation of free mCherry can be reliably assayed using immunoblot analysis to infer autophagic flux (Mauvezin et al., 2014; Nagy et al., 2015; Klionsky et al., 2016). Transgenic females expressing mCherry-Atg8a were subjected to nutrient limitation and CQ treatment and, the protein from the ovaries was assayed for mCherry liberation using an immunoblot assay. In fed, starved and CQ treatment, both mCherry-Atg8a-I (∼44 kDa) and mCherry-Atg8a-II (∼41 kDa) forms were detected. As expected there was an increase in the formation of mCherry-Atg8a-II and corresponding increase in liberation of free mCherry (∼29 kDa) in starved conditions. As compared to fed and starved conditions, in CQ treated animals, the mCherry-Atg8a-II form was detected at significantly higher levels, however, the liberation of free mCherry was inhibited indicating impaired lysosomal destruction of mCherry-Atg8a (**Figure 3C** and **Supplementary Figures S8C,D**). These data suggest that the mCherry-Atg8a transgenic lines could be used to monitor autophagy during oogenesis.

We tested if mCherry positive punctate structures expressed during oogenesis from the transgene are positive for Atg8a. To test this, anti-GABARAP antibodies which also detect *Drosophila* Atg8a were utilized (Lörincz et al., 2017; Tusco et al., 2017). mCherry positive puncta colocalized with anti-GABARAP positive puncta both in the germarium and late stage egg chamber confirming expression of mCherry-Atg8a fusion protein (**Supplementary Figure S4A**).

UASp-mCherry-Atg8a autophagy reporter has been previously described and has been demonstrated to monitor autophagy in the nurse cells during oogenesis (Jacomin and Nezis, 2016). In this study, we compared UASp-mCherry-Atg8a (nanos-Gal4VP16 X UASp-mCherry-Atg8a) and nosP-mCherry-Atg8a simultaneously in starvation-induced autophagy assay in the ovaries. mCherry-Atg8a punctae are not significantly different between germaria dissected from nanos-Gal4 driven UASp-mCherry-Atg8a females and nosP-mCherry-Atg8a females (**Supplementary Figures S4D,E**). nanos-Gal4VP16 X UASp-mCherry-Atg8a germaria possess higher levels of cytoplasmic mCherry-Atg8a in region 2 (4, 8 and 16 cell cysts) of germaria. Upon nutrient limitation, the increase in the number and fluorescence intensity of mCherry-Atg8a punctae in both nanos-Gal4 X UASp-mCherry-Atg8a expressing germaria and nanosP-mCherry-Atg8a germaria is comparable (**Supplementary Figures S4D,E**).

We checked if nosP-mCherry-Atg8a transgene can rescue lethality associated with $Atg8a^{KG07569}$ transposon insertion. Homozygous $Atg8a^{KG07569}$ insertion mutants lack detectable levels of Atg8a protein as tested by western blotting technique (Chang et al., 2013). The rescue experiment is designed to test if the expression level of mCherry-Atg8a from nanos promoter is adequate enough for the complementing the deficiency of Atg8a in homozygous $Atg8a^{KG07569}$ mutant. Two separate nosP-mCherry-Atg8a insertions were tested for rescue of lethality of $Atg8a^{KG07569}$ mutant. Our data indicate that both transgenes were capable of complementing the deficiency of Atg8a in homozygous $Atg8a^{KG07569}$ mutant. Mendelian ratios of inheritance were observed (**Supplementary Figure S5B**). Taken together, these data suggest that nosP-mCherry-Atg8a transgenic line could be used to monitor autophagy in GSCs, germaria and nurse cells during oogenesis.

Measurement of Autophagy Flux

Autophagic flux is a measure of degradation of autophagic cargo within the lysosomes. There are several methods to measure autophagic flux. Atg8a based assays measure autophagic carrier flux and not autophagic cargo/substrate flux *per se* (Tanida et al., 2005; Klionsky et al., 2016). While Ref(2)P based assays are considered to be a better measure of autophagic flux. This is due to the fact that Ref(2)P has ability to bind to polyubiquitinated proteins/substrates which allows for their delivery to the autophagosome. In addition, upon starvation and rapamycin treatment, Ref(2)P displays the largest degree of change which can be quantified reliably (Klionsky et al., 2016). To test if transgenic lines expressing GFP-Ref(2)P and mCherry-Atg8a individually could be used for assaying flux we stained them for CathepsinL. CathepsinL is cysteine protease that is a component of the lysosomal acid proteases and used as a lysosomal marker (Klionsky et al., 2016). GFP-Ref(2)P (green) puncta localized close to CathepsinL (red) dots in the germarium and nurse cell cytoplasm in late egg chambers (**Figure 4A** and **Supplementary Figure S6A**). As expected three different types of puncta were visible viz. green puncta (GFP-Ref(2)P aggregates alone or vesicle-bound), and red puncta (lysosomes) could be detected along with yellow puncta (autolysosomes). Pearson's coefficient of GFP-Ref(2)P and CathepsinL colocalization was found to be significantly lower in starved conditions as compared to fed conditions (**Figures 4A,C**). In contrast, mCherry-Atg8a (red) puncta colocalized with CathepsinL (green dots) stained lysosomes. In this experiment, three different puncta were visible: red puncta which represent autophagosomes, yellow puncta which correspond to autolysosomes, and green dots that depict lysosomes. Pearson's coefficient showed a significant increase of mCherry-Atg8a and CathepsinL colocalization in starved vs. fed conditions (**Figures 4B,D** and **Supplementary Figure S6B**).

To get a better estimate of autophagic flux transgenic lines expressing both GFP-Ref(2)P and mCherry-Ag8a were crossed together and ovaries were dissected and stained for CathepsinL. Several puncta positive for GFP-Ref(2)P, mCherry-Atg8a and CathepsinL were visible in fed germaria (**Figure 4E** and **Supplementary Figure S6C**). Taken together, our analyses suggest that these transgenic lines could be used to measure changes in autophagic flux in combination with CathepsinL.

The Utility of Transgenes in Genetic Screens

One of the advantages of the *Drosophila* model system is the ability to conduct rapid forward and reverse genetic screens (St Johnston, 2002). We examined whether the GFP-Ref(2)P and mCherry-Atg8a reporters could be of utility in genetic screens. A double-stranded inverse-repeat (IR)

