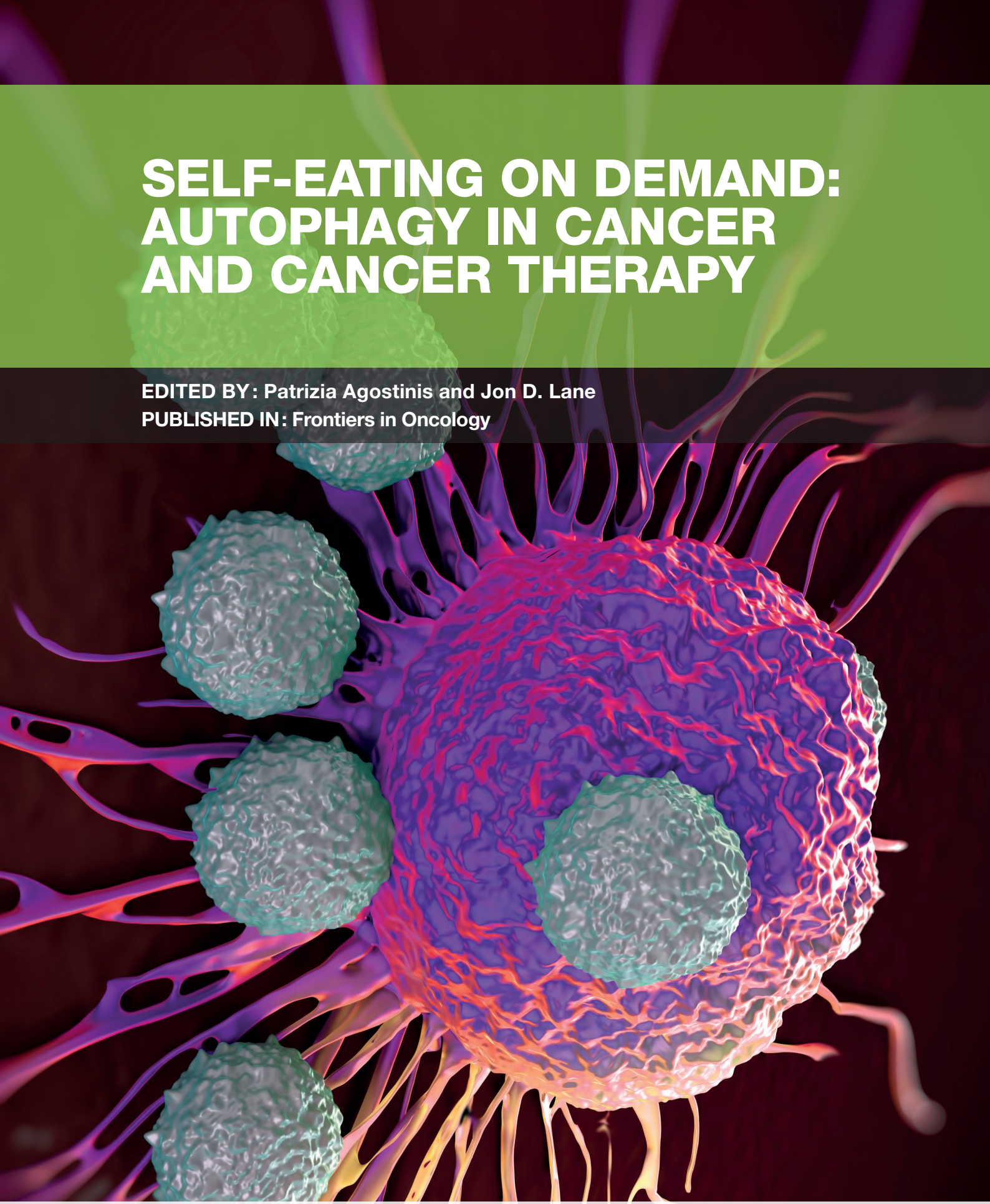
SELF-EATING ON DEMAND: AUTOPHAGY IN CANCER AND CANCER THERAPY

EDITED BY: Patrizia Agostinis and Jon D. Lane

PUBLISHED IN: Frontiers in Oncology



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ISSN 1664-8714
ISBN 978-2-88945-422-8
DOI 10.3389/978-2-88945-422-8

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SELF-EATING ON DEMAND: AUTOPHAGY IN CANCER AND CANCER THERAPY

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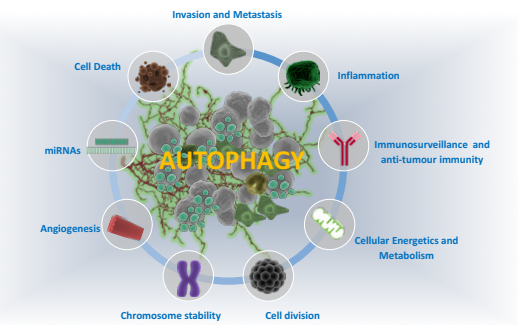

Cancer associated Autophagy controls the main hallmarks of cancer. Image: Patrizia Agostinis and Jon D. Lane.

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Macroautophagy, the major lysosomal pathway for recycling intracellular components including whole organelles, has emerged as a key process modulating tumorigenesis, tumor–stroma interactions, and cancer therapy. An impressive number of studies over the past decade have unraveled the plastic role of autophagy during tumor development and dissemination. The discoveries that autophagy may either support or repress neoplastic growth and contextually favor or weaken resistance and impact antitumor immunity have spurred efforts from many laboratories trying to conceptualize the complex role of autophagy in cancer using cellular and preclinical models. This complexity is further accentuated by recent findings highlighting that various autophagy-related genes have roles beyond this catabolic mechanism and interface with oncogenic pathways, other trafficking and degradation mechanisms and the cell death machinery. From a therapeutic perspective, knowledge of how autophagy modulates the tumor microenvironment is crucial to devise autophagy-targeting strategies using smart combination of drugs or anticancer modalities. This eBook contains a collection of reviews by autophagy researchers and provides a background to the state-of-the-art in the field of autophagy in cancer, focusing on various aspects of autophagy regulation ranging from its molecular components to its cell autonomous role, e.g. in cell division and oncogenesis, miRNAs regulation, cross-talk with cell death pathways as well as cell non-autonomous role, e.g. in secretion, interface with tumor stroma and clinical prospects of autophagy-based biomarkers and autophagy modulators in anticancer therapy.

This eBook is part of the TransAutophagy initiative to better understand the clinical implications of autophagy in cancer.

Citation: Agostinis, P., Lane, J. D., eds. (2018). Self-Eating on Demand: Autophagy in Cancer and Cancer Therapy. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-422-8

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frontiers in Oncology

EDITORIAL
published: 07 December 2017
doi: 10.3389/fonc.2017.00302

Editorial: Self-Eating on Demand: Autophagy in Cancer and Cancer Therapy

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Keywords: calcium signaling, tumor microenvironment, secretion, microRNAs, hypoxia, melanoma, pancreatic cancer, immunosurveillance

Editorial on the Research Topic

Self-Eating on Demand: Autophagy in Cancer and Cancer Therapy

OPEN ACCESS

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *10 October 2017*
Accepted: *24 November 2017*
Published: *07 December 2017*

Citation:
Lane JD and Agostinis P (2017) Editorial: Self-Eating on Demand: Autophagy in Cancer and Cancer Therapy. Front. Oncol. 7:302. doi: 10.3389/fonc.2017.00302

The field of autophagy has grown enormously over the past 10–15 years, with rapid advances in our understanding of the regulatory mechanisms that control autophagy pathways in mammalian systems, and an improved understanding of the physiological influences of autophagy in health and disease. Supporting such progress, there has been substantial diversification in assessment and modulation tools (1), assisted by the advancement of model reporter systems. Indeed, we are now starting to realize the potential for autophagy control for novel practical applications, including disease intervention and biotechnology. With the aim of promoting, supporting, and streamlining cooperative European research networks to realize the enormous potential of autophagy in the clinic and in industry, a collaborative consortium—called TransAutophagy—was approved in November 2015 in the framework of the Horizon 2020 Program as a European Union CO-operation in Science and Technology (COST) Action (CA15138[1]) (2). Sponsored for 4 years, this network includes more than 250 scientists from 21 countries, with each participant actively engaged in basic and/or translational autophagy research.

TransAutophagy comprises five different thematic Working Groups with activities designed to synergize and support translation of our ever-advancing basic autophagy knowledge into biomedical and biotechnological applications (2). Targeting the complex physiological and metabolic changes inherent within cancer cells during transformation, tumor growth, and metastasis, through manipulation of autophagy regulatory networks, is a key objective because emerging evidence indicates that autophagy capability underpins a cancer cell's ability to face the increasingly hostile tumor microenvironment. Here, poor nutrient availability and elevated cellular stress place demands upon the cancer cell for an increased capability to adapt and survive. Several lines of evidence have established that cancer cells use autophagy as a highly plastic and dynamic mechanism to either repress initial steps in carcinogenesis or to support the survival and growth of established tumors (3). Moreover, it is becoming increasingly clear that autophagy regulates the intersection between cancer and stromal cells in tumors. The tumor-suppressing role of autophagy involves, e.g., (i) maintenance of genetic/

[1] http://cost-transautophagy.eu.

genomic stability; (ii) preservation of bioenergetics; (iii) reduction and control of (mutagenic/damaging) reactive oxygen species; (iv) degradation of oncogenic proteins; (v) activation of tumor-suppressing mechanisms like oncogene-induced senescence and autophagic cell death; (vi) reduction of chronic inflammation; and (vii) regulation of immunosurveillance mechanisms [reviewed in Ref. (3, 4)]. This collection of reviews—comprising this research topic—addresses emerging traits highlighting how autophagy shapes the cancer cell-tumor microenvironment crosstalk.

The review of Mathiassen et al. (Cecconi's lab) discusses mounting evidence for new regulatory intersections between autophagy and the cell cycle, which need to be urgently validated *in vivo*. At the mechanistic level, the tumor suppressor role of autophagy has been ascribed to its vital cell-autonomous functions in mitigating damage and maintaining cellular integrity during metabolic stress. An emerging and intriguing link, which is discussed in the review of Kania et al. (Bultynck's/Parys's labs), is the regulation of autophagy in cancer cells through Ca^{2+} transfer from the ER to mitochondria *via* the inositol 1,4,5-trisphosphate receptor (IP3R) at ER–mitochondria contact sites. In a developing research area with enormous potential, the impact of miRNA-mediated autophagy regulation on the tumor microenvironment and cancer growth, and their potential as cancer biomarkers and therapeutic targets, is discussed in the review of Gozuacik et al. (Gozuacik's lab).

In established tumors, elevated levels of autophagy are often associated with poorly oxygenated regions where the demand for nutrients and the need to withstand diverse metabolic stresses are increased. As further discussed in the review of Viry et al. (Janji's lab), cancer cell-associated autophagy in hypoxic tumors plays a crucial role in modulating immunosurveillance and in fostering the immunosuppressive tumor microenvironment, by suppressing key mechanisms of innate and adaptive antitumor immunity, thus favoring tumor outgrow and dissemination. Consistent with this pro-tumorigenic role, advanced tumors often display an "autophagy-lysosomal addiction," which appears to be required to maintain their energy balance through the recycling of intracellular components into biosynthetic pathways or ATP synthesis and to regulate secretion of pro-tumorigenic factors. In the review of New et al. (Tooze's lab), the idea that advanced and aggressive mutant *KRAS*-driven tumors (such as pancreatic ductal adenocarcinomas) exploit a heightened autophagy–lysosomal pathway under the transcriptional control of the MiF/TFE factors to support energy metabolism and to allow growth under conditions of energy deficit and metabolic stress is discussed (5). Furthermore, the review of Iovanna (Iovanna's lab) highlights the key role played by the pancreatitis-associated vacuolar protein 1 (VMP1) in pancreatic acinar cells and how its elevated expression drives early autophagy and cooperates with the *KRAS* oncogene to promote carcinogenesis in the pancreas.

Another emerging aspect linking autophagy to tumor progression, discussed in the review of Keulers et al. (Rouschop's lab), is the ability of advanced cancer cells to use autophagy as a trafficking and export mechanism of pro-tumorigenic factors, such as pro-inflammatory/pro-angiogenic cytokines or chemotactic/pro-invasive molecules. This cancer cell-autonomous trait further illustrates the plasticity of tumor-associated autophagy, which can enable and modulate the crosstalk between cancer and stromal cells thereby affecting the tumor microenvironment, a property that needs to be taken into consideration when considering therapeutic approaches. Based on the growing relevance of tumor-associated autophagy, many labs are developing and testing the effects of autophagy modulators in cancer therapy. The recognition of the prevalent—albeit not unique—cytoprotective and stress adaptation roles of autophagy in advanced cancers has led to the assumption—as supported by *in vitro* and preclinical data—that blocking cancer cell-intrinsic autophagy may curtail cancer cell resistance to chemotherapy, thereby improving therapy outcome. Thus, the first-generation autophagy blockers, e.g., chloroquine and its derivative hydroxychloroquine (6, 7) are currently being tested in different clinical trials to potentiate patients' responses to a variety of anticancer regimens.[2] On the other hand, as autophagy can control both cell death and survival programs, the induction of autophagic cancer cell death elicited by certain anticancer therapies, may offer a therapeutically attractive strategy, especially when cancer cells display resistance to apoptosis, as discussed in the review by Fulda (Fulda's lab). Finally, although autophagy is a highly dynamic process, the expression of certain autophagy genes in aggressive tumors like melanoma, may provide novel independent prognostic biomarkers for early stage neoplasms, as discussed in the review of Tang et al. (Lovat's lab). This may help to identify patients at risk of disease progression, thus facilitating earlier patient therapeutic intervention and stratification for personalized therapeutic approaches.

AUTHOR CONTRIBUTIONS

Authors contributed equally.

ACKNOWLEDGMENTS

The authors would like to thank all the authors who contributed to this research topic as well as various reviewers/editors of the respective manuscripts, for their efforts, timely responses and enthusiasm. We also thank the Frontiers Editorial Office for their assistance and support. This work was supported by KU Leuven C16/15/073 and FWO grant G0584.12 to PA, and by Parkinson's UK (G1402), Alzheimer's Society, BBSRC BrisSYnBio project grants, and a Wellcome Trust Ph.D. studentship (awarded through the Dynamic Cell Biology programme: no. 083474) to JL.

[2]https://clinicaltrials.gov/.