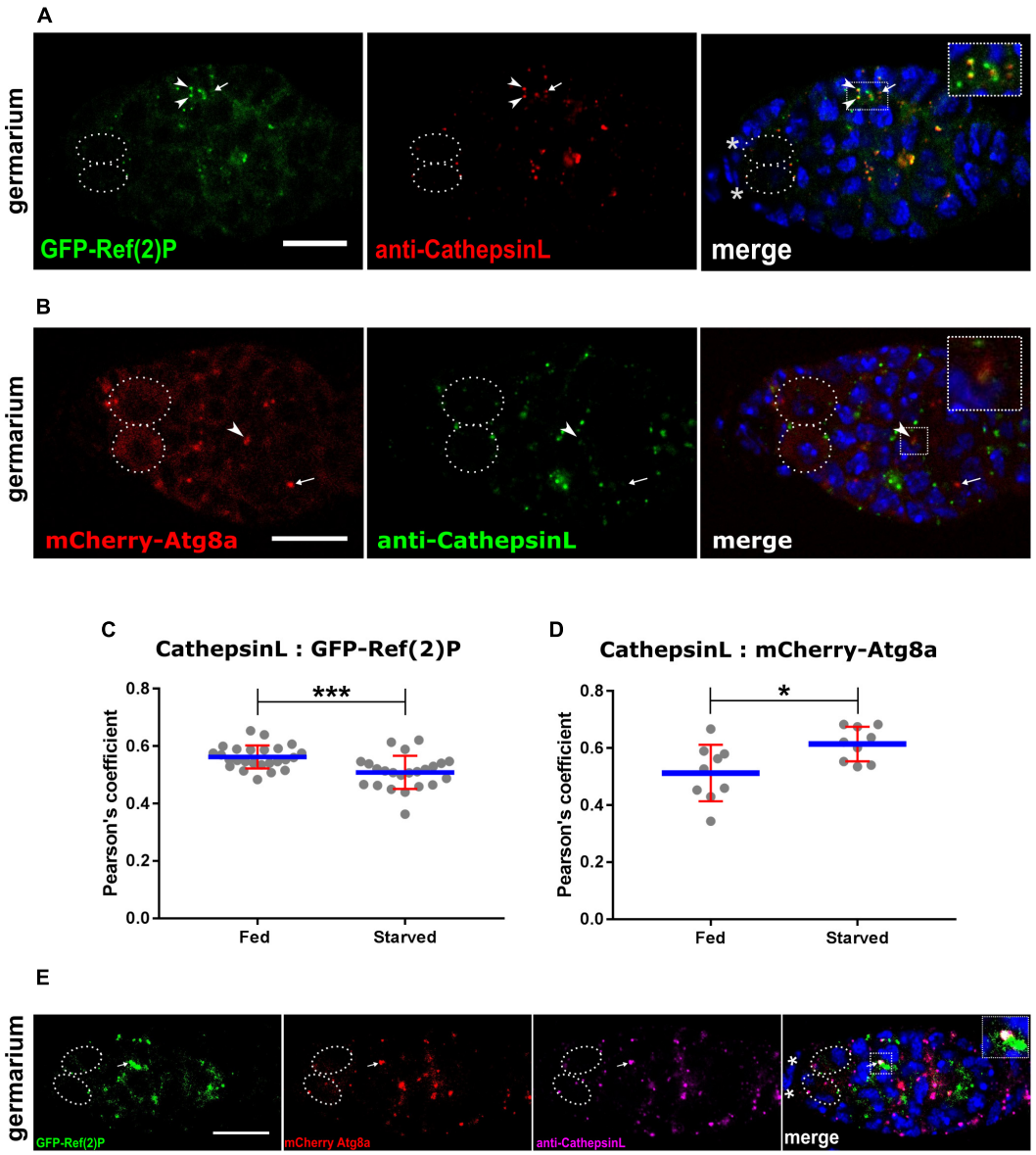


FIGURE 4 | Measurement of autophagy flux together with CathepsinL. **(A)** GFP-Ref(2)P punctae colocalizing with CathepsinL region (lysosomes). Arrowheads point to colocalized punctae and arrows indicate Cathepsin-L alone punctae. Inset shows enlarged region of Ref(2)P sequestered by lysosomes. Dotted ovals mark the GSCs and the asterisks mark the cap cells. Scale bar 10 μm. **(B)** mCherry-Atg8a punctae colocalizing with CathepsinL region (lysosomes). Arrowheads point to colocalized punctae (autolysosomes) and arrows indicate mCherry-Atg8a alone punctae (autophagosomes). Inset shows enlarged region with an autolysosome. Dotted ovals mark the GSCs and the asterisks mark the cap cells. Scale bar 10 μm. **(C)** Starvation induced autophagic degradation of Ref(2)P. Interleaved scatter graph showing Pearson's coefficient as measure of colocalization of Cathepsin-L and GFP-Ref(2)P in germarium of fed and starved nosP-GFP-Ref(2)P transgenic flies immunostained with anti-Cathepsin-L antibody. Error bars represent SD in red and the mean is blue. $n = 25$ for fed (6 days) and $n = 23$ for starved (2 days fed + 4 days starved). ***$p < 0.001$. **(D)** Induction of autophagy upon starvation. Interleaved scatter graph showing Pearson's coefficient as measure of colocalization (autolysosome) of Cathepsin-L (lysosome) and mCherry-Atg8a (autophagosomes) in germarium of fed and starved nosP-mCherry-Atg8a transgenic flies immunostained with anti-Cathepsin-L antibody. Error bars represent SD in red and the mean is blue. $n = 9$ for both fed and starved. *$p < 0.05$. **(E)** Ref(2)P sequestered by autophagosomes and fused to lysosomes. Germarium of mCherry-Atg8a; GFP-Ref(2)P flies immunostained for Cathepsin-L. Inset shows enlarged region having a puncta (arrow) positive for GFP, mCherry as well as Cathepsin-L. Dotted ovals mark the GSCs and the asterisks mark the cap cells. Scale bar 10 μm.

construct designed to target and knockdown Atg5 (Atg5IR) was expressed specifically in the germline cells using nanos-Gal4VP16 (Doren et al., 1998; Ni et al., 2008). The expression and cytoplasmic localization of GFP-Ref(2)P and mCherry-Atg8a was monitored in Atg5 knockdown cells. Indeed, as compared to control, germaria expressing Atg5IR failed to degrade GFP-Ref(2)P as seen from the accumulation of GFP positive punctate structures. Moreover, these GFP-Ref(2)P puncta appeared to be larger (GFP-Ref(2)P aggregates) as compared to GFP-Ref(2)P in control germaria indicating

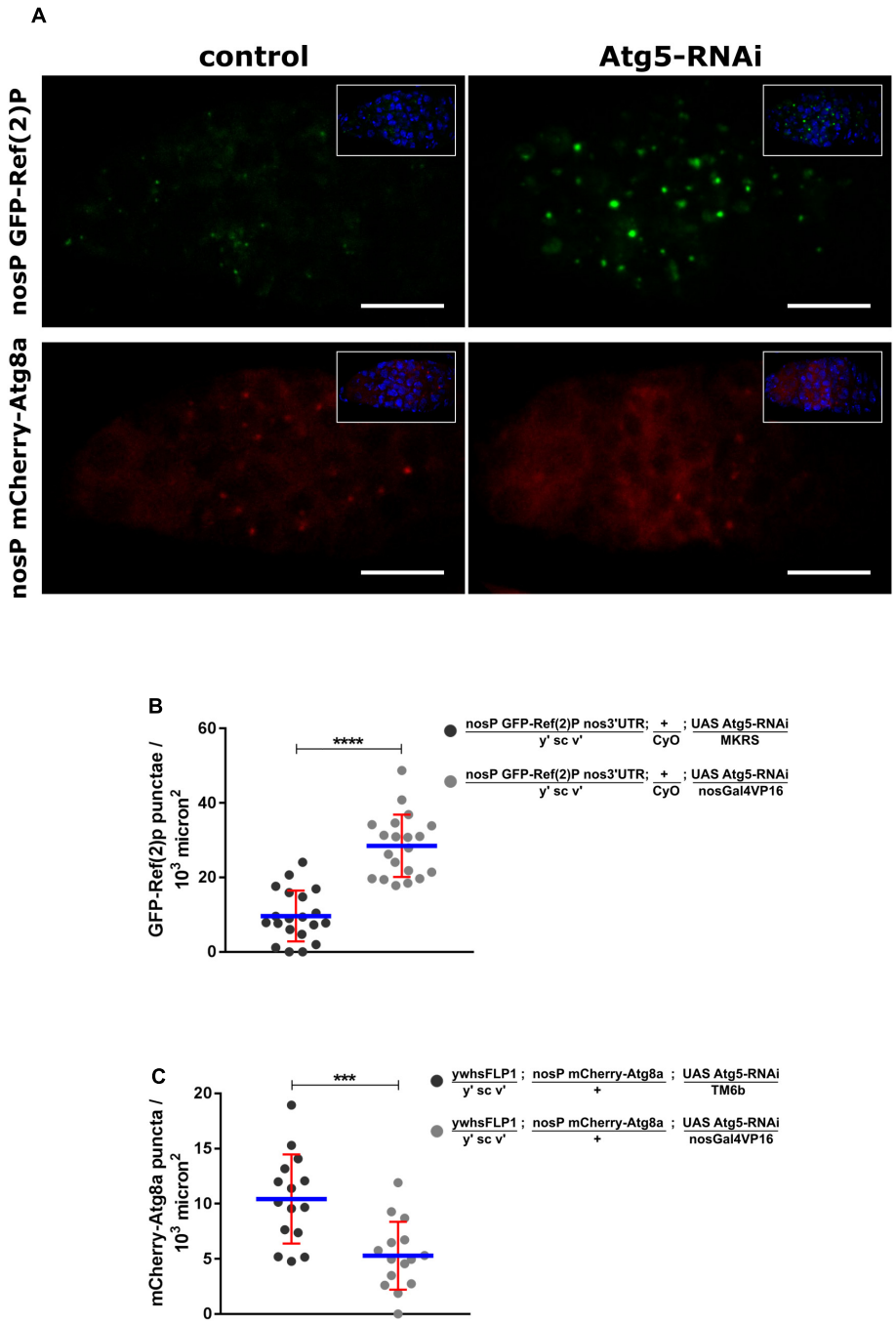


FIGURE 5 | Utility of transgenic reporter lines in RNAi based genetic screen. **(A)** Germaria of nosP GFP-Ref(2)P nos 3′UTR and nosP mCherry-Atg8a nos 3′UTR transgenic flies in background of either no knockdown (control) or knockdown of Atg5 by RNAi in the germline. Scale bar 10 μm. **(B)** Interleaved scatter graph showing the increase in GFP-Ref(2)P punctae upon Atg5 knockdown. Error bars represent SD in red and the mean is blue. $n = 20$ for both the data sets. ****$p < 0.0001$. **(C)** Interleaved scatter graph showing the decrease in mCherry-Atg8a punctae upon Atg5 knockdown. Error bars represent SD in red and the mean is blue. $n = 15$ for both the data sets. ***$p < 0.001$.

impaired autophagy (**Figures 5A,B**). Knockdown of Atg5 in germaria led to the disruption of punctate localization of mCherry-Atg8a indicating reduced autophagosome formation when compared to control germaria. mCherry-Atg8a was predominantly cytoplasmic in Atg5IR expressing germaria further supporting a decrease in autophagosome formation (**Figures 5A,C**). Similar results were obtained when Atg8aIR was expressed in the germ cells (**Supplementary Figure S5A**). Taken together, these data suggest that both GFP-Ref(2)P and mCherry-Atg8a could be used in conducting genetic screens

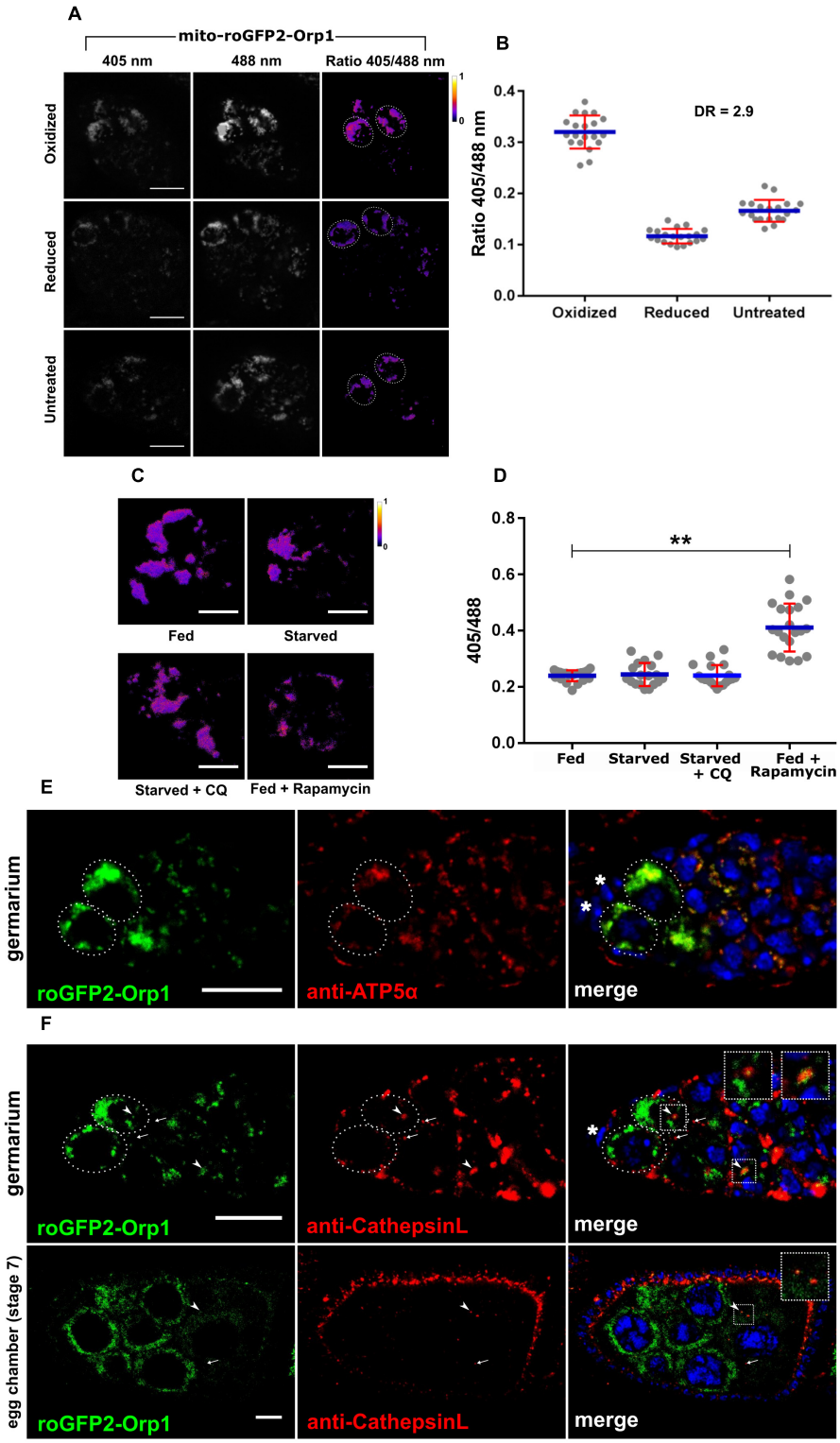


FIGURE 6 | Expression and function of nosP-mito-roGFP2-Orp1-nos 3′UTR reporter. **(A)** mito-roGFP2-Orp1 monitors the redox state in the GSCs. Ratio of emission at 405 and 488 nm obtained as a response of the reporter to applied oxidant (DA) or reductant (DTT). Dotted ovals mark the GSCs. Scale bar 10 μm. **(B)** Interleaved scatter graph showing ratiometric shift of excitation. Error bars represent SD in red and the mean is blue. *n* = 20 for each condition. DR stands for dynamic range. **(C)** Redox state of GSCs of mito-roGFP2-Orp1 transgenic flies for fed (for 6 days), fed + starved (2 days fed + 4 days), starved + Chloroquine (2 days fed + 4 days), fed + rapamycin (2 days fed + 4 days) treatment. Scale bar 5 μm. **(D)** Interleaved scatter graph showing ratiometric shift of excitation fed (for 6 days), fed + starved

(Continued)

FIGURE 6 | Continued
(2 days fed + 4 days), starved + Chloroquine (2 days fed + 4 days), fed + rapamycin (2 days fed + 4 days) treatment. Error bars represent SD in red and the mean is blue. n = 20, 18, 19, and 21 for fed, fed + starved, starved + Chloroquine, fed + rapamycin respectively. $^{**}p < 0.01$. **(E)** Germarium of mito-roGFP2-Orp1 transgenic flies immunostained with anti-ATP5α antibody that marks the mitochondria. Dotted ovals mark the GSCs and the asterisks mark the cap cells. Scale bar 10 μm. **(F)** mito-roGFP2-Orp1 (green) in the germarium and a stage 7 egg chamber shows co-localization with lysosomes marked by Cathepsin-L (red). Some lysosomes are GFP positive (yellow) marked by arrow heads while the arrows mark GFP negative lysosomes. Inset shows the enlarged region of the fusion of mitochondrion (presumably in the autophagosome) and lysosome. Dotted ovals mark the GSCs and the asterisk marks the cap cell. Scale bar 10 μm.

to identify modules contributing to the maintenance and induction of autophagy.

Expression Analysis of mito-roGFP2-Orp1

Mitochondria are susceptible to oxidative damage due to the production of mitochondrial reactive oxygen species (mROS) which include OH^-, O_2^- and H_2O_2 (Dröge, 2002; Dan Dunn et al., 2015). Damaged mitochondria lose their redox potential and are subsequently cleared via mitophagy. mROS are products of normal as well as altered cell physiology and can provide valuable information of cellular health (Dröge, 2002; Shadel and Horvath, 2015; Tan et al., 2017). We generated a germline mitochondria-specific sensor for H_2O_2, termed here as mito-roGFP2-Orp1.

The expression of this H_2O_2 sensor was tested in the larval and adult ovaries. mito-roGFP2-Orp1 was detected in the entire germarium with very strong expression in the GSCs (Tan et al., 2017). mito-roGFP2-Orp1 distribution in GSCs appeared to be predominantly at the periphery of the GSC nucleus with random distribution in cysts present in region 2 of the germarium. In late stage egg chambers, mito-roGFP2-Orp1 was also detected surrounding the nurse cell nucleus. The follicle cells were devoid of any GFP expression (**Figures 6A,F**). mito-roGFP2-Orp1 can be detected in the larval ovaries with predominant expression within the developing GSCs. GFP signal was not detected in differentiating cells of the larval ovaries or in the fat tissue surrounding the larval ovaries (**Supplementary Figure S7**).

We tested if mito-roGFP2-Orp1 could be used to sense the mitochondrial redox potential within the germarium and GSCs as the mito-roGFP2-Orp1 cassette carries a mitochondrial localization signal at the N-terminal. This reporter senses H_2O_2 first via Orp1 which oxidizes when exposed to H_2O_2 and relays the oxidation state to roGFP2 which leads to shift in its excitation maxima of roGFP2 from 488 to 405 nm (Albrecht et al., 2011, 2014). The ovarian tissue was subjected to complete oxidation and reduction using chemical agents, and the excitation maxima of the reporter was tested in the GSCs. As shown in **Figures 6A,B** this redox sensor has the ability to sense both reduced and oxidation states of mitochondria within the GSCs. The dynamic range of the reporter that measure the maximum oxidation and maximum reduction and was found to be 2.9 in the GSCs (**Figure 6B**).

To determine if mito-roGFP2-Orp1 expression or its distribution changes in response to different stress stimuli, we subjected mito-roGFP2-Orp1 expressing females to nutrient limitation, CQ treatment and rapamycin exposure. As compared to nutrient-rich conditions, starvation of the mito-roGFP2-Orp1 expressing females led to reduced roGFP2 expression within the GSCs indicating turnover of mitochondria. In contrast, exposure to CQ during nutrient limitation restored the roGFP2 expression in the GSCs indicating disruption of mitophagy. Rapamycin treated germaria exhibited reduced roGFP2 expression indicating upregulation of mitophagy leading to clearance of mitochondria within the GSCs (**Figure 6C**). We further examined the shift in excitation maxima of roGFP2 from 488 to 405 nm in response to these stress stimuli. A shift in excitation maxima of roGFP2 was not detected when females expressing the transgene were grown on nutrient-rich, nutrient-deprived or in presence of CQ. However, we observed a significant shift in excitation maxima of roGFP2 from 488 to 405 nm indicating increased oxidative stress in GSCs upon rapamycin exposure (**Figure 6D**).

We confirmed that the mito-roGFP2-Orp1 expression is indeed mitochondrial by co-staining with an anti-ATP5α antibody. Our data suggest near complete overlap of the roGFP2 signal with anti-ATP5α suggesting mitochondrial-specific expression of the mito-roGFP2-Orp1 transgene (**Figure 6E**). To test if mito-roGFP2-Orp1 could be used to track mitophagy we stained ovaries dissected from flies expressing mito-roGFP2-Orp1 with CathepsinL. As seen in **Figure 6F** we could detect colocalization of GFP (green mitochondria) and CathepsinL (red lysosomes) in germarium and stage 7 egg chambers suggesting occurrence of mitophagy.

DISCUSSION

Here we describe generation and characterization of three transgenic lines that aid in assaying for autophagy, mitochondrial H_2O_2 production and mitophagy specifically in the GSCs. The purpose of generating these lines was multifold. First, to avoid problems linked with Gal4/UASp mediated overexpression of GFP-Ref(2)P, mCherry-Atg8a and mito-roGFP2-Orp1. Second, to validate expression and localization of GFP-Ref(2)P, mCherry-Atg8a and assay for H_2O_2 production in the GSC and their progeny. Third, to document any differences in expression in different cell types within the germarium including GSCs. And finally, to test the utility of the transgenes in the measurement of autophagy flux and mitophagy.