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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.

frontiers in Oncology

REVIEW
published: 31 March 2017
doi: 10.3389/fonc.2017.00051

Autophagy and the Cell Cycle: A Complex Landscape

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Autophagy is a self-degradation pathway, in which cytoplasmic material is sequestered in double-membrane vesicles and delivered to the lysosome for degradation. Under basal conditions, autophagy plays a homeostatic function. However, in response to various stresses, the pathway can be further induced to mediate cytoprotection. Defective autophagy has been linked to a number of human pathologies, including neoplastic transformation, even though autophagy can also sustain the growth of tumor cells in certain contexts. In recent years, a considerable correlation has emerged between autophagy induction and stress-related cell-cycle responses, as well as unexpected roles for autophagy factors and selective autophagic degradation in the process of cell division. These advances have obvious implications for our understanding of the intricate relationship between autophagy and cancer. In this review, we will discuss our current knowledge of the reciprocal regulation connecting the autophagy pathway and cell-cycle progression. Furthermore, key findings involving nonautophagic functions for autophagy-related factors in cell-cycle regulation will be addressed.

Keywords: autophagy, cancer, cell cycle, cell stress, senescence, mitosis, cytokinesis, p53

OPEN ACCESS

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *01 February 2017*
Accepted: *10 March 2017*
Published: *31 March 2017*

Citation:
Mathiassen SG, De Zio D and Cecconi F (2017) Autophagy and the Cell Cycle: A Complex Landscape. Front. Oncol. 7:51. doi: 10.3389/fonc.2017.00051

Abbreviations: γH2AX, γ-Histone 2AX; 3-MA, 3-methyladenine; AMBRA1, activating molecule in Beclin 1-regulated autophagy; AMPK, AMP-activated protein kinase; APC/C, anaphase-promoting complex/cyclosome; ASPP2, apoptosis-stimulating of p53 protein 2; Atg 3, 5, 7, 8, 9, 12, 14, autophagy-related gene 3, 5, 7, 8, 9, 12, 14; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related protein; Bcl-2, B-cell lymphoma 2; Bcl-X_L, B-cell lymphoma extra large; BIF-1, Bax-interacting factor 1; BNIP3, BCL2-interacting protein 3; CAMKK, calcium/calmodulin-dependent protein kinase kinase; C-Cbl, Casitas B-lineage lymphoma; CCFs, cytoplasmic chromatin fragments; CDK 1, 2, 4, cyclin-dependent kinase 1, 2, 4; CDKIs, cyclin-dependent kinase inhibitors; CDKs, cyclin-dependent kinases; CEP55, centrosomal protein 55; CHMP4B, charged multivesicular body protein 4B; CIN, chromosomal instability; Cip/Kip, CDK interacting protein/kinase inhibitory protein; c-Myc, myelocytomatosis oncogene cellular homolog; DAPK1, death-associated protein kinase 1; Ddit4/Redd1, DNA damage-inducible transcript 4; DRAM, damage-regulated autophagy modulator; Drp1, dynamin-like protein; E2F, E2 factor; Ect2, epithelial cell-transforming sequence 2; ESCRT, endosomal sorting complex required for transport; FBXL20, F-box/LRR-repeat protein 20; GATA4, GATA binding protein 4; GEFs, GDP–GTP exchange factors; GFP, green fluorescence protein; INK4, inhibitors of CDK4; LADs, lamin-associated domains; LAMP2, lysosomal-associated membrane protein 2; LC3, light chain 3; LIR, LC3-interacting region; LKB1, liver kinase B1; MAP1LC3B, microtubule-associated protein 1 light chain 3 beta; Mdm2, mouse double minute 2 homolog; MEFs, murine embryonic fibroblast; MR, midbody ring; MR^d, midbody ring derivative; MRLC, myosin regulatory light chain; mTOR, mammalian target of rapamycin; NBR1, neighbor of BRCA1 gene; NDP52, nuclear dot protein 52 kDa; OIS, oncogene-induced senescence; PAK2, p21-activated protein kinase; PE, phosphatidylethanolamine; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3; PP2A, protein phosphatase 2 A; PPP1R12C, protein phosphatase 1 regulatory subunit 12C; PRKAB1, 2, protein kinase AMP-activated noncatalytic subunit beta 1, 2; PtdIns3K, class III phosphatidylinositol 3-kinase; PtdIns3P, phosphatidylinositol 3-phosphate; PTEN, phosphatase and tensin homolog; RAS, rat sarcoma; Rb, retinoblastoma protein; RB1CC1/FIP200, RB1 inducible coiled-coil 1/FAK family kinase-interacting protein of 200 kDa; RhoA, Ras homolog family member A; SASP, senescence-associated secretion phenotype; Skp1, S-phase kinase-associated protein 1; smARF, small mitochondrial ARF; SQSTM1, sequestosome 1; TASCC, mTOR-autophagy spatial coupling compartment; TIS, therapy-induced senescence; TSC2, tuberous sclerosis 2; ULK1, 2, 3, unc-51-like autophagy-activating kinase 1, 2, 3; USP 10, 13, ubiquitin-specific peptidase 10, 13; V-ATPase, vacuolar-type H^+-ATPase; UVRAG, UV irradiation resistance-associated gene; Vps 15, 34, vacuolar protein sorting 15, 34.

INTRODUCTION

Macroautophagy (herein referred to as autophagy) is a highly conserved catabolic pathway that mediates the sequestration and delivery of cytoplasmic material to the lysosome for degradation. This is achieved by the formation and expansion of an isolation membrane (or phagophore) that fuses to engulf cytoplasmic constituents in a double-membrane autophagic vacuole (the autophagosome). The autophagosome finally undergoes fusion with lysosomes whereby the enclosed cargo is degraded and subsequently released and recycled to support cellular metabolism. In physiological conditions, autophagy proceeds at a basal level to ensure the turnover of superfluous or damaged components, including organelles and long-lived proteins, to maintain cellular homeostasis. Moreover, the autophagic flux can be upregulated in response to a wide range of stresses, such as nutrient deprivation, reactive oxygen species, DNA damage, protein aggregates, damaged organelles, or intracellular pathogens, whereby it functions as an adaptive cytoprotective response (1, 2).

The molecular pathway that orchestrates the initiation and execution of autophagy has been comprehensively reviewed elsewhere (3–5). In short, the initiation phase of autophagy is governed by two main complexes: the unc-51-like autophagy-activating kinase (ULK) complex and the class III phosphatidylinositol 3-kinase (PtdIns3K) complex (**Figure 1A**). The PtdIns3K complex produces phosphatidylinositol 3-phosphate (PtdIns3P) for recruitment of additional autophagy factors to the phagophore and is partially comprised of the key autophagy regulators vacuolar protein sorting 34 (Vps34), Beclin 1, vacuolar protein sorting 15 (Vps15), and activating molecule in Beclin 1-regulated autophagy (AMBRA1). Downstream of these complexes are two ubiquitin-like conjugation systems that mediate vesicle expansion [the autophagy-related gene 8 (Atg8) and autophagy-related gene 12 (Atg12) systems]. Both systems require the E1-like protein autophagy-related gene 7 (Atg7) for activation of the ubiquitin-like proteins Atg8 [light chain 3 (LC3) in mammals] and Atg12. In the Atg8 system, Atg8/LC3 undergoes proteolytic processing and covalent attachment to the lipid phosphatidylethanolamine (in mammalian cells, the precursor form is termed LC3-I and the lipidated form LC3-II), by which it becomes associated with the phagophore membrane. Consequently, autophagy can be detected biochemically (by assessing the generation of LC3-II) or microscopically (by observing the formation of LC3 puncta, representative of LC3 redistribution to the developing autophagosomes). Apart from these systems, the pathway includes the transmembrane protein autophagy-related gene 9 (Atg9), as well as factors involved in autophagosome–lysosome fusion [e.g., lysosomal-associated membrane protein 2 (LAMP2)], vacuolar permeases mediating the efflux of amino acids from the lysosome, and lysosomal enzymes required for cargo degradation (3–7). Furthermore, while originally considered a largely unspecific process, recent years have revealed the existence of selective autophagy pathways, in which specific cargoes can be targeted to the emerging autophagosomes for engulfment and degradation. Cargoes destined for selective autophagy are often ubiquitinated and recognized by autophagy receptors [i.e., p62/sequestosome 1, neighbor of BRCA1 gene (NBR1), nuclear dot protein 52 kDa (NDP52), optineurin, or C-Cbl] that contain ubiquitin-binding domains as well as LC3-interacting region (LIR) motifs for recruitment to the inner phagophore membrane (8, 9) (**Figure 1A**).

Autophagy induction is controlled upstream by energy-sensing proteins, a key regulator being the mammalian target of rapamycin (mTOR), which provides the major inhibitory signal that shuts off autophagy in the presence of abundant nutrients. A key inhibitor of mTOR AMP-activated protein kinase (AMPK) is activated upon energy stress that increases the AMP/ATP ratio. Once activated, AMPK downregulates ATP-consuming (anabolic) pathways and upregulates ATP-generating (catabolic) pathways, such as autophagy, to maintain cellular energy homeostasis. Besides inhibiting the catalytic activity of mTOR, AMPK also directly stimulates autophagy by phosphorylating upstream autophagy factors [e.g., unc-51-like autophagy-activating kinase 1 (ULK1) and Beclin 1] (1, 3, 4) (**Figure 1A**).

In recent years, the notion that autophagy may represent a bona fide tumor suppressor pathway has obtained increasing support. Autophagy-deficient animal models are often prone to tumor formation (10–16) and autophagy deficiency is associated with increased DNA damage and chromosomal instability (CIN) (17). Thus, autophagy is thought to constitute a barrier against malignant transformation by preserving intracellular homeostasis, even though the exact mechanism of autophagy-mediated oncosuppression is not well-understood. Autophagy can conversely sustain the survival and proliferation of neoplastic cells exposed to intracellular and environmental stresses, such as hypoxia and chemotherapy, and thereby supports tumor growth and progression. Hence, depending on the context, autophagy can act either as a tumor-suppressive or a tumor-promoting pathway (2, 18, 19).

As many signaling pathways exhibit opposing effects on autophagy and cell-cycle progression (20), these are often considered mutually exclusive processes. Accumulating evidence suggests that this opposing regulation may be coordinated and that an interplay between the two processes exists. This is exemplified by the scaffold protein AMBRA1, a pro-autophagic protein that is also able to negatively regulate the oncogene c-Myc (10). AMBRA1 interacts with the catalytic subunit of the protein phosphatase 2 A (PP2A) and facilitates PP2A-mediated dephosphorylation and subsequent proteasomal degradation of c-Myc, thus resulting in inhibition of proliferation and in tumor suppression (10). Both the role of AMBRA1 in promoting c-Myc degradation, as well as in AMBRA1-dependent autophagy, is controlled upstream by mTOR (10, 21), which argues for a coordinated regulation of autophagy and cell-cycle progression.

In the present review, we will focus on various aspects of the reciprocal regulation connecting autophagy and the cell cycle. Cell-cycle progression is governed by cyclin-dependent kinases (CDKs). CDK activity is coordinated by binding of their essential regulatory subunits, cyclins, which are synthesized and degraded at specified times during the cell cycle to coordinate timely CDK activation and drive cell-cycle progression (**Figure 1B**). The

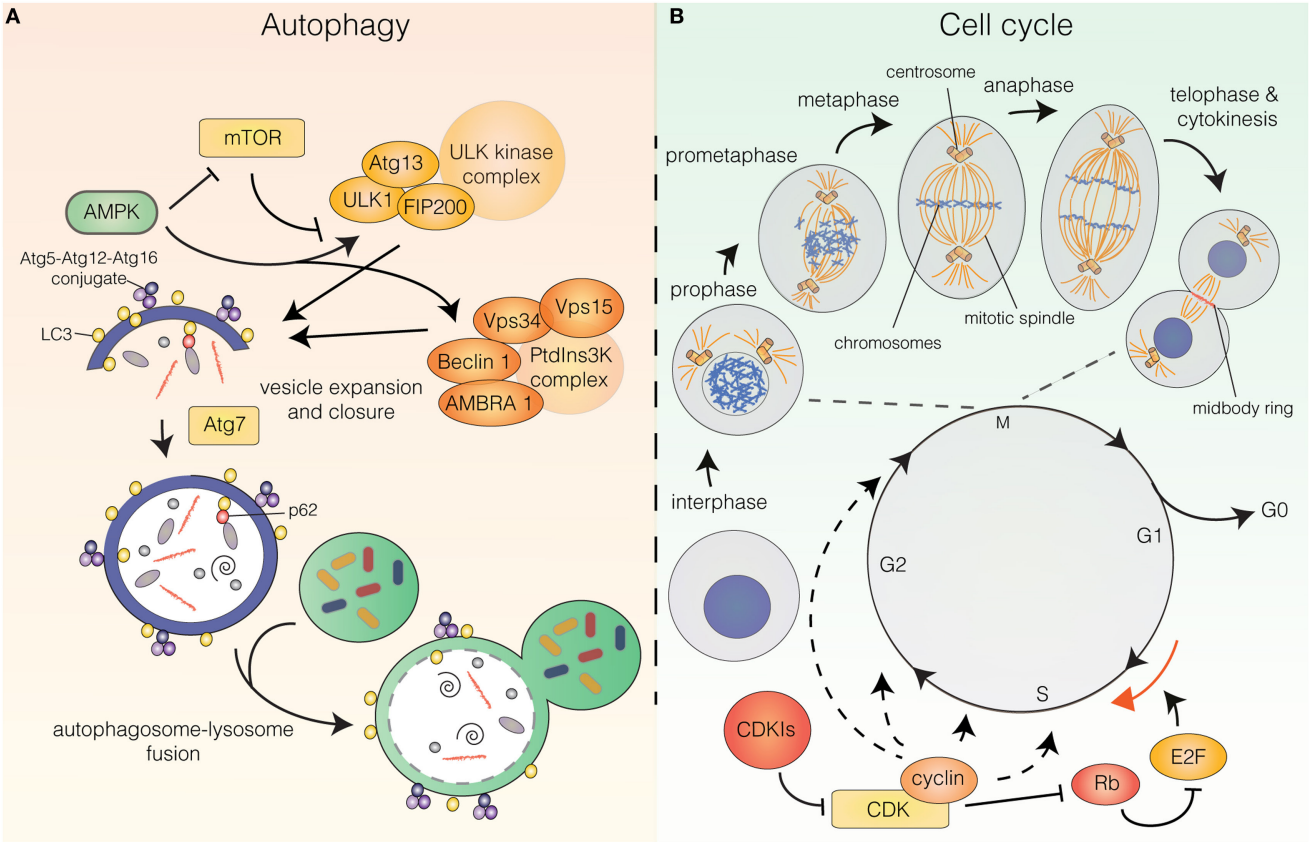

FIGURE 1 | **(A)** The autophagy pathway. Autophagy induction is controlled upstream by energy sensors, mammalian target of rapamycin (mTOR), and AMP-activated protein kinase (AMPK). mTOR shuts off autophagy in the presence of abundant nutrients, while AMPK is activated upon energy stress. AMPK induces autophagy by inhibiting mTOR and stimulating upstream autophagy factors of the unc-51-like autophagy-activating kinase (ULK) and class III phosphatidylinositol 3-kinase (PtdIns3K) complexes. Vesicle expansion requires the autophagy-related gene 8 (Atg8)/light chain 3 (LC3) and autophagy-related gene 12 (Atg12) ubiquitin-like conjugation systems. Autophagy receptors (e.g., p62) can mediate selective recruitment of cargo to the inner vesicle membrane. Following vesicle closure, the autophagosome fuses with the lysosome whereby the engulfed material is degraded. **(B)** The cell cycle. The cell cycle can be divided into G0, G1, S, G2 (interphase), and M-phase (mitosis and cytokinesis). Mitosis can be subdivided into prophase (DNA condensation is initiated), prometaphase (the mitotic spindle starts to form and the nuclear envelope has been dissolved), metaphase (the chromosomes are aligned at the metaphase plate), anaphase (separation of the sisterchromatids) and telophase (DNA decondenses, the nuclear envelope reforms, the contractile ring starts forming) and is followed by cytokinesis (physical separation of the daughter cells). Cell-cycle progression is governed by cyclin-dependent kinase (CDK) holoenzymes. CDK activity can be inhibited by cyclin-dependent kinase inhibitors. For G1/S transition cyclin-CDKs phosphorylate retinoblastoma protein (Rb), which releases E2 factor (E2F) transcription factors from inhibitory binding, leading them to induce transcription of targets for G1/S transition.