Although Gal4/UASp is a powerful tool to express genes of interest in the germline cells, it has limitations similar to those documented in the case of GAL4/UAS. Gal4/UASp driven transgenes have mosaic/patchy expression within the tissue (Brand and Perrimon, 1993). This leads to significant differences in the expression levels of the transgenes and in addition the variance is dependent on cell type within the tissue (Duffy, 2002). Our analyses with nanosGal4/UASp-mCherry-Atg8a in

the germarium confirms the patchy mCherry-Atg8a expression (**Supplementary Figure S4B**, fed panel vs. germarium panel). Since Gal4/UASp too is a binary system the utility of this system in genetic screens is reduced as the transgene is expressed only in the F1 generation. This can be disadvantageous as a potential positive hit identified from a genetic screen needs additional experimentation in F2 generation for confirmation. Further, GAL4/UASp driven transgenes may lead to over-production of the protein leading to erroneous results and complicate image analyses (**Supplementary Figure S4B**, note the excess deposition of mCherry-Atga in the developing cysts). The nanos promoter driven transgenes provide a superior alternative to Gal4/UASp driven transgenes, as the transgenes are expressed directly from nanos promoter. This leads to production of moderate to low levels of mRNA and protein within the germline cells and it is possible to conduct F1 genetic screens (**Figures 2**, **3**, **6**).

The earliest expression of GFP-Ref(2)P in the larval ovaries was detected in the GSCs (**Supplementary Figure S1**). Fat body cells surrounding the larval ovaries also exhibited detectable levels of GFP-Ref(2)P expression. This was unexpected as *nanos* promoter is specific to germ cells and it has not been reported to be active in fat cells (Rørth, 1998; Gelbart and Emmert, 2013). However, we cannot rule out prolonged persistence of GFP-Ref(2)P deposited in the egg through larval development. *Drosophila* Ref(2)P was first characterized for its role in sigma rhabdovirus multiplication. Ref(2)P is mammalian homolog of p62, and impaired autophagy causes an accumulation of Ref(2)P- aggregates, indicating that it can be used as a marker of autophagic activity (Nezis et al., 2008, 2010b; Bartlett et al., 2011). Nezis et al. (2008) have also showed Ref(2)P expression in the nurse cells as well as in the follicle cells during oogenesis. Our data show that GFP-Ref(2)P is expressed in the GSCs and nurse cells in late egg chambers with punctate distribution in fed conditions (Devorkin and Gorski, 2014; Jacomin and Nezis, 2016). It is worth noting that a certain percentage of germaria analyzed did not bear any GFP-Ref(2)P puncta. Interestingly, in few germaria and nurse cells from late stage egg chambers, Ref(2)P was found to be localized in rod shaped form described in Nezis (2012). We also report occurrence of these rod-shaped structures in the germarium (**Supplementary Figure S1D**). Further, GFP-Ref(2)P puncta were found to be concentrated in region 2a and 2b of germarium. This region-specific localization was also observed in germaria from starved flies but was significantly lower. These results support earlier reports that GFP-Ref(2)P in combination with immunoblot assays can be used to measure autophagic flux in *Drosophila* ovaries (Pircs et al., 2012; Devorkin and Gorski, 2014; Mauvezin et al., 2014; Lörincz et al., 2017).

GFP-Ref(2)P levels are maintained at steady state in fed conditions (Pircs et al., 2012). Upon starvation, in addition to the expected band of GFP-Ref(2)P at 130kd, high molecular weight bands were also observed indicating formation of GFP-Ref(2)P aggregates (**Figure 2C** and **Supplementary Figures S8A,B**). This also corelated with increased turnover of GFP-Ref(2)P as GFP-Ref(2)P intermediates could be detected at 12, 24, and 48 h of starvation (**Figure 2** and **Supplementary Figure S8**). ∼ 60 kDa GFP-Ref(2)P intermediate species was seen to be predominant form at 48 h of starvation and its abundance decreased upon CQ treatment indicating inhibition of lysosomal activity. This intermediate form of GFP-Ref(2)P was also reported by Juhasz group (Pircs et al., 2012). Surprisingly, free GFP was not detected at 12, 24, and 48 h of nutrient-limitation and CQ treatment. This could be due to altered configuration of GFP due to exposure to acidic pH that does not allow for binding of the antibodies in the polyclonal serum. Alternatively, this could be due to a reduction in the autophagy carrier flux or due to efficient turnover of the GFP protein. Additionally, the possibility of degradation of a fraction of GFP-Ref2P pool through the proteosomal pathway cannot be ruled out (Pircs et al., 2012; Devorkin and Gorski, 2014; Klionsky et al., 2016).

Both mCherry-Atg8a-I and mCherry-Atg8a-II were detected in nutrient-rich conditions indicating basal autophagic activity during oogenesis. Correspondingly, low levels of free mCherry were detected in fed conditions as seen with the appearance of ∼29 kDa band (free mCherry). In starved ovaries, along with mCherry-Atg8a-I and mCherry-Atg8a-II forms, increased levels of free mCherry could be detected indicative of upregulation of autophagy and increase turnover of mCherry-Atg8a within the autolysosomes. These changes could be detected as early as 12 h post nutrient-deprivation. Upon CQ treatment, mCherry-Atg8a-II form was found to accumulate as compared to starved condition, with a corresponding decrease in the formation of free mCherry indicating impaired degradation of mCherry-Atg8a within the autolysosomes. These data indicate that the ovarian tissue responds to nutrient and pharmacological stress by upregulating or downregulating autophagy as expected (**Figure 3** and **Supplementary Figure S8**; Barth et al., 2011).

We also detected CathepsinL in germaria expressing GFP-Ref(2)P and estimated the colocalization status of the two proteins. Our data suggest a significant overlap between GFP-Ref(2)P and CathepsinL indicating that Ref(2)P positive protein aggregates are indeed targeted to the lysosomes. Also, the measure of overlap decreases in germarium of starved flies suggesting increased degradation/turnover of Ref(2)P.

Previous studies have established transgenes of Atg8a that express in the germline. The UASp-mCherry-Atg8a transgene can be expressed only in presence of a germline driver (nosGal4VP16) and may lead to complications in interpretation of autophagy due to overloading of the cells with mCherry-Atg8a protein (**Supplementary Figure S4**; Nezis et al., 2009; Jacomin and Nezis, 2016). We have previously generated and characterized a mCherry-Atg8a fusion transgene which expresses under the endogenous promoter of Atg8a as part of investigating regulatory genetic elements of autophagy genes (Bali and Shravage, 2017). However, the expression levels of mCherry-Atg8a from the endogenous promoter appeared to have weak expression in the germline (unpublished). The mCherry-Atg8a transgenic line presented in this study has a strong expression in the germline cells and their progeny. As reported previously mCherry-Atg8a is predominantly found in region 2a and 2b of the germarium (Nezis et al., 2009). mCherry-Atg8a could also be detected in GSCs and hence could be used to monitor autophagy in the GSCs (Barth et al., 2011; Zhao et al., 2018). Upon starvation, mCherry-Atg8a puncta were found to be abundant in region 2a and 2b of germarium. Interestingly, we did

not detect any increased mCherry-Atg8a puncta in GSCs upon starvation suggesting that GSC may be protected from starvation-induced autophagy. However, mCherry-Atg8a intensity appeared to be elevated in starved GSCs vs. Fed GSCs, where Atg8a may be participating in non-autophagic functions (Subramani and Malhotra, 2013). Additional experiments need to be performed to get better insights into this interesting phenomenon. mCherry-Atg8a puncta accumulated in germaria upon nutrient deprivation and in response to CQ treatment. Further rapamycin treatment too activated autophagy leading to increase in mCherry-Atg8a puncta. Rapamycin treatment stimulated the strongest response in germaria. These data suggest that nosP-mCherry-Atg8a transgene could be used to monitor basal as well as stress induced autophagy during oogenesis. Increased colocalization of mCherry-Atg8a and CathepsinL in starved germaria support the utility of this transgene for quantifying autophagy flux. Taken together, we believe that these germline specific Ref(2)P and Atg8a reporter will aid in measurement of autophagy flux during gametogenesis and under different conditions of stress. It will also allow measuring autophagy during early embryogenesis as these proteins are deposited in the egg (Forrest and Gavis, 2003).

This is the first report of development of a H_2O_2 sensor for female germline in *Drosophila*. The expression analysis suggest that this sensor could be used for detecting H_2O_2 in the germline cells. In fact, untreated germaria show roGFP2 excitation ratio profile close to that of reduced germarium indicating that normal physiological conditions are indeed reducing. Colocalization of roGFP2-Orp1 protein with ATP5α indicated that the protein is indeed targeted to the mitochondria. roGFP2-Orp1 expression in the GSCs was rather uncharacteristic when females were exposed to limited nutrients, rapamycin and chloroquine. It is well established that starvation induces oxidative stress (Filomeni et al., 2015). However, roGFP2-Orp1 fluorescence excitation did not shift from 488 to 405 nm in starved and CQ treated animals. In contrast, rapamycin treatment induced excitation shift of roGFP2-Orp1 which could be due to induction of mitochondrial remodeling, however, it needs further investigation (Chiao et al., 2016). Colocalization experiments with CathepsinL suggested that this sensor could also be used to monitor mitophagy in the germline cells. Thus, mito-roGFP2-Orp1 sensor could be used as a dual reporter for mitophagy and for measuring H_2O_2 production during oogenesis. We hope that these transgenic lines will provide a valuable tool that can be used in performing genetic screens that aid in identifying novel regulators of autophagy flux and redox homeostasis during oogenesis.

AUTHOR CONTRIBUTIONS

KN, NM, GS, and BS designed and performed the experiments. All authors wrote the manuscript and commented on it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00047/full#supplementary-material

FIGURE S1 | (A) Expression of nosP GFP-Ref(2)P nos 3′UTR in larval ovaries and fat body. Dotted line marks the boundary of larval ovary. Scale bar 10 μm. **(B)** Expression of nosP GFP-Ref(2)P nos 3′UTR in the germarium. The germarium is immunostained with anti-α-spectrin antibody. Arrows point to spectrosomes, arrowheads point to GFP-Ref(2)P punctae, asterisks mark the cap cells and the dotted line marks the GSCs. Scale bar 10 μm. **(C)** Whole ovaries of nosP GFP-Ref(2)P transgenic flies exposed to pharmacological conditions. Scale bar 500 μm. **(D)** GFP-Ref(2)P rod shaped structures pointed by arrow heads in germarium and a stage 6 egg chamber. Dotted ovals mark the GSCs. Scale bar 10 μm for germarium and 20 μm for egg chamber.