decision to enter or exit the cell cycle depends on the nutrient and mitogen availability and is also affected by stress-stimuli that may block the cell cycle transiently or irreversibly. Once committed to cell-cycle progression, the cell undergoes a series of regulated events (i.e., cell growth, DNA replication, and quality control checkpoints) culminating in the highly orchestrated process of cell division. Dysregulation of proteins controlling the frequency and fidelity of proliferation is inextricably linked to neoplastic transformation (22–24).

Herein, we will address the activation of autophagy during normal and abnormal cell-cycle progression as well as the coordinated induction of autophagy and cell-cycle responses following exposure to various stresses. Finally, the involvement of autophagy and autophagy-related proteins in the regulation of cell division will be discussed.

AUTOPHAGY STATUS DURING CELL-CYCLE PROGRESSION

Only few studies have focused on a putative correlation between autophagy flux and cell-cycle progression. The cell cycle can be divided into five major phases: G_0, G_1, S, G_2, and M-phase (**Figure 1B**). G_0, G_1, S, and G_2 are collectively referred to as interphase, while M-phase is comprised of mitosis and cytokinesis, the processes by which the duplicated genome and other cellular constituents are distributed to the two daughter cells and the subsequent separation of these. Mitosis is traditionally subdivided into five phases: prophase (DNA condensation is initiated), prometaphase (the mitotic spindle starts to form and the nuclear envelope is dissolved), metaphase (the chromosomes are aligned at the metaphase plate), anaphase (separation of

the sister chromatids to separate chromosomes) and telophase (DNA decondenses, the nuclear envelope is reformed and the contractile ring at the intercellular bridge between the two nuclei starts forming). This is followed by cytokinesis, in which the two daughter cells are physically separated (25) (**Figure 1B**).

Autophagy and Interphase

The question of differential regulation of autophagy during cell-cycle progression was initially addressed by Tasdemir et al., prompted by their observation that autophagy-inducing treatment of unsynchronized cell populations only induced green fluorescence protein (GFP)–LC3 aggregation in approximately 50% of cells (26). To understand if autophagy preferentially occurs in certain cell-cycle phases, immunocytochemical approaches were employed to monitor cytoplasmic GFP–LC3 aggregation in connection with cell-cycle progression (26). Using a panel of autophagy activators, including the BH3 mimetic ABT737, lithium, rapamycin, tunicamycin, or starvation, autophagy induction was observed to preferentially occur in the G1 and S phases of the cell cycle (26). More recently, Kaminskyy et al. developed another strategy to monitor autophagosome accumulation by extracting membrane-unbound LC3-I from cells, followed by flow cytometric detection of the remaining autophagosomal membrane-associated fraction of LC3-II. This was combined with propidium iodide staining for detection of cell-cycle status (27). By using this approach, basal autophagy was detected in G1, S, and G2/M phases. Furthermore, autophagy induction by starvation or rapamycin treatment resulted in LC3-II accumulation in all stages (27), suggesting the absence of cell-cycle-dependent autophagy regulation. The contradictory findings may be the result of the variant experimental approaches. Thus, further studies are required to determine if autophagy activation is preferentially linked to specific cell-cycle phases.

Autophagy and Mitosis

As the above studies do not allow discrimination between G2 and M phase, this leaves the question of autophagy status during mitosis. Two elegant studies have reported a striking decrease in autophagic activity during mitosis (28, 29). By means of electron microscopy and stereology to quantify the presence of autophagic vacuoles in mitotic cells, Eskelinen et al. found a strong reduction in autophagosomal content in both (pro)metaphase and anaphase cells (28). Furuya et al. expanded on these findings revealing that mitotic autophagy inhibition depends on cyclin-dependent kinase 1 (CDK1)-mediated phosphorylation of Vps34 on Thr159 during mitosis (29). This phosphorylation event negatively regulates the interaction between Vps34 and Beclin 1, thereby inhibiting PtdIns3K activity, PtdIns3P production, and autophagy induction (29). Of note, during mitosis, cells undergo extensive structural rearrangements and the inhibition of autophagy has been speculated to function as a protective mechanism to prevent unintended loss of organelles and chromosomes. Indeed, break down of the nuclear envelope during mitosis leaves the condensed chromosomes potentially vulnerable to the cytoplasmic autophagy machinery. Accordingly, Eskelinen et al. observed that re-appearance of autophagosomes occurred in telophase/G1 after formation of the new nuclear envelopes (28). Furthermore, autophagosomal engulfment of mitotic chromosomes was reported in mitotic cells undergoing programmed cell death (30), suggesting that autophagy inhibition may, indeed, protect the condensed genome from accidental autophagic engulfment. Moreover, during cell division, mitochondria and the Golgi apparatus become fragmented to facilitate their distribution between the two daughter cells (31, 32). While elongated mitochondria are spared from autophagic degradation (33, 34), the smaller size of fragmented mitochondria facilitates their uptake by autophagosomes (35, 36). Mitotic fragmentation of mitochondria is mediated by CDK1-dependent phosphorylation and activation of the dynamin-like protein (Drp1), involved in mitochondrial fission (37). Interestingly, cells arrested in mitosis by abrogated Cyclin B1 degradation, exhibit a gradual decline in mitochondrial mass due to ongoing mitophagic degradation (38). Prevention of mitophagy by depletion of Drp1 or key autophagy proteins delayed cell death by mitotic arrest; thus, mitophagy may facilitate mitotic cell death during prolonged mitotic block (38). The resistance to mitotic cell death acquired upon Drp1 knock-down supports the speculated vulnerability of fragmented mitotic mitochondria to autophagic degradation. Ongoing mitophagy during mitotic arrest may simply represent leaky degradation from incompletely blocked autophagy, which is functionally relevant during prolonged mitotic arrest but likely negligible during normal mitotic progression. However, this mechanism may also participate in pushing cells with mitotic abnormalities toward cell death.

In accordance with the reported ongoing mitophagy in arrested mitotic cells (38), LC3 puncta have been observed in mitotic cells, although at a significantly decreased level compared to interphase cells (28, 29, 39, 40). While these may also represent inefficient autophagy inhibition, Loukil et al. observed LC3, p62, and lysosomal markers colocalizing with Cyclin A2 foci during mitosis and found that autophagy partially contributes to mediating mitotic Cyclin A2 degradation (40). Thus, an intriguing although highly controversial theory is the existence of distinct sites of active autophagy during cell division. Treatment with autophagy inducers or lysosomal inhibitors has been shown to result in accumulation of LC3 puncta in mitotic cells, which was suggested as an indication of active autophagy flux in mitosis (39, 41). The short duration of mitosis, however, poses technical challenges in employing these treatments, as it is difficult to rule out autophagosome accumulation from interphase. Live-cell imaging using GFP–LC3 cell lines or preferably cell lines carrying endogenously tagged autophagy proteins may help in determinining the degree of autophagy inhibition as well as the potential presence of active autophagic compartments in mitosis.

INTERPLAY BETWEEN AUTOPHAGY AND CELL-CYCLE ARREST

In response to unfavorable or stressful conditions, cells are able to arrest the cell cycle transiently or irreversibly. This ability helps regulate proliferation during development and differentiation, and prevents the expansion of potentially harmful cell populations (23, 42). Autophagy, like cell cycle arrest, is induced in response to a variety of stress conditions, where it plays a pivotal

role in preserving cellular viability (2). While the correlative induction of autophagy and cell-cycle arrest has been extensively documented, the molecular mechanisms linking them together are still debated and largely unknown.

Autophagy Regulation by Cyclin-Dependent Kinase Inhibitors (CDKIs) and Retinoblastoma Protein (Rb)/E2 Factor (E2F) Activity

Cell-cycle arrest often relies on the action of various cell-cycle inhibitors. An important class of those are CDKIs that inhibit CDK activity by direct interaction with CDKs or cyclin-CDK holoenzymes (43) (**Figure 1B**). CDKIs can be categorized into two main families: the inhibitors of CDK4 (INK4) family consisting of $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$; and the Cip/Kip family composed of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (23, 42). In spite of their similar modes of action, CDKIs are speculated to have functionally distinct roles and appear to be activated by different stimuli (42). Thus, while p21 is most strongly linked to stress and DNA damage signaling, downstream of p53-mediated pathways, p27 is more often associated with cell-cycle arrest in response to low nutrient and mitogen conditions (42). CDKIs were originally strictly linked to proliferation control, but they are now demonstrated to have a wide range of alternative functions in processes including transcription, apoptosis, migration (42), as well as autophagy induction (44–46). Cell-cycle arrest can also occur by repression of E2F transcription factors that mediate transcriptional induction of a plethora of targets, including cyclins and replication regulators required for G1/S transition and cell-cycle progression (47, 48) (**Figure 1B**). E2F activity is controlled by binding of the Rb protein or other Rb family members (49). Upon mitogenic stimuli, Rb is gradually phosphorylated by cyclin-CDK complexes whereby E2F is released to induce transcription of its target genes, pushing cells to pass the G1/S boundary (47, 49). CDKIs, through their ability to inhibit CDKs, are also important indirect promoters of Rb/E2F interactions (48) (**Figure 1B**).

A number of CDKIs, including p16, p21, and p27 have been reported to induce autophagy (44–46), suggesting the existence of coordinated stress responses linking autophagy induction and cell-cycle arrest. Liang et al. showed that in response to starvation, p27 is activated by the liver kinase B1 (LKB1)–AMPK nutrient-sensing pathway through phosphorylation of Thr198, thus resulting in p27 stabilization (46). Interestingly, p27 was required for efficient starvation-induced autophagy in murine embryonic fibroblast and protected from cell death resulting from metabolic stress (46), indicating a critical role for p27 in autophagy activation under starvation conditions. The increased stability of $p\text{-}p27^{Thr198}$ implies a function for the LKB1–AMPK pathway in mediating p27-dependent cell-cycle arrest. Accordingly, a non-phosphorylatable $p27^{T198A}$ mutant was less efficient than wild type p27 or a phospho-mimicking $p27^{T198D}$ mutant at inhibiting colony formation (46). This is in line with previous reports arguing for a central role for p27 in starvation-induced cell-cycle arrest (50, 51). p27 is upregulated in response to serum starvation (50) and its depletion allows serum-starved cells to evade cell-cycle arrest and continue proliferation (50, 51). Thus, p27 may be a key effector of the cellular response to metabolic stress, functioning downstream of the LKB1–AMPK axis to mediate both cell-cycle arrest and autophagy induction. Accordingly, p27 is degraded by caspases during growth-factor deprivation-induced apoptosis (52).

The mechanism by which p27 mediates autophagy induction and the relevance of its CDK inhibitory function in this context is, however, not clear. Nonetheless, it has been reported that the cyclin-binding region of p27 is required for autophagy induction (46, 53) and that depletion of CDK2 and CDK4 partially reproduces p27-induced effects on autophagy and apoptosis (46). In this context, indirect activation of Rb by p27 could be a contributing factor, as this has been reported for p16 (45). Overexpression of p16 is able to induce autophagy in an Rb-dependent manner through promoting Rb/E2F interaction (45), which suggests negative regulation of autophagy by E2Fs. This supports a model in which p16-mediated CDK inhibition facilitates Rb/E2F interaction and consequent E2F inhibition, resulting in activation of autophagy through an unspecified mechanism. However, while autophagy induction by p16 appears to largely depend on Rb/E2F regulation, p27-induced autophagy was only mildly affected by Rb status (45), suggesting varying mechanisms of autophagy activation between CDKIs. Intriguingly, in budding yeast, the CDK Pho85 is able to both induce or inhibit autophagy, depending on its associated cyclin partner (54).

The literature linking Rb/E2F and autophagy is complex, as positive regulation of autophagy by E2Fs has also been reported. Using an inducible E2F activation system, Polager et al. demonstrated that several autophagy genes such as LC3, ULK1, and DRAM were direct targets of E2F transcription factors (55). Moreover, E2Fs were shown to bind the promoter region of Beclin 1 (56), even though the functional significance of this binding remains to be demonstrated. E2F downstream targets such as smARF or the hypoxia-inducible B-cell lymphoma 2 (Bcl-2) family member BCL2 interacting protein 3 (BNIP3) have also been shown to induce autophagy (44, 57, 58). BNIP3 was demonstrated to be required for efficient hypoxia-induced autophagy activation (58) and E2F1 to be required for efficient DNA-damage-induced autophagy (55). This evidence indicates a potential role for E2Fs in mediating autophagy during acute stress responses, rather than during normal cell-cycle progression. E2F-mediated autophagy induction may therefore depend on the context and stimuli. Furthermore, as the E2F family comprises eight family members that can both transactivate and repress gene expression (47), E2F contribution to autophagy regulation likely depends on the involved E2F factor.