FIGURE S2 | (A) nosP GFP-Ref(2)P nos 3′UTR transgenic immunostained for GFP. Dotted ovals mark the GSCs and asterisks mark the cap cells. Arrow heads point to merge of GFP-Ref(2)P and immunostained GFP. Inset shows enlarged region of punctae. Scale bar 10 μm. **(B)** nosP GFP-Ref(2)P nos 3′UTR transgenic immunostained for Ref(2)P. Dotted ovals mark the GSCs and asterisks mark the cap cells. Arrow heads point to merge of GFP-Ref(2)P and immunostained Ref(2)P. Inset shows enlarged region of punctae. Scale bar 10 μm. **(C)** Interleaved scatter graph showing Pearson's coefficient as a measure of colocalization of GFP-Ref(2)P with immunostained GFP and immunostained Ref(2)P. Error bars represent SD in red and the mean is blue. n = 22 and n = 23 for anti-GFP and anti-Ref(2)P respectively. **(D)** Column graph showing the proportion of germarium with and without GFP-Ref(2)P punctae in fed conditions. Total of 94 germaria were analyzed.

FIGURE S3 | (A) Expression of nosP mCherry-Atg8a nos 3′UTR in larval ovaries. Dotted line marks the boundary of larval ovary. Scale bar 10 μm. **(B)** Expression of nosP mCherry-Atg8a nos 3′UTR in the germarium. The germarium is immunostained with anti-α-spectrin antibody. Arrows point to spectrosomes, arrowheads point to mCherry-Atg8a punctae, asterisks mark the cap cells and the dotted line marks the GSCs. Scale bar 10 μm. **(C)** nosP mCherry-Atg8a nos 3′UTR transgenic flies subject to incrementally various days of starvation. Dotted

ovals mark the GSCs. Scale bar 10 μm. **(D)** Interleaved scatter graph showing mCherry-Atg8a punctae per germarium upon starvation for incrementally various days. Error bars represent SD in red and the mean is blue. $n = 8$ for fed, $n = 7$ for 1, 2, 3 days and $n = 13$ for 4 days starvation respectively. $^{**}p < 0.01$, $^{***}p < 0.001$.

FIGURE S4 | (A) nosP mCherry-Atg8a nos 3′UTR transgenic immunostained with anti-GABARAP antibody. Dotted ovals mark the GSCs. Scale bar 10 μm. **(B)** Comparison of UASp.mCherry.Atg8a and nosP mCherry-Atg8a lines. Germarium of UASp.mCherry.Atg8a; nosGal4VP16 and nosP mCherry-Atg8a transgenic subjected to fed and starved condition. Fed (6 days) and starved (2 days fed + 4 days starved). Dotted ovals mark the GSCs. Scale bar 10 μm. **(C)** Germarium of UASp.mCherry.Atg8a; nosGal4VP16 (left) and nosP mCherry-Atg8a (right) where mCherry-Atg8a punctae present in GSCs are pointed by arrowheads. Dotted ovals mark the GSCs. Scale bar 10 μm. **(D)** Interleaved scatter graph showing mCherry-Atg8a punctae in germarium of UASp.mCherry.Atg8a; nosGal4VP16 and nosP mCherry-Atg8a transgenic subjected to fed and starved condition. Fed (6 days) and starved (2 days fed + 4 days starved) $^{**}p < 0.01$, $^{***}p < 0.001$. **(E)** Interleaved scatter graph plotted for the mean intensity of mCherry-Atg8a in UASp.mCherry.Atg8a; nosGal4VP16 and nosP mCherry-Atg8a flies as a function of fed and starved condition. Fed (6 days) and starved (2 days fed + 4 days starved) $^{***}p < 0.001$. For **(D,E)** 20 germarium were analyzed each for fed and starved for UASp.mCherry.Atg8a; nosGal4VP16. For nosP mCherry-Atg8a transgenic, 25 and 26 germarium were analyzed for fed (6 days) and starved (2 days fed + 4 days starved) respectively. Error bars represent SD in red and the mean is blue.

FIGURE S5 | (A) Interleaved scatter graph showing mCherry-Atg8a punctae in germarium of nosP mCherry-Atg8a (control) flies and nosP mCherry-Atg8a in combination with RNAi for Atg8a. The number of germarium analyzed are 22 for control and 24 for Atg8a-RNAi. Error bars represent SD in red and the mean is blue. $^{****}p < 0.0001$. **(B)** Rescue of male lethality of Atg8a^{KG07569} insertion mutant by nosP mCherry Atg8a. Schematic and genotypes of the cross performed for the rescue experiment along with number and proportion of viable F1 imagoes. Columns for rescued males are highlighted with red dotted boxes.

FIGURE S6 | (A) Colocalization (yellow) of GFP-Ref(2)P (green) with CathepsinL (red) marked by arrow heads. Cathepsin-L marks the lysosomes. Arrow points to lysosome alone. Inset shows enlarged region of colocalization. Scale bar 20 μm. **(B)** mCherry-Atg8a can be used to visualize and distinguish between autophagosomes and autophagolysosomes. Arrow heads mark the autophagolysosomes (yellow) which are fusion of autophagosomes marked by mcherry-Atg8a (red) and lysosomes marked by Cathepsin-L (green). Arrows mark the autophagosomes. Inset shows enlarged region of colocalization. Scale bar 10 μm. **(C)** Stage 9 egg chamber of mCherry-Atg8a; GFP-Ref(2)P fly immunostained for Cathepsin-L. Inset shows enlarged region having a puncta (arrow) positive for GFP, mCherry as well as Cathepsin-L. Scale bar 10 μm.

FIGURE S7 | Expression of nosP mito-roGFP2-Orp1 nos 3'UTR in larval ovaries. Dotted line marks the boundary of larval ovary. Scale bar 10 μm.

FIGURE S8 | Western blot analysis using anti-GFP antibodies of ovarian extracts expressing GFP-Ref(2)P. 130 kDa GFP-Ref(2)P band could be detected in fed, starved and starved + CQ treated ovaries for 12 h **(A)** and 24 h **(B)**. Loading control actin is shown below for the same samples. Western blot analysis using anti-mCherry antibodies of ovarian extracts expressing mCherry-Atg8a. 44, 41, and 29 kDa bands corresponding to mCherry-Atg8a-I, mCherry-Atg8a-II and free mCherry respectively could be detected in fed, starved and starved + CQ treated ovaries for 12 h **(C)** and 24 h **(D)**. Loading control actin is shown below for the same samples.

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Transcriptional Regulation of Autophagy: Mechanisms and Diseases

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Macro (Autophagy) is a catabolic process that relies on the cooperative function of two organelles: the lysosome and the autophagosome. The recent discovery of a transcriptional gene network that co-regulates the biogenesis and function of these two organelles, and the identification of transcription factors, miRNAs and epigenetic regulators of autophagy, demonstrated that this catabolic process is controlled by both transcriptional and post-transcriptional mechanisms. In this review article, we discuss the nuclear events that control autophagy, focusing particularly on the role of the MiT/TFE transcription factor family. In addition, we will discuss evidence suggesting that the transcriptional regulation of autophagy could be targeted for the treatment of human genetic diseases, such as lysosomal storage disorders (LSDs) and neurodegeneration.

Keywords: autophagy, TFEB, genetic diseases, nucleus, transcription, lysosomal storage disease

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INTRODUCTION

Autophagy is an evolutionary conserved catabolic process devoted to the degradation of intracellular components. Three main types of autophagy have been described to date: macroautophagy, microautophagy, and chaperon-mediated autophagy. Macroautophagy involves the formation of a double-membrane vesicle, the autophagosome, which captures cytoplasmic contents and then fuses with lysosomes to generate autophagolysosomes, structures in which cargo substrates are degraded by lysosomal enzymes (Mizushima et al., 2008; He and Klionsky, 2009; Hurley and Schulman, 2014). In microautophagy, cytoplasmic constituents are directly imported into the lysosome and degraded (Ahlberg et al., 1982; Mijaljica et al., 2011; Sahu et al., 2011), while chaperon-mediated autophagy is characterized by the translocation of cytosolic proteins harboring the pentapeptide KFERQ sequence across the lysosomal membrane for degradation (Kaushik and Cuervo, 2012). Thus, the three types of autophagy rely on functional lysosomes to digest intracellular cargos.

Macroautophagy (herein referred to as autophagy) is constitutively active, albeit at low levels, in most cells of our body as part of the constitutive turnover of cytosolic components (Mizushima and Komatsu, 2011). This is generally referred as "basal autophagy." In addition, different cellular stimuli, in particular nutrient starvation, can potently stimulate autophagy to enhance the degradation of cytosolic components to generate energy (Kaur and Debnath, 2015). Two nutrient-responsive kinases, mTORC1 and AMPK, rapidly respond to nutrient fluctuations and phosphorylate critical regulators of autophagosome biogenesis and maturation (e.g., fusion with lysosomes) (Egan et al., 2011). In particular, in the presence of nutrients, mTORC1 phosphorylates two fundamental autophagy initiation proteins, unc-51-like autophagy activating kinase (ULK)1

and ATG13, inhibiting their pro-autophagic activity (Hosokawa et al., 2009). Conversely, nutrient depletion inactivates mTORC1 and concomitantly activates AMPK, which phosphorylates ULK1 and ATG13 on specific amino acid residues promoting ULK1/ATG13 complex activity and autophagy initiation (Shang et al., 2011). In addition, several other mechanisms of post-translational regulation of autophagy in response to nutrient fluctuations have been described and reviewed elsewhere [see for example reviews (He and Klionsky, 2009; Kuma and Mizushima, 2010; Rabinowitz and White, 2010; Mizushima et al., 2011)].