Autophagy and p53

The most well-documented connection between autophagy and stress-induced cell-cycle responses is likely the link between p53 and autophagy regulation. p53 is one of the most extensively characterized tumor suppressor proteins and a central coordinator of the cellular response to acute stress (59, 60). Under basal conditions, p53 levels are strictly controlled by mouse double minute 2 homolog (Mdm2)-mediated ubiquitination and proteasomal degradation, while in response to a wide range of stresses (e.g., DNA damage, oncogene expression or nutrient deprivation), p53 undergoes rapid post-translational modifications that allow for

its stabilization and activation (59) (**Figure 2**). Upon activation, p53 orchestrates the induction of appropriate cellular responses, be it apoptosis, cell-cycle arrest, DNA repair, metabolic adaptation, or autophagy, with the purpose of limiting the expansion of damaged and potentially harmful cells (59, 60) (**Figure 2**). The shared involvement of p53 and autophagy in stress-related processes, as well as their relevance for neoplastic transformation has motivated great efforts to understand the role of autophagy ablation in the context of p53-deficient and -proficient animal models of human cancers, reviewed in Ref. (61). In this article, we will focus our attention on the molecular mechanisms linking p53 to autophagy regulation.

Autophagy Modulation by Nuclear p53

A number of reports have demonstrated autophagy induction by p53 (18, 62, 63). The ability of p53 to stimulate autophagy appears to rely on its function as a stress-induced transcription factor, as p53 can transactivate a wide range of autophagy-related genes (18, 62, 63) (**Figure 2**). Activation of some of these genes converges on activation of AMPK and inhibition of mTOR. These include genes encoding the AMPKβ1 and β2 subunits (64), the AMPK activators Sestrin 1 and Sestrin 2 (65, 66), as well as negative regulators of mTORC1, tuberous sclerosis 2 (TSC2), phosphatase and tensin homolog (PTEN), and DNA damage-inducible transcript 4 (Ddit4) (64, 67, 68). Accordingly, Feng et al. reported that p53-induced autophagy following DNA damage relied on AMPK-mediated inhibition of mTOR (69). Other p53 responsive genes include ULK1 and unc-51-like autophagy-activating kinase 2 (ULK2) (70), genes encoding various BH3-only proteins and death-associated protein kinase 1 (DAPK1), all of which stimulate autophagy by favoring the displacement of Beclin 1 from inhibitory interactions with Bcl-2 and Bcl-X_L (71–74), as well as the gene coding for DRAM (75), a highly conserved lysosomal protein, which was also suggested

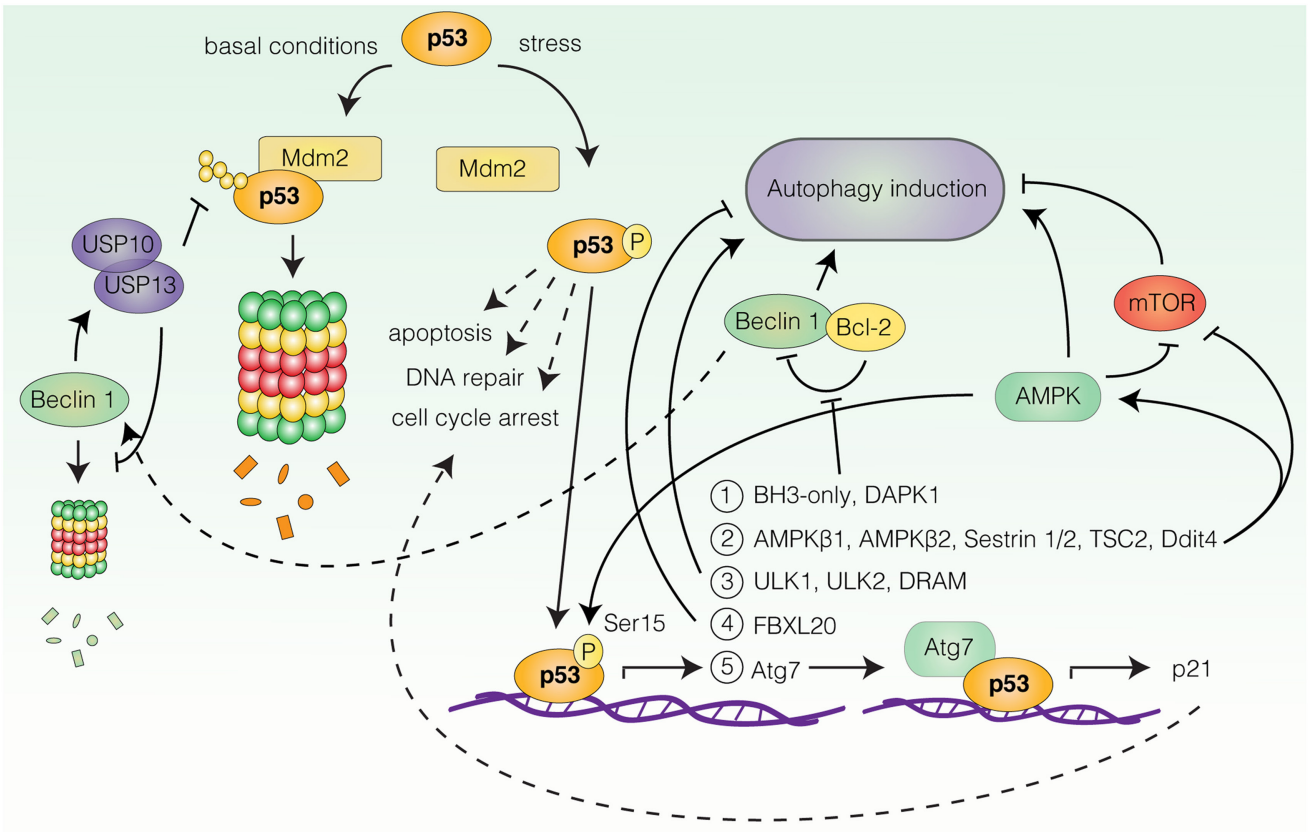

FIGURE 2 | Transcriptional regulation of autophagy by p53. Under basal conditions p53 is degraded by mouse double minute 2 homolog (Mdm2)-mediated proteasomal degradation. In response to stress, p53 undergoes post-translational modifications leading to its stabilization and activation. Upon activation, p53 can induce transcription of autophagy-related genes (only a selection is represented here). Group 1: BH3-only proteins and death-associated protein kinase 1 (DAPK1), all stimulate autophagy by favoring Beclin 1 displacement from B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra large (Bcl-X_L). Beclin 1 can contribute to p53 stabilization by stabilizing the deubiquitinating enzymes ubiquitin-specific peptidase 10/13 (USP10/13). Group 2: AMP-activated protein kinase (AMPK) subunits β1 and β2, AMPK activators Sestrin 1/2, negative regulators of mammalian target of rapamycin (mTOR), tuberous sclerosis 2 (TSC2), and DNA damage-inducible transcript 4 (Ddit4), all promote autophagy induction. AMPK can in turn phosphorylate and activate p53. Group 3: unc-51-like autophagy-activating kinase 1 (ULK1), unc-51-like autophagy-activating kinase 2 (ULK2), and damage-regulated autophagy modulator. Target 4: F-box/LRR-repeat protein 20 (FBXL20) negatively regulates autophagy by promoting vacuolar protein sorting 34 (Vps34) degradation. Target 5: Key autophagy protein autophagy-related gene 7 (Atg7) cooperates with p53 for p21 induction.

to be required for p53-dependent autophagy induction in response to DNA damage (75). Furthermore, Kenzelmann Broz et al. utilized a high-throughput approach to uncover novel p53 transcriptional targets in response to DNA damage (67). This approach identified extensive transactivation of the autophagy network, encompassing both upstream autophagy regulators, members of the autophagy core machinery, and lysosomal proteins by all three p53 family members; p53, p63, and p73 (67). Interestingly, one of the identified targets, Atg7 (67), has been reported to bind the promoter of p21, collaborating with p53 for efficient p21 upregulation in a nonautophagy-dependent manner (76) (**Figure 2**). Thus, p53-dependent upregulation of Atg7 may function as an effector mechanism boosting the p53 response through p21 production. Similarly, AMPK can activate p53 upon glucose deprivation by phosphorylation of Ser15, which is required for AMPK-mediated cell-cycle arrest in this context (77) (**Figure 2**). Surprisingly, Beclin 1 can also contribute to p53 stabilization by promoting the stabilization of deubiquitinating enzymes ubiquitin-specific peptidase 10/13 (USP10/13) (78), which counteract the Mdm2-mediated degradation of p53 (78, 79), as well as degradation of Beclin 1 itself (78) (**Figure 2**). Thus, autophagy and p53 pathways may potentiate and sustain each other in establishing efficient stress-related cell-cycle programs.

Interestingly, activated p53 is also able to decrease autophagy, as the p53-responsive gene F-box/LRR-repeat protein 20 (FBXL20) is able to mediate the degradation of Vps34 following DNA damage, resulting in autophagy inhibition (80). In which context p53 activation results in autophagy stimulation and inhibition, respectively, is not understood. Furthermore, the effect of p53-induced autophagy is not clear, but in several contexts autophagy surprisingly appears to function as an effector of p53-mediated cell death rather than as a survival mechanism (67, 70, 75).

Autophagy Inhibition by Cytosolic p53

Contrasting the proautophagic transcriptional activity of nuclear p53, the cytoplasmic pool of p53 has been demonstrated to suppress autophagy (81). Knockout, depletion, or pharmacological inhibition of p53 in human, mouse as well as nematode cells, can induce autophagy in a manner appearing to depend on the AMPK/mTOR pathway (81). Correspondingly, p53 restricted to the cytosol but not nucleus-restricted p53 inhibited autophagy, a regulation that also persisted in enucleated cells (81). Accordingly, suppression of autophagy by p53 correlated with its nuclear-to-cytosolic distribution in a panel of cancer-associated p53 mutants (82). Surprisingly, several distinct proautophagic stimuli, including nutrient deprivation and mTOR inhibition by rapamycin were found to induce Mdm2-dependent proteasomal degradation of p53. Inhibition of proteasomal activity, Mdm2 depletion, or pharmacological inhibition of Mdm2 reduced autophagy induction in response to these stimuli (81), suggesting the requirement of p53 degradation for efficient autophagy activation. The molecular mechanism underlying this p53-mediated autophagy suppression is not understood, but has been suggested to involve negative regulation of the upstream autophagy factor RB1 inducible coiled-coil 1/FAK family kinase-interacting protein of 200 kDa (RB1CC1/FIP200) through a physical interaction with p53 (83). How the contradictory regimes of cytoplasmic versus nuclear p53-mediated autophagy regulation can be reconciled remains to be determined.

Autophagy and Senescence

While several lines of evidence suggest coordinated induction of autophagy and cell-cycle arrest pathways, another issue remains the involvement of autophagy in the execution of cell-cycle exit programs, in particular, *senescence*. The terms *quiescence* and *senescence* are often used interchangeably to describe cell-cycle arrest, although they refer to distinct cell states (84). *Quiescence* represents a reversible cell-cycle arrest often caused by lack of nutrients and/or mitogens and growth factors, while *senescence* is an irreversible state of cell-cycle arrest that is more often induced in abnormal (potentially cancerous), DNA-damaged, or aging cells as a stress response (84–86). While it is clear that autophagy and senescence are often parallel processes, the question of their interdependence is a subject of much debate. It is beyond the scope of the present review to comprehensively recapitulate the literature involving this topic, and for more on this subject, we refer to Ref. (84, 87, 88). In this article, we will focus our attention on key findings and recent publications that offer mechanistic insight to the relationship between autophagy and senescence.

Autophagy and Senescence Transition

In recent years, a number of studies have argued for a more direct link between autophagy and senescence that goes beyond their correlative induction, by showing that inhibition of autophagy delays senescence transition (89–93). Young et al. employed models of oncogene-induced and DNA damage-induced senescence to study autophagy activation during senescence transition (93). In the applied model of oncogene-induced senescence (OIS), an initial "mitotic phase" of proliferative burst occurs around day 1. This is followed by a "transition phase," preceding the "senescence phase," which is achieved after 5–6 days. Autophagy was induced specifically in the senescence transition phase in a manner that correlated with inhibition of mTOR activity. Importantly, Young et al. observed that depletion of the autophagy proteins autophagy-related gene 5 (Atg5) or Atg7 resulted in delayed senescence transition (93), thus indicating that autophagy contributes to the establishment of senescence. Similar results were obtained in a system of therapy-induced senescence, in which pharmacological or genetic inhibition of autophagy delayed senescence acquisition in response to treatment with the chemotherapeutic drugs adriamycin or camptothecin (90). In accordance with these findings, a recent study expands on a putative mechanism of autophagy-mediated senescence transition, as Dou et al. found that autophagy facilitates OIS by degrading the nuclear lamina constituent, Lamin B1, and associated heterochromatin domains called lamin-associated domains (LADs) (89). Degradation was a result of nuclear blebbing of Lamin B1 regions and a direct interaction between Lamin B1 and LC3, and preferentially occurred in response to oncogenic transformation, oxidative stress, and DNA damage, but not starvation (89), indicating that the degradation event is specific to a subset of stresses. Senescence was delayed upon expression of Lamin B1 mutants unable to bind LC3 and undergo autophagic degradation (89). Thus, autophagic Lamin B1 degradation may be of key importance during senescence

transition. Interestingly, senescent cells have previously been shown to exhibit a gradual decline in histone mass that was dependent on lysosomal activity (94). Whether the degradation of Lamin B1-associated chromatin is of relevance for senescence transition is an interesting point for further investigation. Furthermore, autophagy was found to mediate the selective degradation of Δ133p53α (95), a p53 isoform suppressing the action of full-length p53 (96, 97), for induction of replicative senescence but not OIS (95, 97). Interestingly, overexpression of autophagy proteins is, in some cases, sufficient to stimulate coordinated induction of autophagy and premature senescence (93, 98). Nonetheless, as autophagy inhibition, in most cases, delays rather than fully abrogates senescence, it has been argued that autophagy is not required for senescence transition, but may function in potentiating and accelerating the response (87).