The modulation of autophagy in the maintenance of cellular homeostasis goes far beyond the response to nutrient fluctuation, as cells exploit autophagy to eliminate damaged organelles, misfolded proteins, and invading organisms (Deretic et al., 2006; Mizushima et al., 2008). Deregulation of these autophagy-dependent cytoprotective functions has been associated to different pathologies, including immune disorders, neurodegenerative diseases, cancer and aging (Deretic et al., 2006; Hara et al., 2006; Komatsu et al., 2006; Harris and Rubinsztein, 2011; Mizushima and Komatsu, 2011; White, 2015).

For a long time, autophagy was considered as a pathway exclusively regulated by cytosolic processes. This concept was supported by the observation that enucleated cells still form autophagosomes (Morselli et al., 2011). However, increasing amounts of evidence collected in the last decade clearly indicate that nuclear transcriptional and epigenetic events play a major role in autophagy regulation. This review aims to summarize the "nuclear" control of autophagy, focusing in particular on the co-regulation of autophagy and lysosome biogenesis by the transcription factor EB (TFEB).

TRANSCRIPTIONAL REGULATION OF AUTOPHAGY

The first observation that autophagy can be induced at the transcriptional level was made in yeast in Kirisako et al. (1999), who reported that nitrogen starvation induced the upregulation of the essential autophagy gene Apg8p, the homologous of mammalian LC3. In the last 10 years several laboratories demonstrated that transcription factors that enhance the expression of autophagy genes (even few of them) increase autophagy and the degradation of unwanted substrates [see below and (Lapierre et al., 2015; Füllgrabe et al., 2016)]. These observations opened a new, unexpected, scenario indicating that autophagy activity could in fact be modulated from the nucleus.

TFEB AND MiT FACTORS

Transcription factor EB is a member of the microphthalmia/transcription factor E (MiT/TFE) family of transcription factors (TFs) that also includes MITF, TFE3, and TFEC proteins (Hemesath et al., 1994). They belong to the larger family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors, such as MYC, MAD, and MAX, and share a basic DNA-binding domain, and an HLH plus a leucine zipper domain important for dimerization (Beckmann et al., 1990; Sato et al., 1997; Steingrimsson et al., 2002). The homo- or hetero- dimerization is necessary to activate transcription. MiT/TFE members can only form heterodimers among each other due to structural constraints in their leucine zipper domain (Hemesath et al., 1994; Pogenberg et al., 2012). Binding to DNA is mediated by the recognition of a common DNA hexanucleotide sequence (CACGTG) known as the E-box (Hemesath et al., 1994). This sequence conforms to the canonical CANNTG motif, recognized by other bHLH-Zip transcription factors, however, specific nucleotide residues that flank this motif characterize the coordinated lysosomal expression and regulation (CLEAR) motif (GTCACGTGAC) that is preferentially recognized by MiT/TFE members (Sardiello et al., 2009; Palmieri et al., 2011; Martina et al., 2014). Bioinformatic analysis identified one or more CLEAR motifs in the promoter region of many lysosomal genes. Notably, these genes belong to different functional lysosomal categories, (ion channels, hydrolases, and transmembrane proteins, etc.) so that TFEB activation leads to a global enhancement of lysosomal catabolic efficiency (Sardiello et al., 2009).

In addition, TFEB also regulates the expression of genes involved in different steps of the autophagy process, such as genes important for autophagy initiation (*BECN1, WIPI1, ATG9B*, and *NRBF2*) autophagosome membrane elongation (*GABARAP, MAP1LC3B*, and *ATG5*), but also genes important for substrate capture (*SQSTM1*) and for autophagosomes trafficking and fusion with lysosomes (*UVRAG, RAB7*) (Palmieri et al., 2011; Settembre et al., 2011). As a result, TFEB activation induces a striking increase in autophagy flux. Similarly, TFE3 and MITF were successively identified as regulators of autophagy and lysosomal biogenesis (Martina et al., 2014; Ploper et al., 2015).

Transcription factor EB activity is largely controlled by its subcellular localization, which is mainly regulated by phosphorylation (Puertollano et al., 2018). Phosphorylated TFEB is sequestered into the cytosol, hence the transcriptional induction of its target genes is inhibited. Conversely, upon nutrient starvation, TFEB is dephosphorylated and rapidly translocates into the nucleus where it binds to the promoter of target genes (Settembre et al., 2011). To date, different kinases that phosphorylate TFEB have been identified. mTOR, as part of the protein complex mTORC1, represents the main kinase responsible for TFEB phosphorylation in presence of amino acids (Peña-Llopis et al., 2011; Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Inhibition of TFEB activity via phosphorylation of conserved amino acid residues (Ser 142, Ser 211, Ser122, and Ser138) is part of a larger metabolic response mediated by mTORC1 aimed to shut-off catabolic pathways while turning on anabolic ones when nutrients are available (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Vega-Rubin-de-Celis et al., 2017; Napolitano G. et al., 2018). Similarly, mTORC1 also regulates the nuclear localization of TFE3 and some isoforms of MITF, thus efficiently inhibiting transcriptional induction of lysosome biogenesis and autophagy (Martina et al., 2014).

In addition, mTORC1 can inhibit TFEB transcriptional activity by modulating the zinc finger transcription factors

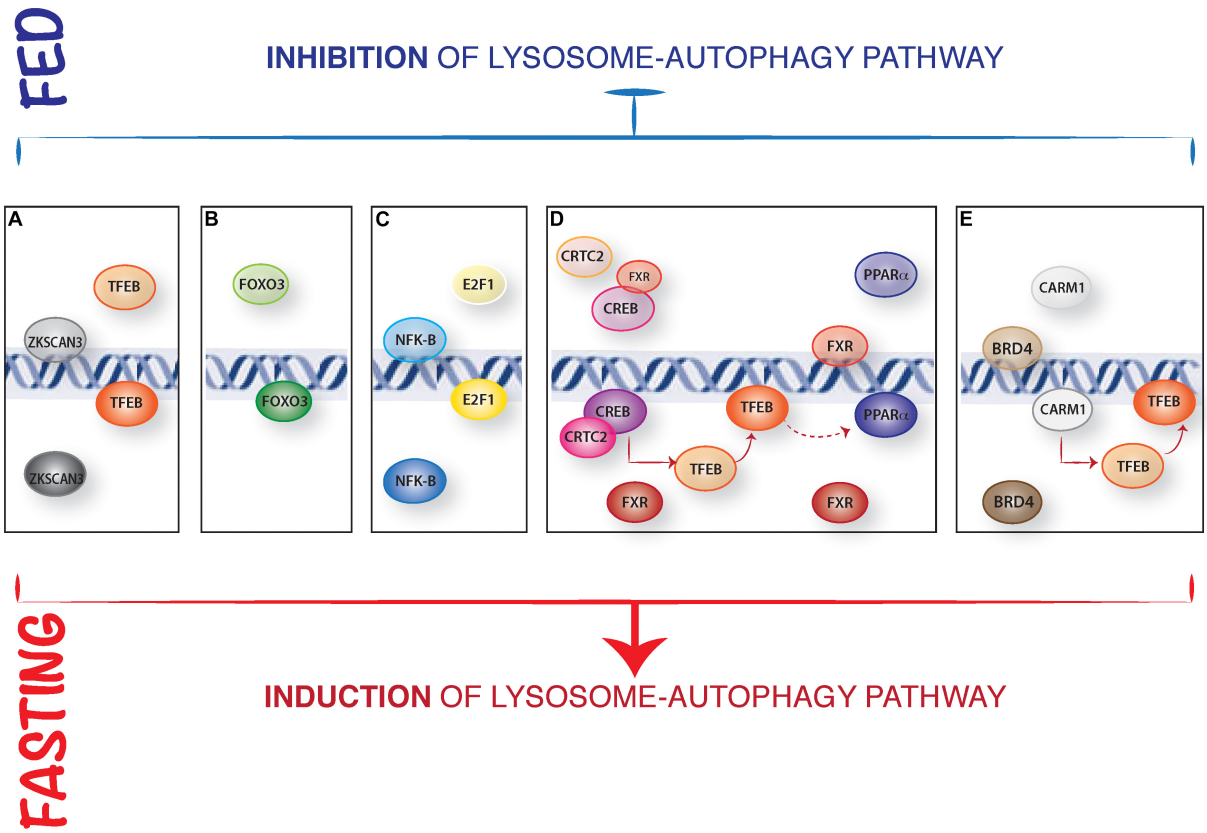


FIGURE 1 | Representative model of the nuclear control of lysosome-autophagy pathway. **(A)** Opposed regulation of ZKSCAN3 and TFEB. In presence of nutrients, TFEB is cytosolic, and the transcription factor ZKSCAN3 localizes in the nucleus, inhibiting lysosome gene expression. During starvation, ZKSCAN3 translocates into the cytosol and TFEB translocates into the nucleus where activates lysosome-autophagy gene expression. **(B)** The nuclear translocation and activation of FOXOs transcription factors is induced IN serum starved condition. **(C)** NFKB binds to the promoter and represses Bnip3 expression in fed conditions, while during starvation Bnip3 expression is promoted by E2F1. **(D)** In presence of nutrients, FXR inhibits autophagy by preventing the binding of PPARα to DNA and by inhibiting CREB interaction with its coactivator CRTC2. Conversely, during starvation FXR activity is inhibited, and CREB-CRTC2 complex is formed and binds to the promoters of lysosomal autophagy genes and of TFEB; similarly, starvation-mediated inhibition of FXR allows PPARα binding to the DR1 elements in the promoters of autophagy genes. **(E)** Epigenetic regulation of autophagy: in fed status, CARM1 is inactive and BRD4 represses the expression of autophagic and lysosomal genes regulating Histone3 lysine9 methylation. In fast state, BRD4 is inactive and CARM1 translocates into the nucleus promoting lysosomal-autophagy gene expression via a positive Histone3 Arginine17 methylation and inducing TFEB transcriptional activity.

harboring Kruppel-associated box (KRAB) and SCAN domain (ZKSCAN3) activity (Chauhan et al., 2013). ZKSCAN3 represses a large group of lysosomal and autophagy genes when nutrients, in particular amino acids, are present in the cell. Conversely, treatment with the mTOR inhibitor Torin1 induced ZKSCAN3 nuclear exclusion. Silencing of ZKSCAN3 augmented TFEB-mediated lysosomal and autophagic activation suggesting that these two transcription factors act in opposite ways to regulate autophagy in response to nutrient fluctuations (**Figure 1A**). While this mechanism appears to be relevant in cell culture experiments, its relevance *in vivo* is unclear (Pan et al., 2017).