It should also be noted that active mTOR is demonstrated to have a key role in favoring senescence over quiescence and may even be a requirement for senescence transition and/or maintenance in many contexts (99–104). In fact, the main characteristics of senescent cells include hyperactive features such as cellular hypertrophy and the senescence-associated secretion phenotype, which require high metabolic activity (84, 104), and have been speculated to be in part the result of uncoupling proliferation and mTOR activity (85, 105). It should therefore follow that an intrinsic feature of senescent cells would be decreased autophagic activity, as has indeed been demonstrated in some reports (106). However, Narita et al. intriguingly described the formation of a compartment termed the mTOR-autophagy spatial coupling compartment (TASCC) upon OIS, in which mTOR and lysosomes are enriched in the vicinity of the rough endoplasmic reticulum–Golgi apparatus (107). The TASCC was speculated to shield mTOR from the upstream autophagy factors it usually inhibits (4, 107), allowing for concurrence of protein synthesis and degradation, while strategically situating mTOR and lysosomes in a favorable context for mTOR activation on the lysosomal surface (107, 108). In addition, an increasing number of reports have identified pathways and molecules that regulate autophagy independently of mTOR status, as reviewed in Ref. (109). Thus, mTOR activation and autophagy induction are likely not mutually exclusive processes in all contexts.

Decreased Autophagy can Favor Senescence

At variance with the above studies, it has also been reported that inhibition of autophagy promotes senescence (87, 110, 111). Autophagy was reported to counteract senescence by mediating the selective degradation of the transcription factor GATA binding protein 4 (GATA4), which is linked to acquisition of a senescent phenotype in response to DNA damage (112). GATA4 degradation depends on GATA4 recognition by the autophagy receptor p62. Following DNA damage, the p62/GATA4 interaction is reduced, leading to GATA4 stabilization and activation (112). Interestingly, GATA4 activation depends on the DNA damage response regulators, ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and Rad3-related protein (ATR), but not on the traditional senescence effector molecules, p53, and p16 (112). GATA4 may therefore function in DNA damage-induced senescence rather than being a universal senescence-effector molecule.

In addition, a study by Wang et al. adds complexity to the role of autophagy during OIS, as it was reported that genetic ablation of autophagy was permissive rather than restrictive for senescence acquisition during oncogenic RAS-induced senescence (113). In this system, overexpression of Atg5 but not of an autophagy-deficient Atg5 point mutant promoted senescence by-pass, while depletion of Atg5 or Atg3 was permissive for senescence acquisition (113). Induction of OIS was regulated by apoptosis-stimulating of p53 protein 2 (ASPP2) that promoted senescence and inhibited oncogene-induced autophagy through direct disruption of the Atg16–Atg5–Atg12 complex (113), the assembly of which is required for autophagosome formation (114). This suggests a role for ASPP2 in modulating autophagy levels to control the cellular response to oncogene activation. Whether ASPP2 functions in senescence regulation in response to other stimuli remains to be determined. Of note, the ability of autophagy to inhibit OIS appeared not to involve protection from reactive oxygen species or abrogation of p53-activation (113). Understanding the mechanism by which autophagy counteracts senescence in this system may hold the key to combine the contradictory findings on the impact of autophagy on OIS.

Autophagy may also counteract senescence in the context of aging-related senescence and stem-cell maintenance. A study focusing on the regenerative capacity of muscle stem cells using physiologically aged mice, demonstrated that quiescent muscle stem cells preserve their integrity over time through active maintenance of organelle and protein homeostasis by continuous basal autophagy (110). The physiological decline of autophagy in old satellite cells or its genetic impairment in young cells, resulted in accumulation of toxic cellular waste and entry into senescence (110). Similarly, Kang et al. reported that depletion of essential autophagy components resulted in senescence due to build-up of toxic material in primary human fibroblasts (111). The latter studies represent a markedly different experimental system than stress-induced senescence, as they are devoid of external stimuli. Thus, while long-term autophagy inhibition may cause senescence due to accumulation of toxic constituents, autophagy may also function in acute responses to facilitate cellular remodeling in senescence transition in response to conditions such as oncogenic stress or DNA damage.

CELL DIVISION AND AUTOPHAGY

Apart from the complex interplay between autophagy and cell-cycle arrest pathways, several studies have reported more specialized regulatory functions for autophagy or autophagy-related factors in the cell division process. Correct segregation of the duplicated genome during cell division is a prerequisite for preventing CIN and aneuploidy, well-described contributors to cellular transformation (115, 116). Involvement of autophagy factors in regulating the progression or fidelity of cell division may thus be an additional component to consider when discussing the intricate relationship between autophagy and tumorigenesis.

Autophagy and Cytokinesis

In accordance with studies reporting decreased autophagy during mitosis (28, 29), autophagy proteins have primarily been linked

to the final phase of cell division, cytokinesis. Cytokinesis is the process in which the two daughter cells are physically separated following chromosome segregation. This is achieved by the formation of a contractile actomyosin ring that constricts the cytoplasm between the segregated reforming nuclei, thereby generating a narrow intercellular bridge. In the center of the bridge is a dense proteinaceous structure termed the midbody ring (MR), which is thought to function as a targeting platform for cleavage factors. Cytokinesis is completed by plasma membrane fission at the intercellular bridge in a process called abscission (117).

A number of studies have reported cytokinesis failure following knock-down of members of the Vps34 complex including Vps34, Beclin 1, Vps15, Bax-interacting factor 1 (BIF-1), and UV irradiation resistance-associated gene (UVRAG) (118–120). The role of the Vps34 complex in cytokinesis regulation is distinct from its function in autophagy induction as it depends on Vps34-mediated production of PtdIns3P at the MR, which functions as a recruitment signal for the FYVE domain-containing cytokinesis regulator FYVE-CENT (120, 121). Accordingly, PI3Kinase inhibition by 3-methyladenine, but not inhibition of autophagy by the lysosome inhibitor bafilomycin A1 or Atg14 depletion, results in abscission failure (120).

The initiation of cytokinesis and mitotic exit is signaled by the anaphase-promoting complex/cyclosome (APC/C) that promotes proteasomal degradation of mitotic regulators including cyclin B; this, in turn, results in CDK1 inactivation and dephosphorylation of its substrates by counteracting phosphatases (122). Vps34 may be one of such CDK1 substrates that are re-activated during the late stages of mitosis after initially being inhibited (29), to participate in the regulation of mitotic exit, although the timing of Vps34 re-activation is not known. Cytokinesis failure can result in the generation of tetraploid cells with supernumerary centrosomes (123). Such tetraploid cells display CIN due to chromosome segregation defects in subsequent cell divisions and are suggested to exhibit increased tumorigenic potential (123–125). Interestingly, the Vps34 complex members Beclin 1, BIF-1, and UVRAG are amongst the autophagy-related proteins with the most well-substantiated tumor suppressor properties (11–14, 16). A detailed dissection of how the individual roles of the Vps34 complex in regulating cytokinesis and autophagy as well as growth factor receptor degradation (126) each contribute to the tumor suppressor function of these proteins, is an important issue for further investigation.

At variance with the studies discussed above, Belaid et al. reported abscission failure upon depletion of Atg5 and in cells derived from lysosomal vacuolar-type H^+-ATPase *a3*-null mice (127), indicating a function for autophagy in cytokinesis. The cytokinesis defects observed in these systems were attributed to defective turn-over of active RhoA (127), a member of the Rho GTPase family that orchestrates cytokinesis through its ability to regulate the actomyosin contractile network at the cleavage zone (128). Depletion of Atg5 resulted in RhoA enrichment at the intercellular bridge leading to approximately three times broader RhoA activity zones. Consequently, Atg5-depleted cells progressing through mitosis exhibited loose and unstable cleavage furrows and increased generation of multinucleated cells (127). RhoA activity depends on GDP–GTP exchange factors (GEFs) including Ect2, which localizes at the mitotic midbody zone to mediate local RhoA activation and cleavage furrow formation (128, 129). Furthermore, an alternative function for cyclin A2 in potentiating RhoA GTP loading by its GEFs has also been described (130). While the majority of cyclin A2 is degraded by the proteasome in prometaphase (131–133), a small fraction of cyclin A2 was shown to persist in foci later in mitosis and appeared to be subjected to autophagic degradation (40). It is therefore possible that autophagy may have a composite function in controlling appropriate RhoA protein levels and activity at the cytokinesis midzone, by mediating RhoA and Cyclin A2 degradation in late mitosis.

The apparent discrepancies between the reported Vps34 and autophagy-mediated cytokinesis regulation may be most efficiently addressed by expanding these studies to a wider panel of cell systems and autophagy-related proteins. Understanding the contribution of these pathways to cytokinesis completion also *in vivo* is vital for evaluating the potential relevance of these mechanisms in the context of autophagy-related tumor suppression.

In addition, an autophagy-independent function for unc-51-like autophagy-activating kinase 3 (ULK3) as an abscission regulator has been reported (134). Abscission is mediated by the endosomal sorting complexes required for transport (ESCRT) machinery, which mediates membrane remodeling in a number of processes including cytokinesis, viral budding, and autophagy (135). The timing of abscission is regulated by the abscission checkpoint, which delays abscission in response to a number of mitotic abnormalities (136). Interestingly, ULK3 was shown to function in the abscission checkpoint to delay abscission by phosphorylating and binding ESCRT-III subunits in response to lagging chromosomes, nuclear pore defects, and tension forces at the midbody (134). Thus, ULK3 appears to function as an integral part of the abscission checkpoint machinery.

Autophagy and Cell Division Cleanup

In accordance with the more traditional function for autophagy in cellular maintenance, autophagy may also have a role in returning the cell to its interphase state by clearing leftover structures from normal and abnormal cell divisions.

Removal of the MR

Following cytokinesis, the MR is inherited asymmetrically by one of the two daughter cells, and is hereafter often referred to as a MR derivative (MR^d). MR^ds can be eliminated by extrusion to the extracellular space (137–139) or by p62/NBR1-mediated selective autophagy (140–142). The NBR1-dependent pathway relies on the interaction between NBR1 and the midbody protein centrosomal protein 55 (CEP55) (141), while the mechanism of p62-mediated MR^d degradation and the varying requirement for the two autophagy receptors is not understood. Intriguingly, the MR^d extrusion pathway may also involve CEP55 recognition (138). Which elimination pathway predominates varies between cell lines (138), but how MR^ds are allocated for extrusion or retention and subsequent autophagic degradation is not known. Midbody extrusion likely leads to disposal of both the cytoplasmic and membraneous midbody components, which is

not necessarily the case for autophagic degradation; thus, there could be a functional difference between the two midbody disposal pathways. Accumulation of MR[d]s preferentially occurs in stem cells and cancer cells and was suggested to contribute to an undifferentiated phenotype (138, 141). Cells accumulating MR[d]s show decreased autophagic activity and an ability to evade MR[d] encapsulation and autophagic degradation (141), suggesting a link between autophagy status and MR[d] accumulation. Nonetheless, MR[d]s remain poorly described structures. How they influence cellular differentiation and their potential tumorigenic relevance is an interesting open question.

Removal of Micronuclei

If a cell fails to incorporate all chromosomes and chromosome fragments in the reforming nuclei during cell division, micronuclei can be generated (143). Two studies have observed micronuclei associated with LC3 and LAMP2-stained structures (144, 145), and also colocalizing with charged multivesicular body protein 4B (CHMP4B) (145), a member of the ESCRT machinery. Rello-Varona et al. treated U2OS cells with various cell-cycle inhibitors to increase formation of micronuclei, 2–5% of which colocalized with LC3 and p62, and partially with the lysosome marker LAMP2 (144). Importantly, LC3 colocalization was abrogated upon depletion of Atg5 and Atg7, and electron microscopy further confirmed the presence of micronuclei sequestered within double-membrane vesicles (autophagosomes). LC3-positive micronuclei contained less dense chromatin and discontinuous Lamin B1-stained nuclear envelopes (144), suggesting ongoing digestion. How the autophagy machinery is recruited to micronuclei is, however, not known. Furthermore, as only a small fraction of micronuclei appears to be targeted by autophagy, it remains to be investigated to what extent autophagy contributes to their elimination in comparison to other mechanisms of micronuclei removal, such as extrusion (143).

Of note, the formation of extranuclear chromatin entities does not strictly occur as a result of abnormal mitosis (143). Indeed, Ivanov et al. observed the formation of what was referred to as cytoplasmic chromatin fragments (CCFs) in senescent cells (94). CCFs, in contrast to micronuclei generated from malfunctioning mitosis, were negative for the nuclear lamin A/C and positive for the DNA damage marker γ-Histone 2AX and were generated by nuclear blebbing. CCFs were suggested to be identical to the Lamin B1-associated LADs that were later identified in senescent cells by Dou et al. (89), and intriguingly, both are degraded by autophagy (89, 94). These studies suggest a more general role for autophagy in disposing of extranuclear chromatin.

Autophagy in Mitotic Arrest and Mitotic Life/Death Decisions

Upon starvation, eukaryotic cells usually arrest in G1 (22). Nonetheless, it has been reported that nitrogen starved budding yeast, lacking essential autophagy genes arrest at the G2/M transition or in mitosis (146, 147). Matsui et al. reported that also nitrogen-starved wild type yeast exhibits a transient G2/M arrest (147). Recovery and progression from this arrest for subsequent G1 block requires autophagy-dependent supplementation of selected amino acids required for cell growth (147). Following replenishment with a nitrogen source, the previously arrested autophagy-deficient cells showed abnormal mitosis associated with a higher incidence of aneuploidy (147). This suggests a role for autophagy in maintaining genome stability by securing arrest in G1 during starvation, at least in budding yeast. Surprisingly, budding yeast may also require autophagy for completing cytokinesis and mitotic exit during nitrogen starvation (146, 147), even though the importance of amino acid supplementation in this context and the relevance of this phenotype in relation to the described mammalian autophagy-related cytokinesis regulation is not fully understood.

In mammalian cells, autophagy may have an important role in determining cell survival during mitotic arrest and mitotic catastrophe. Mitotic catastrophe is a complex oncosuppressive mechanism that is thought to sense mitotic failure and respond by driving cells toward an irreversible fate, be it apoptosis, necrosis, or senescence (148). Autophagy has been shown to facilitate cell survival during mitotic catastrophe (149, 150). Interestingly, during DNA damage-activated mitotic arrest, the previously identified mitosis-related CDK1-mediated phosphorylation of Vps34 on Thr159 (118) promotes Vps34 ubiquitination and proteasomal degradation (80). Degradation is mediated by the p53-responsive gene FBXL20 and the associated Skp1-Cullin-1 complex, and leads to inhibition of autophagy and receptor endocytosis (80). Thus, mitotic Vps34 phosphorylation in the context of p53 activation appears to promote Vps34 degradation (80). Such a mechanism may prevent survival of defective mitotic cells in a dual fashion, by potentially impeding both cytokinesis completion (118) as well as autophagy-dependent cell survival during mitotic arrest.