In addition to mTORC1, other growth-regulating kinases control TFEB nuclear localization. ERK2 was the first kinase to be associated with TFEB phosphorylation in response to nutrients availability (Settembre et al., 2011). In particular, ERK2 mediated phosphorylation of TFEB at Ser142 inhibited TFEB nuclear translocation thus limiting transcriptional activation of its downstream target genes (Settembre et al., 2011; Li et al., 2018, 2019). Subsequently, the glycogen synthase kinase 3 beta (GSK3B) was identified as the kinase responsible for TFEB phosphorylation at Ser134 and 138 (Li et al., 2016). This event, coupled to phosphorylation at Ser142 by ERK2 and mTORC1, unmasks a nuclear export localization signal required for TFEB cytosolic accumulation (Li et al., 2018). Moreover, the Akt and the PKCβ kinases phosphorylate TFEB at c-terminal critical serines, but this phosphorylation seems to control TFEB stability rather than its nuclear localization (Ferron et al., 2013; Palmieri et al., 2017).

Transcription factor EB nuclear translocation can also be triggered by activation of the calcium and calmodulin dependent serine/threonine phosphatase calcineurin (Medina et al., 2015). Notably, the calcium efflux through the lysosomal cation channel Mucolipin1 triggers calcineurin-mediated TFEB dephosphorylation and activation, hence providing a mechanistic explanation of autophagy regulation by calcium signaling.

More recently, the protein phosphatase 2A (PP2A) has been shown to dephosphorylate TFEB upon induction of acute oxidative stress by sodium arsenite (Martina and Puertollano, 2018).

To date, the mechanisms controlling TFEB nuclear export are less characterized but seem to be dependent on the CRM1 exportin and on the presence of a TFEB nuclear export sequence (Napolitano G. et al., 2018). Intriguingly, mTOR-dependent TFEB re-phosphorylation in the nucleus seems to play a major role in TFEB nuclear export.

These studies indicate that several signaling events regulate TFEB subcellular localization, thus placing the transcriptional activation of the lysosomal-autophagy pathway as a general response to cope with different types of cellular stresses.

FOXO FACTORS

The class O of forkhead box transcription factors (FOXO) family has an established role in autophagy regulation (Webb and Brunet, 2014). In mammals, this family includes four members: FOXO1, FOXO3, FOXO4, and FOXO6. The activity of three out of four members (FOXO1, FOXO3, and FOXO4) is mainly regulated by AKT phosphorylation in response to growth factors and insulin stimulation. FOXO3 was the first FOXO member identified as a transcriptional regulator of several autophagy genes (*ATG4, ATG12, BECN1, BNIP3, LC3, ULK1, ULK2*, and *VPS34*) in muscle (Mammucari et al., 2007; Zhao et al., 2007; Sanchez et al., 2012). Similar to what reported for MiT/TFE family of transcription factors, FOXO3 transcriptional activity is mostly regulated by a nuclear/cytosolic shuttling. Once activated by growth factors, AKT phosphorylates FOXO3 and this results in its cytoplasmic retention, thus inhibiting transcriptional activation of its target genes. Later on, another member of this family, FOXO1, was also described as a transcriptional regulator of different autophagy genes (Liu et al., 2009; Xu et al., 2011; Xiong et al., 2012). However, FOXO1 also induces autophagy in a transcriptional-independent way: in response to oxidative stress or serum starvation, FOXO1 is acetylated in the cytosol and binds to Atg7 thus favoring autophagy induction by direct interaction with key regulators of autophagosome biogenesis (Zhao et al., 2010; **Figure 1B**). More recently FOXO transcription factors have been shown to cooperatively control autophagy in cartilage and protect against osteoarthritis (Matsuzaki et al., 2018).

Most notably, a study using *Caenorhabditis elegans* demonstrated that DAF16 (FOXO in mammals) physically and functionally cooperates with HLH30 (TFEB in mammals) to ensure appropriate expression of target genes during organismal responses to stressors (Lin et al., 2018). It will be important to understand whether a FOXO-TFEB cooperation occurs also in mammals.

P53

Different studies suggest that P53, the most studied tumor suppressor protein, is an inducer of the autophagy pathway. P53 was initially described to promote autophagy by inhibiting the mTORC1 pathway, through transcriptional induction of Sestrin proteins, which activate AMPK while inhibiting mTORC1 lysosomal recruitment (Budanov and Karin, 2008; Chantranupong et al., 2014), and by inducing the expression of the Damaged-regulated- modulator DRAM, a lysosomal protein, which induces autophagy through a yet not identified mechanism (Crighton et al., 2006). Subsequently, a combined CHIP-SEQ and RNA-SEQ analysis performed on mouse embryonic fibroblasts (MEFs) upon DNA-damage, revealed that P53 controls the expression of several genes essential for autophagy induction (*LKB1, ULK1/2*), and autophagosome maturation (*ATG4, ATG7*, and *ATG10*) (Kenzelmann Broz et al., 2013). Moreover, P53 regulates both FOXO3a expression and activity (You et al., 2004; Fu et al., 2009; Miyaguchi et al., 2009; Renault et al., 2011), and promotes TFEB/TFE3 nuclear translocation upon DNA damage (Jeong et al., 2018), thus controlling key upstream modulators of the autophagy pathway.

However, cytoplasmatic P53 may also act as a negative regulator of autophagy, although the mechanisms underlying this inhibitory regulation are still elusive (Green and Kroemer, 2009; Comel et al., 2014). Further studies are needed to fully define the role of P53 in the regulation of autophagy pathway.

E2F1/NF-kB AXIS

The transcription factors E2F1 and NF-kB regulate autophagy through the regulation of BNIP3 expression (Tracy et al., 2007; Gang et al., 2011). BNIP3 is a hypoxia-induced activator of autophagy that disrupts the inhibitory binding of B-cell lymphoma 2 (BCL-2) to Beclin1, a component of the class III phosphatidylinositol-3-OH kinase (PI3K) complex, that promotes autophagosome biogenesis. During normoxia, NF-kB constitutively binds to the promoter of BNIP3 repressing its expression (Shaw et al., 2008). Hypoxia reduces the occupancy of NF-kB on the BNIP3 promoter thus allowing E2F1 to induce its expression and activate autophagy (**Figure 1C**). In addition, E2F1 can also promote the expression of other autophagy genes, such as *ULK1, LC3*, and *ATG5* (Polager et al., 2008).

CREB-FXR AND PPARα-FXR CIRCUITS

The farnesoid X receptor (FXR) represses liver autophagy during feeding conditions (Thomas et al., 2008; Calkin and Tontonoz, 2012). FXR is activated by increased bile acid levels after feeding and transcriptionally represses several autophagy genes through two apparently independent mechanisms. Seok et al. (2014) proposed that FXR inhibits the transcriptional activity of the fasting-activated cAMP response element-binding protein (CREB) by impeding the interaction between CREB and its coactivator CRTC2. Upon fasting, FXR inhibition is relieved thus allowing the CREB-CRTC2 complex to form and induce the expression of many autophagy genes, including *ATG7, ULK1*, and *TFEB* (**Figure 1D**). Interestingly, TFEB

also regulates the expression of genes important for lipid metabolism in the liver, suggesting that its role in the FXR-CREB axis might be not limited to autophagy regulation (Settembre et al., 2013). In addition, Lee et al. (2014) identified the nuclear receptor Peroxisome Proliferator Activated Receptor alpha (PPARα) as the transcriptional activator that opposes FXR in response to nutrient availability. FXR and PPARα share the ability to bind to specific DNA sites (DR1 elements) in the promoter regions of many autophagy-related genes, so that these two nuclear receptors compete for the binding to the same target genes. Fasting activates PPARα while inhibiting FXR, thus inducing transcriptional activation of autophagy genes in liver (**Figure 1D**). Notably, TFEB transcriptionally enhances the expression of *PPARα* and its coactivator peroxisome proliferator activated receptor gamma 1 alpha (*PGC1α*) (Settembre et al., 2013), suggesting that the induction of *TFEB* expression by CREB could in turn potentiate PPARα activity. Thus, it is possible that both the FXR-CREB and FXR-PPARα circuits coexist and participate to the coordination of autophagy with other metabolic processes (e.g., lipid degradation) occurring in the liver.

EPIGENETIC REGULATION OF AUTOPHAGY

Histone post-translational modifications, such as methylation, acetylation, and deacetylation, influence the overall chromatin structure, thus affecting the accessibility of transcription factors to chromatin (Lawrence et al., 2016). To date, several examples of epigenetic regulations of the autophagy pathway have been described.