An alternative function for the autophagy-related protein Atg5 in mitotic catastrophe has also been demonstrated (150). Atg5 was found to be both necessary and sufficient for induction of mitotic catastrophe resulting from sublethal concentrations of DNA-damaging drugs (150). Following these insults, Atg5 translocates to the nucleus, where it physically interacts with survivin and causes the displacement of elements of the chromosomal passenger complex during mitosis, thus resulting in chromosome misalignment and segregation defects, representative of mitotic catastrophe (150). Atg5-mediated mitotic catastrophe does not depend on Atg5–Atg12 conjugation and is unaffected by pharmacological inhibition of autophagy (150); thus, Atg5-mediated mitotic catastrophe occurs independently of its role in autophagy regulation. While the applied drug concentrations only resulted in modest cell death, pharmacological inhibition of the autophagy pathway shifted the response to early caspase-dependent cell death (150), suggesting that in response to DNA damage, cytoplasmic Atg5 and nuclear Atg5 have distinct roles in autophagy induction and mitotic catastrophe, respectively.

Autophagy may under some conditions also participate in promoting mitotic cell death. Doménech et al. reported that, during mitotic arrest caused by abrogation of cyclin B1 degradation, autophagy promotes cell death through ongoing mitophagy (38). The gradual decline in the mitochondrial mass and oxidative respiration, however, resulted in a metabolic switch through activation of AMPK and subsequent induction of glycolysis

in a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3-(PFKFB3)-dependent manner. Inhibition of glycolysis in breast cancer cells resulted in accelerated death of mitotic cells caused by microtubule poisons (38). This system represents a nonstress-induced mitotic arrest and is likely devoid of p53 activation. Thus, while autophagy induction occurred both during prolonged mitotic arrest (38) as well as in response to DNA damage-induced mitotic catastrophe (150), the resulting effect on cell survival may depend on the nature and severity of the stimulus leading to mitotic block. Of note, exploring how metabolic pathways influence life and death decisions of mitotically arrested cells is of particular interest in the context of cancer treatments, such as DNA-damaging agents or microtubule poisons, which affect the progression and fidelity of mitosis.

Nutrient Sensing and Cell Division: Involvement of AMPK in Mitosis Regulation

Surprisingly, in recent years, an unexpected mitotic role for the nutrient sensing and autophagy-inducing factor, AMPK, has been discovered. AMPK depletion results in mitotic abnormalities, including spindle misorientation and cytokinesis failure in *Drosophila Melanogaster* S2 cells and human cell lines (151–153). Furthermore, *Drosophila* AMPK-null embryos display severe abnormalities in cell polarity and mitosis (154). AMPK activation, evaluated by AMPK Thr172 phosphorylation (p-AMPK^{Thr172}), correlates with induction of mitosis (151, 153), during which p-AMPK^{Thr172} is enriched specifically at centrosomes and at the cleavage furrow (153, 155). Furthermore, an elegant chemical genetics screen designed to identify novel substrates of AMPKα2 provided additional emphasis to a mitotic function for AMPK as it revealed 28 previously unidentified putative AMPK substrates enriched for proteins involved in chromosomal segregation, mitosis, cytokinesis, and cytoskeletal reorganization (151). These evidence indicate a role for AMPK in regulating mitosis through phosphorylation of mitosis-specific substrates. Nonetheless, there appears to be a considerable overlap between the pathways governing AMPK induction and responses during mitosis and during nutrient stress.

Several reports have implicated myosin regulatory light chain (MRLC) as a key target of AMPK-mediated mitosis regulation (151, 153, 154). AMPK facilitates the phosphorylation of MRLC at Serine 19 (151, 154), a phosphorylation event known to stimulate the Mg^{2+}-ATPase activity of myosin II leading to actin-based regulation of mitosis, cell migration, and cell polarity (156–159). Accordingly, AMPK depletion decreases the level of p-MRLC^{Ser19} at spindle poles and reduces overall mitotic p-MRLC^{Ser19} levels. MRLC has been suggested to be a direct target of AMPK in *Drosophila* (154), but mammalian cells may employ alternative strategies for AMPK-mediated p-MRLC^{Ser19} regulation. Protein phosphatase 1 regulatory subunit 12C (PPP1R12C) and p21-activated protein kinase (PAK2), both regulators of MRLC phosphorylation status (160–163), were identified as direct targets of AMPK (151). AMPK phosphorylation of these substrates indirectly induces MRLC Ser19 phosphorylation (151). Of note, the AMPK substrate and upstream autophagy regulator ULK1 has also been implicated in the regulation of MRLC phosphorylation (164). Thus, AMPK-induced MRLC phosphorylation may also involve autophagy factors. While Banko et al. identified a number of well-known mitotic regulators as putative AMPK substrates (151), MRLC regulation appears to be a major contributing factor, as depletion of MRLC partially reproduces AMPK depletion phenotypes (153). Moreover, the expression of a phosphomimetic mutant of MRLC is able to rescue AMPK-null-related cell polarity and mitosis defects in *Drosophila* (154). Whether AMPK regulates other substrates during mitosis remains to be determined.

Perhaps, the most intriguing questions in this context remains whether AMPK activation during mitosis is coordinated with its nutrient sensing ability, and if not, which mitosis-specific signals facilitate AMPK activation. Starvation or stress-induced AMPK activation involves allosteric activation by AMP and phosphorylation by upstream kinases on Thr172 in the activation loop of the catalytic α subunit (165). In mammals, the primary kinases performing this task are LKB1 (166–168) and calcium/calmodulin-dependent protein kinase kinase (CAMKK) (169, 170). LKB1 deficiency reproduces the mitotic abnormalities of AMPK deficiency (152, 153, 171), although CAMKK can also promote mitotic AMPK activation in LKB1-deficient systems (153). Thus, mitotic AMPK activation appears to rely on mechanisms resembling those governing starvation-induced AMPK activation. Interestingly, phosphorylation of PPP1R12C, PAK2, and MRLC also occurs in response to energy deprivation (151, 154, 164), indicating that regulation of these factors may be a general response to AMPK-activating stimuli rather than mitosis-specific. Intriguingly, myosin II activation, as indicated by MRLC phosphorylation, is reported to participate in autophagy induction by modulating Atg9 trafficking during starvation (164). Whether autophagy is induced in response to mitotic AMPK activation remains to be investigated.

It is entirely possible that AMPK regulation of mitosis represents a novel function that is unaffected by the cellular energy status, potentially involving selective AMPK activation at specific subcellular localizations during cell division. However, it has also been speculated that AMPK could alternatively promote the completion of already initiated cell cycles in response to energy deprivation to secure proper cell-cycle arrest in the ensuing G1 phase (165). This theory would imply a role for AMPK in initiating responses similar to those reported in yeast, in which autophagy supplies amino acids required for mitotic completion during starvation (147). Thus, intriguing questions for further investigation include understanding the exact mechanism governing mitotic AMPK activation and the requirement for AMPK (and possibly autophagy) for mitotic progression in response to diverse nutrient conditions.

CONCLUDING REMARKS

Autophagy, being traditionally viewed as a bulk process, was initially rarely linked to strictly structured processes, such as cell-cycle progression. Recent advances in the field, however, clearly suggest a strong correlation between autophagy activation

and the induction and possibly execution of cell-cycle arrest programs, as well as autophagy (factor) regulation of the cell division process. Cell-cycle stress responses and resulting senescence acquisition constitute important anticancer barriers. Therefore, the relevance of autophagy in executing these responses and the role of autophagy in determining cellular life and death decisions in these contexts are of discernible interest. The role of autophagy and autophagy-related factors in regulating the fidelity of cell division is also potentially of substantial relevance, as findings on this topic suggest that the genomic instability observed upon ablation of autophagy (or specific autophagy components) may be partially attributed to dysregulation of this process. Furthermore, as an increasing number of autophagy proteins are being demonstrated to mediate alternative nonautophagic functions (i.e., PtdIns3K components, Atg5, Atg7, AMPK, AMBRA1, ULK1), we may need to more frequently consider autophagy factors individually. Of note, most evidence linking autophagy and cell-cycle regulation has been obtained in yeast and mammalian cell culture systems and remains to be tested *in vivo*. Thus, an important topic for future investigation includes evaluating the contribution of cell-cycle arrest programs and mitosis regulation to tumor progression or prevention in autophagy-manipulated animal models. While considering cell-cycle (dys)regulation as a factor surely adds to the complexity, it may also open up new avenues for improving our understanding of the intricate relationship between autophagy and tumorigenesis.

AUTHOR CONTRIBUTIONS

SM wrote the manuscript and drafted the figures. FC and DZ provided senior supervision and critically revised the manuscript.

ACKNOWLEDGMENTS

The authors are grateful to Emiliano Maiani for helping with figures and to Laila Fischer for secretarial work.

FUNDING

The lab of Francesco Cecconi is supported by grants from the Telethon Foundation (GGP14202), AIRC (IG2016-18906), FISM (2013), KBVU from the Danish Cancer Society (R146-A9364), the Bjarne Saxhof Foundation, the Novo Nordisk Foundation (7559, 22544), and the European Union (Horizon 2020 MEL-PLEX, grant agreement 642295). Further, FC lab in Copenhagen is part of the Center of Excellence for Autophagy, Recycling and Disease (CARD), funded by the Danish National Research Foundation.

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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.

frontiers in Oncology

REVIEW
published: 15 June 2017
doi: 10.3389/fonc.2017.00128

Autophagy in Cancer Therapy

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Autophagy represents a catabolic program involved in the degradation of cellular components *via* lysosomes. It serves to mitigate cellular stress and to provide metabolic precursors especially upon starvation. Thereby, autophagy can support the survival of cancer cells. In addition, there is now convincing evidence showing that under certain conditions autophagy can also foster cell death. This dual function of autophagy is also relevant upon anticancer treatment, as many chemotherapeutic agents engage autophagy. A better understanding of the molecular mechanisms that are critical for mediating autophagic cell death in cancer cells will be instrumental to selectively interfere with this cellular program in order to increase the cancer cell's response to cytotoxic drugs. This review illustrates how anticancer drug-induced autophagy is involved in mediating cell death.

Keywords: autophagy, cell death, cancer, autophagic cell death, cancer therapy

OPEN ACCESS

Edited by:
Patrizia Agostinis, State University of Leuven, Belgium

Reviewed by:
Boris Zhivotovsky, Karolinska Institutet, Sweden
Alessandro Rimessi, University of Ferrara, Italy

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *28 April 2017*
Accepted: *01 June 2017*
Published: *15 June 2017*

Citation:
Fulda S (2017) Autophagy in Cancer Therapy. Front. Oncol. 7:128. doi: 10.3389/fonc.2017.00128

INTRODUCTION

Autophagy is a basic cellular process that serves as a quality control checkpoint during physiological and pathophysiological conditions to cope with cellular stress. Autophagy regulates the turnover of damaged cellular elements that are degraded, for example, *via* lysosomal enzymes after engulfment into autophagosomes (1, 2). There are different forms of autophagy including macroautophagy (hereafter referred to as autophagy), microautophagy, and chaperone-mediated autophagy (3). A panel of genes involved in the regulation of autophagy has been identified over the last decades illustrating that autophagy represents a genetically controlled process (4). As far as cancer is concerned, autophagy may function as both a tumor suppressor and tumor promoter (5). One explanation is the dual function of autophagy being either cytoprotective or cytotoxic in a context-dependent fashion. By definition, autophagic cell death (ACD) refers to a mode of cell death that is inhibited *via* specific blockage of the autophagic pathway (6).

Anticancer treatments can engage autophagy in cancer cells on the one side as part of a cytoprotective answer in response to a toxic insult with the aim to mitigate cellular stress (7). On the other side, anticancer therapy can stimulate autophagy pathways that mediate ACD (8). In the following, prototypic examples of ACD upon anticancer treatments will be discussed.

ANTICANCER DRUG-INDUCED ACD

Chemotherapeutic Drugs

Several chemotherapeutic drugs have been reported to engage autophagy (9–12). While chemotherapy-mediated autophagy has mostly been linked to a cytoprotective response that allows cancer cells to cope with the cellular stress imposed upon anticancer drug treatment, there are also cases

of ACD. For example, the DNA-alkylating agent temozolomide (TMZ) has been implicated to elicit ACD. TMZ belongs to the class of DNA-alkylating drugs that triggers the formation of *O*-6-methylguanine in DNA, which causes DNA damage during the following cycle of DNA replication by mispairing with thymine. TMZ has been shown to trigger the recruitment of LC3 to autophagosomal membranes (9). Pharmacological inhibition of autophagy by 3-methyladenine (3-MA) resulted in reduced formation of autophagosomes and attenuated TMZ-mediated cytotoxicity (9). In addition, genetic inhibition of autophagy by RNAi-mediated gene silencing of ATG5 and Beclin 1 (BECN1) impaired cell death upon treatment with TMZ alone or in combination with (−)-gossypol (13), underscoring that autophagy may contribute to TMZ-imposed cytotoxicity. However, a cytoprotective function of autophagy in the course of chemotherapy has also been proposed. This conclusion is based on data showing that a TMZ-induced and autophagy-dependent increase of ATP counteracts cell death of malignant glioma upon exposure to TMZ (14). Consistently, knockdown of core elements of autophagy signaling such as BECN1 enhanced the sensitivity of malignant glioma cells to TMZ-imposed reduction of colony formation after TMZ treatment (14). However, the question as to whether or not TMZ induces ACD remains a controversially discussed issue, since TMZ has also been reported to trigger apoptosis (10). Thus, further studies are required to determine the functional relevance of autophagy in the course of TMZ-induced antitumor activity in malignant glioma cells.

BH3 Mimetics

BH3 mimetics that antagonize antiapoptotic BCL-2 family proteins have been reported to engage ACD by disrupting a complex of BECN1/ATG6 together with BCL-2 or BCL-x_L (15–17). One example is gossypol, a natural compound derived from cotton seeds that functions as a pan-BCL-2 inhibitor by neutralizing BCL-2, BCL-x_L, MCL-1, and BCL-w. (−)-Gossypol (also known as AT-101) proved to be the more potent enantiomer of gossypol to suppress tumor growth as compared to (+)-gossypol. In apoptosis-deficient cancer cells, gossypol has been reported to induce ACD (13, 18), while it triggered apoptotic cell death in cells that can undergo apoptosis (19–22). In glioblastoma cells, (−)-gossypol reportedly triggered ACD alone and in combination with the alkylating agent TMZ, which was accompanied by translocation of LC3 to autophagosomes, and lysosomal activity (13). ACD was supported by rescue experiments demonstrating that knockdown of BECN1 or ATG5 significantly reduced (−)-gossypol-induced cell death alone and combined treatment with TMZ (13). Besides glioblastoma, (−)-gossypol was shown to trigger ACD in apoptosis-resistant prostate cancer and breast carcinoma cells, as silencing of ATG5 and BECN1 significantly rescued (−)-gossypol-mediated cell death.

Obatoclax

Furthermore, obatoclax has been implicated in triggering ACD and the conclusion that it is in fact ACD contributing to obatoclax-induced cell death was drawn on findings showing that genetic silencing of essential autophagy genes such as BECN1, ATG5, or ATG7 inhibits obatoclax-mediated cell death. For example, obatoclax has been shown to exert antileukemic activity in pediatric acute lymphoblastic leukemia including glucocorticoid-resistant cases by engaging autophagy and cell death (23). Parallel silencing of autophagy-related genes such as BECN1 or ATG7 provided protection against obatoclax, underscoring that autophagy is indeed necessary for the observed antileukemic activity (23). Moreover, obatoclax has been reported to stimulate the assembly of the necrosome on autophagosomes, thereby linking autophagy to necroptotic cell death (24). Silencing of ATG5 or ATG7 rescued cells from obatoclax-mediated cell death, highlighting their requirement for cell death upon treatment with obatoclax (24). Also, obatoclax has been described to trigger the conversion of LC3 and cell death in a BECN-dependent manner in B-cell lymphoma (25). Besides autophagy, obatoclax has also been shown to engage apoptosis (26, 27).

Cannabinoids

Tetrahydrocannabinol (THC), which is considered as the main active component of cannabinoids, has been shown to act as a stimulus for ACD, for example, in hepatocellular carcinoma (HCC) and glioblastoma (28, 29). Knockdown of ULK1, ATG5, or Ambra-1 conferred protection of glioblastoma cells from THC-induced cell death (28). Similarly, ATG5 knockout fibroblasts were shown to be refractory to THC-stimulated cytotoxicity (28). In addition, ATG5 deficiency rescued THC-triggered antitumor activity in a tumor xenograft model *in vivo* (28). These studies confirmed the contribution of autophagy to THC-mediated antitumor activity both *in vitro* and *in vivo*.

Molecular studies revealed that THC causes ER stress *via* accumulation of ceramide and phosphorylation of eukaryotic translation initiation factor 2 alpha, resulting in upregulation of CHOP and tribbles homolog 3 (TRB3), two ER stress-related proteins (28). TRB3 then engages autophagy by blocking AKT/mTOR signaling (28). In sharp contrast to THC-triggered ACD in various types of cancer cells, THC did not possess a similar cytotoxicity against normal non-malignant cells (28). This indicates that THC preferentially targets cancer rather than normal cells and thus may offer a therapeutic window that could be exploited for cancer therapy.

JWH-015 is a cannabinoid receptor 2-selective agonist that has been shown to engage ACD in HCC that involved AMPK activation and inhibition of AKT/mTOR signaling (29). Of note, ATG5 silencing or 3-MA protected from JWH-015-induced reduction of HCC growth (29).

HISTONE DEACETYLASE INHIBITORS (HDACIs)

Histone deacetylase inhibitors represent another class of anticancer agents that have been reported to engage autophagy associated with the induction of cell death in chondrosarcoma cell lines. Suberoylanilide hydroxamic acid (SAHA) has been shown to stimulate autophagy-associated cell death accompanied by ultrastructural changes in autophagosome formation and increased lipidation of LC3 (30). Pharmacological inhibition of autophagy using 3-MA significantly protected from SAHA-mediated loss of cell viability (30). However, no genetic evidence has been provided

in this study to confirm that autophagy is indeed required for the induction of cell death. Thus, it remains to be confirmed that SAHA in fact triggers ACD in chondrosarcoma cells.

In HeLa cervical carcinoma cells, SAHA has been reported to induce characteristic autophagic features including morphological changes and LC3-II conversion (31). Genetic silencing of BECN1 and ATG7 inhibited SAHA-stimulated autophagy (31). However, the question as to whether or not autophagy genes are also required for SAHA-induced cell death has not yet been answered (31).

In HCC, HDACIs including SAHA and OSU-HDAC-42 have been described to trigger ACD based on both genetic- and pharmacological blocking experiments underscoring that SAHA- or OSU-HDAC-42-stimulated autophagy is required for the induction of cell death, as either silencing of ATG5 or 3-MA protected cells from the cytotoxicity of SAHA (32). Also, autophagosome formation, LC3 lipidation, and downregulation of p62 have been observed upon treatment with SAHA (32). SAHA and OSU-HDAC-42 might stimulate autophagy by blocking the mTOR pathway, as they suppress AKT/mTOR activity (32).

NEW COMBINATIONS

The tricyclic antidepressant (TCA) imipramine (IM) and the anticoagulant ticlopidine (TIC), two drugs approved by the US Food and Drug Administration, have been shown to synergistically trigger autophagy and cell death in glioblastoma cells (33). In addition, this combination proved to be effective to suppress glioblastoma growth in a murine *in vivo* model. ACD was emphasized by genetic knockdown of ATG7 that significantly rescued combination treatment-induced cell death. The authors went on to show that IM and TIC increase the autophagic flux by upregulating 3′-5′-cyclic adenosine monophosphate (cAMP) levels *via* distinct mechanisms. While IM treatment activates adenylate cyclase and induces cAMP-mediated autophagy, the addition of the $P2Y_{12}$ inhibitor TIC short circuits the ADP/ATP-induced feedback inhibition of adenylate cyclase (33). Together, this increases cAMP levels and elicits hyperactivated autophagy and subsequent cell death. It is interesting to note that the clinical use of TCAs has previously been associated with a decreased incidence of glioblastoma.

CONCLUSION

There is ample evidence showing that some cytotoxic drugs used for the treatment of cancer can engage ACD. Since this property can in principle be exploited for cancer therapy, it is critical to understand the pathways regulating these events. However, as autophagy can control both cell death and survival programs, induction of autophagy in cancer cells represents a double-edged sword. It will therefore be critical to develop novel approaches that will allow selective engagement of the pro-death branch of autophagy.

AUTHOR CONTRIBUTIONS

SF has solely contributed to the conception and design of the work, has drafted and revised the work, and has given final approval of the version to be published.

ACKNOWLEDGMENTS

The expert secretarial assistance of C. Hugenberg is greatly appreciated.

FUNDING

This work has been partly supported by a grant from the BMBF (to SF).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 26 October 2016
doi: 10.3389/fonc.2016.00226

Autophagy Induced during Pancreatitis Promotes *KRAS*-Dependent Transformation in the Pancreas

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Pancreatitis is an inflammatory disease that both facilitates and accelerates the transformation of pancreatic cells upon activation of the *KRAS* oncogene. Autophagy is proposed to be one of the cellular mechanisms contributing to pancreatic carcinogenesis, especially during initial stages in which the *KRAS* oncogene appears to play a key role. Autophagy is also strongly induced during pancreatitis by the overexpression of *VMP1*. We recently developed a genetically engineered mouse model in which the VMP1 protein is induced simultaneously with the activation of the oncogene $Kras^{G12D}$ specifically in the pancreas, by the addition of doxycycline to a water drink. Using this sophisticated animal model, we can affirm that pancreatic autophagy, induced during pancreatitis by the overexpression of *VMP1,* promotes the development of precancerous lesions when induced by the mutated *KRAS*. In addition, the treatment of these mice with chloroquine, an inhibitor of autophagic flux, reverses the effects of VMP1 in pancreatic cancer induced by the *KRAS* oncogene. Overall, these results bear both mechanistic and biomedical relevance for further understanding and potentially targeting pathways that are critical for initiating pancreatic carcinogenesis, particularly if associated with pancreatitis.

Keywords: VMP1, pancreatic cancer, pancreatitis, autophagy, chloroquine, *KRAS*

OPEN ACCESS

Edited by:
Patrizia Agostinis, University of Leuven, Belgium

Reviewed by:
Alessandro Rimessi, University of Ferrara, Italy
Francisco X. Real, Spanish National Cancer Research Centre, Spain
Jun Yan, Nanjing University, China

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *30 August 2016*
Accepted: *10 October 2016*
Published: *26 October 2016*

Citation:
Iovanna JL (2016) Autophagy Induced during Pancreatitis Promotes KRAS-Dependent Transformation in the Pancreas. Front. Oncol. 6:226. doi: 10.3389/fonc.2016.00226

PANCREATIC DUCTAL ADENOCARCINOMA

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the Western world, with prediction curves demonstrating it will become the second leading cause of death by cancer in 2030, just after lung cancer (1). Both the initiation and progression of this pathology result from the interaction of complex genetic events with multiple less characterized factors (2, 3). Genetic alterations that contribute to the pathogenesis of pancreatic adenocarcinoma have been widely studied and definitively determined. Among these alterations, oncogenic mutations in the *KRAS* gene have been frequently detected (more than 90% of cases), not only in the established disease but also in preneoplastic lesions known as pancreatic intraductal neoplasia (PanINs). Activation of the oncogene *KRAS* signals pancreatic cells to undergo acinar-to-ductal metaplasia, an essential step in the formation of premalignant lesions, which together with the inactivation of tumor suppressor genes, such as *CDKN2A*, *TP53*, and *SMAD4*, allow the progression of premalignant lesions to invasive cancer (4). As the activating mutation in the *KRAS* oncogene is almost systematically associated with PDAC, its role in cancer development has been the subject of numerous studies (5).

Autophagy has been proposed as a cellular process contributing to pancreatic carcinogenesis, particularly in the initial stages in which the *KRAS* oncogene is a key element (6–9). Indeed, activation

of the pathway controlled by the *KRAS* oncogene generates a metabolic stress, characterized by a temporary deficit in energy, which must be compensated by an increase in metabolism, through activation of autophagy (6–10). Although this concept appears clear and simple, the role of autophagy in protumor or antitumor development is still debated in the context of PDAC, since multiple factors appear to modulate this process, such as regulatory pathways, the genomic status of transformed pancreatic cells, as well as the physiological and pathological contexts in which the process is enabled (11, 12).

PANCREATITIS-ASSOCIATED AUTOPHAGY PROMOTES THE PROTUMORAL EFFECT OF THE *KRAS* ONCOGENE

Pancreatitis, an inflammatory disease of the pancreas, enables and accelerates the transformation of pancreatic cells when the *KRAS* oncogene is activated (13). Exactly how pancreatitis promotes the development of PDAC is a fundamental question in the field of pancreatology, which has not yet been clearly answered. However, this has been partly answered by studies showing that the systematic activation of autophagy during pancreatitis, often for the protection of pancreatic cells, decreases disease progression and aids the recovery phase (14, 15). We have demonstrated that induction of autophagy in pancreatic acinar cells is accompanied by the overexpression of the *VMP1* gene. VMP1 mRNA encodes a transmembrane protein that we cloned in 2002 due to its extraordinary pancreatic activation during the acute phase of pancreatitis (16). Overexpression of VMP1 triggers autophagy in numerous types of cells (16–19). Concerning its mechanistic activity, VMP1 is involved in the formation of the phagophore (18) following a direct interaction with beclin 1 (17), TP53INP2, a scaffold protein (20), and possibly its homolog, TP53INP1 (21). The main physiological role of autophagy during pancreatitis is the removal of damaged organelles to maintain cellular homeostasis and ensure improved survival of pancreatic cells (22). It is likely that the protective effect of autophagy during the acute phase of the disease is at least partly related to the sequestration of zymogen granules that contain digestive enzymes responsible for autodigestion during pancreatitis. This may have a dual effect on pancreatic cells: first, zymophagy (autophagy of zymogen granules) could reduce the availability of digestive enzymes, which when released into the pancreatic parenchyma destroys the pancreatic gland by necrosis; second, these organelles could meet the unique metabolic needs that accompany cell growth during the regeneration phase (23).

AUTOPHAGY INDUCED BY OVEREXPRESSION OF VMP1 ENHANCES TRANSFORMATION OF PANCREATIC CELLS

It is interesting to note that the expression of VMP1 is also transcriptionally activated by the mutated *KRAS* oncogene through a mechanism dependent on GLI3 and p300 (24). The *KRAS* oncogene possibly induces VMP1 expression to meet the increased energy needs of the cell during the transformation process. Expression of the VMP1 protein, and its triggered autophagy, is therefore induced and maintained by mutation of the *KRAS* oncogene, which is strongly reinforced during the course of pancreatitis. The most likely hypothesis is that autophagy induced by pancreatitis, and mediated by overexpression of VMP1, provides the energy required of cells harboring an activating mutation in the *KRAS* oncogene, therefore allowing their transformation. To test this hypothesis, we have recently developed an animal model wherein the genetically modified VMP1 protein is induced simultaneously with the activation of the oncogene $Kras^{G12D}$ specifically in the pancreas, by the addition of doxycycline to a water drink (25). This model was developed with the objective to first assess the effects of overexpressed VMP1 on initiation of pancreatic cancer, and second, to define the role of pharmacological inhibitors of autophagy in the development of pancreatic cancer. The results of these experiments in mice affirm our hypothesis that autophagy, induced by overexpressing VMP1 in the pancreas, significantly increases the protumor effect of the *KRAS* oncogene (**Figure 1**). In addition, we demonstrated that chloroquine, a classical inhibitor of autophagic flux (26), can reverse the effect of VMP1 overexpression on pancreatic cancer induced by the *KRAS* oncogene in a preclinical trial using our mouse model (25). Overall, these observations support the idea that pathways activated by pancreatitis, particularly those regulating autophagy, can promote pancreatic carcinogenesis. Finally, the results support the concept that inhibition of autophagy could be used to prevent the progression of pancreatic pre-tumoral lesions to pancreatic cancer.