Histone Methylation

The epigenetic reader Bromodomain-containing protein 4 (BRD4) has been identified as a repressor of a transcriptional program that promotes autophagy and lysosome biogenesis (Sakamaki et al., 2017). In presence of nutrients, BRD4 represses the expression of several autophagic and lysosomal genes by recruiting the histone lysine methyltransferase G9a, which deposits a repressive H3K9diMe in the promoters of lysosomal and autophagy genes. Conversely, nutrient depletion promotes AMPK-mediated BRD4 inhibition and the expression of lysosomal and autophagic genes through a yet-to be characterized transcriptional regulator.

The co-activator-associated arginine-methyltransferase 1 (CARM1) was recently identified as a key autophagy regulator (Shin et al., 2016). Glucose (but also amino acid) starvation leads to a CARM1-dependent increase in histone H3 Arg17 dimethylation levels at the promoters of autophagy and lysosomal genes and this is critical for proper autophagy activation. Mechanistically, upon starvation CARM1 translocates into the nucleus where binds TFEB and promotes the transcriptional activation of its target genes. CARM1 seems to be essential for TFEB-mediated autophagy activation since TFEB overexpression fails to increase autophagy in cells lacking CARM1 (**Figure 1E**).

Histone Acetylation

Recently, a global decrease in acetylation levels of H4K16 was described upon nutrient starvation and/or mTOR inhibition (Füllgrabe et al., 2013). This downregulation translates into a transcriptional repression of key autophagy genes in order to prevent a chronic autophagy induction, which could be lethal. These responses are dependent on the histone acetyltransferase hMOF/KAT8/MYST1.

The NAD+-dependent deacetylase Sirt1 regulates autophagy through its deacetylase activity on non-histone cytosolic targets (Lee et al., 2008; Bao and Sack, 2010). Sirt1 may induce autophagy directly by deacetylating autophagy proteins such as ATG5, ATG7 and LC3. Sirt1 might also control the stability of mRNAs encoding for lysosomal enzymes (Latifkar et al., 2019). Moreover, Sirt1 deacetylates the transcriptional regulators of autophagy FOXO1 and FOXO3, enhancing their transcriptional activity (Brunet et al., 2004). Finally, Sirt1 promotes autophagy by activating AMPK, via deacetylation of LKB1 (Lan et al., 2008), while inhibiting mTORC1 signaling favoring its interaction with the TSC1/TSC2 complex (Ghosh et al., 2010).

Additional epigenetic modifications related to autophagy induction are H3K9 methylation (Artal-Martinez de Narvajas et al., 2013), H3K56 acetylation (Chen et al., 2012) and H4K20 methylation (Kourmouli et al., 2004). These are associated with suppression of autophagy, even if further studies are required to clarify their regulation.

MiTF FACTORS AND HUMAN DISEASES

The autophagy pathway is important in several processes required to maintain cellular homeostasis, including adaptation to metabolic stress, removal of dangerous cargo, and prevention of DNA damage. If any of these protective functions are impaired, onset and progression of several diseases, such as infection, cancer, neurodegeneration, cardiovascular diseases, and aging may be favored (Mizushima et al., 2008; Harris and Rubinsztein, 2011; Mizushima and Komatsu, 2011; White, 2015). Therefore, it is not surprising that a long list of diseases is associated to mutations in autophagy-related genes [recently reviewed in Levine and Kroemer (2019)]. However, it is important to note that several autophagy proteins participate to other cellular processes, such as vesicular trafficking, phagocytosis, exocytosis, and even cell cycle regulation and immunity, thus the link between disease manifestation and autophagy dysfunction might be difficult to establish (Levine and Kroemer, 2019). This is particularly true for transcription factors, that control the expression of target genes implicated in a number of diverse cellular functions. The activity and/or the localization of TFEB has been reported to be deregulated in several neurodegenerative diseases, such as X-linked spinal and bulbar muscular atrophy (Cortes et al., 2014), Parkinson disease (Decressac et al., 2013), Huntington disease (Tsunemi et al., 2012), and Alzheimer disease (Reddy et al., 2016). These neurodegenerative disorders are characterized by intracellular protein aggregation and autophagy dysfunction, which is predicted to contribute to disease

establishment (Menzies et al., 2015). Notably, forced overexpression of TFEB in cellular and murine models of these disorders significantly reduced protein aggregation attenuating pathological manifestation, suggesting that TFEB represents an appealing target for therapy (Sardiello et al., 2009; Dehay et al., 2010; Tsunemi et al., 2012; Decressac et al., 2013; Polito et al., 2014; Xiao et al., 2014, 2015; Chauhan et al., 2015; Kilpatrick et al., 2015).

Lysosomal storage disorders (LSDs) are a class of rare diseases due to mutations in genes encoding for lysosomal proteins (Ballabio and Gieselmann, 2009; Cox and Cachón-González, 2012; Platt et al., 2018). As a consequence, cells show progressive accumulation of indigested material within lysosomes and, eventually, impaired autophagy flux. Interestingly, TFEB was found to be predominantly nuclear in several LSD cellular models (Sardiello et al., 2009; Bartolomeo et al., 2017). The increased nuclear localization of TFEB may be interpreted as an attempt to compensate for the decreased autophagy flux and lysosomal degradative function. While in this context the physiological induction of the TFEB seems to be unable to fully counteract disease progression, TFEB overexpression in different LSDs, such as multiple sulfatase deficiency and mucopolysaccharidosis IIIA (Medina et al., 2011), Pompe disease (Spampanato et al., 2013), Batten disease (Palmieri et al., 2017), Gaucher and Tay Sachs disease (Song et al., 2013), and cystinosis (Rega et al., 2016) resulted effective in reducing lysosomal storage. This effect is most likely the consequence of TFEB's ability to concomitantly induce lysosomal exocytosis, autophagy and lysosome biogenesis. Similarly, TFEB overexpression in liver had beneficial effects in mouse models of alpha1-antitrypsin deficiency and hepatic hyperammonemia (Pastore et al., 2013; Soria et al., 2018). Notably, by increasing the autophagic degradation of intracellular lipid droplets, TFEB also represents a potential therapeutic target to fight metabolic syndrome associated with obesity (Settembre et al., 2013). Despite the induction of TFEB activity looks as a promising therapeutic tool for several diseases, the side effects of its long-term overexpression must be considered. The over-activation of MiT family of transcription factors is associated with different types of cancer. MITF genomic amplification is frequently found in melanoma, while chromosomal translocations and rearrangements of TFE3 and TFEB are associated with pediatric renal cell carcinomas and alveolar soft part sarcoma (Argani et al., 2001; Haq and Fisher, 2011; Kauffman et al., 2014). Moreover, upregulation of MiT/TFE members has also been observed in pancreatic ductal adenocarcinoma (Perera et al., 2015).

How over-activation of these TFs may favor pro-tumorigenic processes is not completely clear, but recent data indicate that hyper-activation of mTORC1 signaling is a common feature of MiT/TFE associated malignancies (Di Malta et al., 2017). This signaling deregulation depends on the constitutive induction of the essential components of the mTORC1 amino acid sensing machinery RagD and RagC GTPases, direct downstream targets of MiT/TFE TFs. Interestingly, at least in pancreatic ductal adenocarcinoma, the upregulation of MiT/TFE factors leads to simultaneous mTORC1 hyperactivation and autophagy induction and presumably both pathways are exploited by tumor cells to efficiently compete with non-transformed cells (Perera et al., 2015, 2019; Di Malta et al., 2017). In light of the pathological consequences of the constitutive activation of MiT/TFE factors, a pulsatile approach aimed at enhancing TFEB activity only for a certain time-frame could represent a therapeutic strategy for diseases that might benefit of the stimulation of the lysosomal/autophagy pathway.

CONCLUSION

In the last years, several studies provided conclusive evidence that autophagy is a transcriptionally regulated process. However, despite different transcriptional modulators of autophagy have been identified, we still know very little about the physiological relevance of this nuclear regulation. The most likely hypothesis is that transcriptional regulation of autophagy cooperates with the post-translational regulation to achieve a fine tuning of autophagy flux particularly in conditions of prolonged starvation or chronic stress. Indeed, the degradation of autophagy proteins, in particular those serving as cargo receptors, is enhanced during autophagy, and similarly lysosomes are utilized during the formation of autolysosomes. Hence, the transcriptional induction of lysosomal and autophagy genes might counteract the depletion of the correspondent proteins during autophagy. Consistently, the translation of mRNAs encoding for proteins with catabolic roles is spared from the general inhibition of protein synthesis during nutrient starvation (Saikia et al., 2016). Additionally, the transcriptional regulation of autophagy might participate to biological processes that are regulated independently of the nutrient status of the cells, such as cellular differentiation and tissue development (Cinque et al., 2015). It will be important in the next years to understand whether different transcription factors regulate selective types of autophagy in a tissue and time specific fashion and if their modulation can be exploited for therapeutic purposes.

A selective modulation of autophagy might be beneficial for the treatment of several diseases for which there are no currently available therapies. Notably, several therapeutic benefits associated to administration of widely used drugs, such as aspirin and metformin, and food compounds, such as resveratrol and curcumin, might be due their ability to induce TFEB nuclear translocation and autophagy (Bao et al., 2016; Zhang et al., 2016; Wang et al., 2017; Yan et al., 2017; Chandra et al., 2018). Currently, whether these molecules can be repositioned for the treatment of genetic diseases is largely unexplored. Lastly, the use of computational approaches combined to an integrated analysis of omics data represents an invaluable tool to identify novel transcriptional modulators of autophagy (Napolitano F. et al., 2018).

AUTHOR CONTRIBUTIONS

CDM and CS wrote the manuscript. LC prepared the figure and wrote the figure legend.

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

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