MECHANISMS OF ACTION OF VMP1

In light of these clinically relevant results, it is important to review and discuss the identified functions of the VMP1 protein, which will consequently improve the interpretation of its role in pancreatic tumor progression. For example, it has been established that this protein is involved in the initiation of autophagy since cells engineered to be deficient in VMP1 have high levels of PtdIns3P

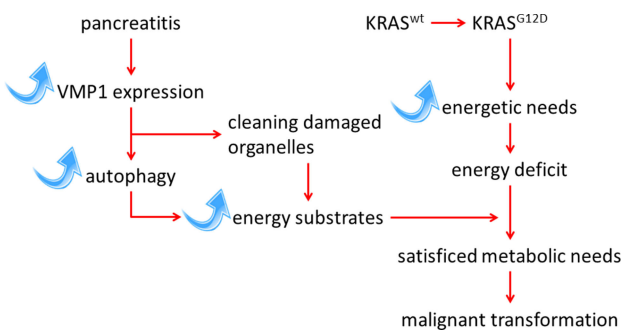

FIGURE 1 | Schematic representation of the interaction between *KRAS*-mediated transformation in PDAC and autophagy induced by the pancreatitis-associated protein VMP1 pathways.

and trigger autophagic signaling by the resulting aberrant endoplasmic reticulum, with subsequent recruitment of ATG18 and other autophagic proteins (19). In addition, although ULK1 and ATG5 are separated in the genetic hierarchy during autophagy, both proteins accumulate synchronously within punctate structures containing VMP1, followed by recruitment of ATG14, ZFYVE1, and WIPI1 (27). Moreover, VMP1 protein directly binds to the BH3 motif of beclin 1 to induce the formation of a complex with hVps34, a key phosphatidylinositol-3 kinase class III regulator of autophagy, on the site where autophagosomes are generated. Importantly, the interaction between beclin 1 and VMP1 proteins leads to the dissociation of the Bcl-2 protein with beclin 1, therefore increasing intracellular levels of beclin 1 available to induce autophagy (28). In addition, the presence of the VMP1 protein regulates the formation of autophagosomes by shortening the training time of the omegasome and therefore significantly accelerating autophagic flux (18). Finally, the production of cells inactivated for VMP1 protein in *Dictyostelium* revealed a massive accumulation of protein aggregates, both poly- and multi-ubiquitinated, containing the autophagic markers ATG8 counterparts and p62 but presenting strong defects in autophagy process. Altogether, these observations demonstrate that expression of the stress protein VMP1 is essential for unloading cells of these protein aggregates by autophagy (29) and recycling them to provide the energy substrate required by the cell under these stress conditions.

It is also important to discuss the broader role that autophagy plays in the development of PDAC as it is so complex and varied. Indeed, it was previously demonstrated that autophagy participates in the transition from mitosis to senescence (30), and certain molecules can induce both autophagy and senescence, such as kinase ULK3 (30). Senescence is known to be an important anticancer pathway set up in response to the oncogenic activation of mutated *KRAS*. In this context, senescence enhanced by activation of autophagy might partially inhibit the oncogenic effect of the *KRAS* oncogene rather than increase it. Furthermore, activation of autophagy in certain tissues, either dependent or independent of VMP1 overexpression, can act as an antiapoptotic factor, according to the biological circumstances (31, 32). Finally, as mentioned earlier, the oncogenic activation of *KRAS* induces a strong metabolic stress to cells due to their exceptional energy requirements that can be partially counterbalanced with the contribution of energy sources through the activation of autophagy. Autophagy can therefore play important roles in either promoting or, on the contrary, antagonizing the development of PDAC, depending on the activated intracellular pathways by cells harboring *KRAS* mutations. This possibly explains the contrasting results reported in the literature on the role of autophagy in cancer. Another important note is that a large majority of these studies were performed *in vitro*, therefore the cellular environment has not, or only partially, been taken into account, possibly causing a bias in data interpretation. Regarding the pancreatic autophagy induced by VMP1 overexpression in mice, we have established that the development of pancreatic precancerous lesions is associated with a significant reduction of apoptosis with a concomitant increase in cell proliferation (25). In other words, autophagy is clearly a pro-tumor cellular event, at least in this context.

Importantly, autophagy has been considered an important mediator of the resistance to radiotherapy and chemotherapy, at least with particular anticancer drugs and for certain cancers (33, 34), although this point still remains controversial. Nevertheless, the fact that cancer treatments systematically induce autophagy has now been clearly established (35). However, the mechanism by which autophagy is involved in resistance to cancer treatments seem to be initiated by the removal of damaged intracellular organelles to improve cell viability. Furthermore, autophagy has also been reported as a mediator of cell death induced by chemotherapy in several cancers (36). Although the mechanism by which autophagy induces cell death is not yet clearly established, it appears to be mediated by the activation of caspase 3 (36). Therefore, in line with such knowledge, co-treatment with chloroquine appears to enhance the effect of many anticancer drugs *in vitro* as well as in some preclinical models (37–41), although a clinical study has yet to confirm its benefit as a co-treatment.

CONCLUSION AND PERSPECTIVES

In conclusion, many aspects concerning the role of autophagy during PDAC development are still not clearly defined. However, we can confirm that pancreatic autophagy induced during pancreatitis through the overexpression of VMP1, a protein associated with pancreatitis, promotes PanINs when activated by the *KRAS* oncogene. In addition, inhibition of autophagic flux by chloroquine almost completely abolishes the *KRAS* pro-tumor effect in the pancreas. Overall, these results bear both mechanistic and biomedical relevance for further understanding and potentially targeting those pathways critical for initiating pancreatic carcinogenesis, particularly if associated with pancreatitis. In the near future, it will be necessary to take into account not only the role of autophagy activation in transformed cells but also in the stromal non-transformed cells. Recently, it was clearly evidenced that the activation of autophagy in cancer-activated fibroblast (CAF cells) is an essential mechanism to produce and secrete non-essential amino acids into the microenvironment, which serves as a major source of energy for transformed cells (42). This may be the starting point of a novel time in which the autophagy may be considered as the fuel source for other cells. All in all, these facts are revealing a more complex scenario than suspected and therefore are opening news ways for treating diseases in which autophagy seems to be strongly involved, such as PDAC. An interesting observation to be noted was recently pointed out by Guo and colleagues who demonstrated that the loss of VMP1 expression in colorectal cancer is associated with a poor prognosis and aggressiveness of the cancer cells. In addition, *in vitro* assays revealed that colon cancer-derived cells in which VMP1 was knocked down gained significant aggressive properties in regards to proliferation and invasion. Remarkably, *in vivo* studies revealed a higher number of formed nodules in mice after intraperitoneal injection of VMP1 knocked down cells (43). Another recent work reports that approximately 10% of

esophageal adenocarcinomas present a RPS6KB1–VMP1 gene fusion as a recurrent event. Notably, esophageal adenocarcinoma cases harboring RPS6KB1–VMP1 fusions exhibited significantly poorer overall survival as compared to fusion-negative cases. Mechanistically, the RPS6KB1–VMP1 fusion protein promotes cell growth *in vitro*, but it is ineffective in triggering autophagy (44). Altogether, these studies suggest that the role of VMP1, and perhaps autophagy, in cancer development and progression is organ or context dependent.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

FUNDING

This work was supported by La Ligue Contre le Cancer, INCa, Canceropôle PACA, DGOS (labellisation SIRIC) and INSERM.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 25 November 2016
doi: 10.3389/fonc.2016.00251

Autophagy-Dependent Secretion: Contribution to Tumor Progression

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Autophagy is best known as a lysosomal degradation and recycling pathway to maintain cellular homeostasis. During autophagy, cytoplasmic content is recognized and packed in autophagic vacuoles, or autophagosomes, and targeted for degradation. However, during the last years, it has become evident that the role of autophagy is not restricted to degradation alone but also mediates unconventional forms of secretion. Furthermore, cells with defects in autophagy apparently are able to reroute their cargo, like mitochondria, to the extracellular environment; effects that contribute to an array of pathologies. In this review, we discuss the current knowledge of the physiological roles of autophagy-dependent secretion, i.e., the effect on inflammation and insulin/hormone secretion. Finally, we focus on the effects of autophagy-dependent secretion on the tumor microenvironment (TME) and tumor progression. The autophagy-mediated secreted factors may stimulate cellular proliferation *via* auto- and paracrine signaling. The autophagy-mediated release of immune modulating proteins changes the immunosuppresive TME and may promote an invasive phenotype. These effects may be either direct or indirect through facilitating formation of the mobilized vesicle, aid in anterograde trafficking, or alterations in homeostasis and/or autonomous cell signaling.

Keywords: autophagy, secretion, autosecretion, tumor progression, tumor microenvironment, unconventional secretion

OPEN ACCESS

Edited by:
Jon Lane,
University of Bristol, UK

Reviewed by:
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Harvard Medical School, USA

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *01 October 2016*
Accepted: *10 November 2016*
Published: *25 November 2016*

Citation:
Keulers TG, Schaaf MBE and Rouschop KMA (2016) Autophagy-Dependent Secretion: Contribution to Tumor Progression. Front. Oncol. 6:251. doi: 10.3389/fonc.2016.00251

AUTOPHAGY

Autophagy is a catabolic process required to maintain cellular homeostasis by lysosomal degradation of aged/damaged organelles (e.g., mitochondria), protein aggregates, and pathogens. Autophagy commences with the formation of an initial cup-shaped membrane (phagophore) that elongates while (non-) selectively capturing cytoplasmic constituents into a double-membrane vesicle (autophagosome). Ultimately, the autophagosome fuses with a hydrolase and protease containing lysosome for degradation of the content. The end-products are recycled into the cytosol and are reused in processes including protein synthesis and ATP production.

During the last decade, extensive research revealed that at least 38 autophagy-related proteins (ATGs) comprise the core autophagy machinery that mediate initiation, elongation, cargo recruitment, and fusion with lysosomes (1). Furthermore, the yeast atg8 orthologs of the LC3/GABARAP protein family fulfill specialized roles in the execution of autophagy (2). This family consists of seven active members [LC3A (two splicing variants; LC3A-a and LC3A-b), LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2], which share a high mutual homology, including a conserved C-terminal glycine residue for phosphatidylethanolamine (PE) conjugation to allow membrane

association. Conjugation of the LC3/GABARAP protein family members to PE is required for expansion and closure of the phagophore. This process is controlled by two ubiquitin-like conjugation systems, a process closely orchestrated the ATG4, -7, and -3 and the ATG12–ATG5–ATG16L1 complex (3).

Despite the high grade of homology, the protein family members harbor individual roles in autophagy and are associated with autophagy unrelated functions, such as receptor trafficking, too (2).

Autophagy (and related defects) is associated with several pathologies, including neuropathologies, Crohns disease, and cancer. In cancer, autophagy is considered a double-edged sword, i.e., in healthy tissue, autophagy plays a cytoprotective role by maintaining homeostasis through degradation of cytotoxic constituents, which otherwise may trigger tumorigenic events. Nevertheless, once a tumor is formed, autophagy contributes to survival of cancer cells in areas deprived of nutrients of oxygen (hypoxia) (4, 5), a common feature of solid tumors that contributes to tumor progression, therapy resistance, and metastases formation (6).

Yet, accumulating research shows that the homeostatic role of autophagy and its related proteins is more elaborate than the degradation of cytoplasmic content alone. Autophagy not only contributes to intracellular homeostasis but also seems to contribute to tissue homeostasis by mediating intercellular communication. Peptides, proteins, and hormones that fail to enter the conventional secretory system due to the lack of a leader/secretion signal sequence can be secreted in an autophagy-dependent manner.

In this review, we list the current knowledge on the role of the autophagy machinery in autophagy-dependent secretion and specifically focus on factors that may influence tumor progression.

(UN)CONVENTIONAL PROTEIN SECRETION

In eukaryotes, a classical route for proteins to be secreted is the endoplasmic reticulum (ER)-to-Golgi route. Herein, proteins are directed toward the ER by their amino terminal signal peptide (leader peptide) and progress through vesicular flow to the Golgi. In brief, after ER translocation, proteins are oligomerized and packed into carrier vesicles that exit the ER at specialized regions. These vesicles assemble into vesiculo-tubular structure intermediates known as the ER-to-Golgi intermediate compartments (ERGIC) that, by lateral communication, sort proteins for further anterograde flow to the Golgi complex. In the Golgi, proteins are glycosylated to ensure proper protein structure, increase stability, and to allow interactions with target proteins (7). In the trans-Golgi network, secretory proteins are sorted into secretory vesicles that deliver their content to the plasma membrane to result in secretion (8). An increasing number of secreted proteins that lack the leader peptide have been identified. These proteins require alternative pathways to be secreted in a regulated fashion. This implies differences in vesicle formation, sorting, and transportation. Multiple alternate, non-classical pathways exist and are commonly referred to as unconventional protein secretion and include both non-vesicular and vesicular mechanisms (9). For example, cytosol-residing proteins, including ABC transporter-mediated yeast a-factor (3) or mammalian fibroblast growth factor 2 (FGF2) are directly transported across the plasma membrane. For FGF2 secretion, this is presumed to be mediated through $PI(4,5)P_2$-induced oligomerization followed by membrane insertion and translocation (10). Other proteins are sorted into vesicular intermediates that fuse with the plasma membrane to release their content into the extracellular space [interleukin 1β (IL-1β) and IL-18 (11)], in part dependent on proteins required for autophagy execution (12, 13). This suggests that autophagy-proteins are required to produce secretory vesicles or that autophagosomes gain exocytic features. These processes in autophagy-mediated secretion are distinct from its degradative compartment [as reviewed in more detail in Ref. (14)]. In this context, the trafficking, tethering, docking, and plasma membrane fusion events would differ from canonical autophagy and resemble exocytic mechanisms. Herein, secretory vesicles are diverted from the endocytic system to be released from multivesiclular bodies (MVB). These secretory vesicles are then transported to the plasma membrane for content release. For example, the interaction between autophagosomes and the endocytic/MVB pathway [reviewed in Ref. (15)] is required for acetyl-CoA-binding protein (Acb1)-secretion and requires the fusion of Acb1-containing autophagosomes with endosomes or MVBs before plasma membrane fusion (16).

The involvement of autophagy-related proteins, in protein secretion indicates that cells utilize these effectors in a surprising mechanism of unconventional secretion. Here, we will discuss the data that support a relation between autophagy or autophagy-related proteins with secretory pathways (**Table 1**). First, we will elaborate on the physiological roles of autophagy in secretory events. Second, cancer cells utilize secretory mechanisms to

TABLE 1 | Autophagy-dependent secreted factors.

Protein	Mechanism	Reference
Acbp	Atg5, Atg7, Atg8, Atg12	(16)
IL-1B	ATG5	(11)
NBR1, P62, OPTN, and CACO2 LC3B-II	Inhibition or depletion of PIKfyve by apilimod or siRNA	(19)
Angiogenin 4, interlectin 1, and relmβ	ATG16L1 ATG5 or ATG7	(25)
MUC5AC	ATG5 and ATG16L1	(26)
Insulin	GABARAP	
NPY	ATG16L1 as RAB33a effector	(33)
von Willebrand factor (vWF), P-selectin, interleukin-8, angiopoietin-2, and endothelin-1	ATG7, ATG5, chloroquine or bafilomycin A1	(34)
Matrix-degrading factors including cathepsin K	ATG5 or ATG7	(35)

communicate with stroma and surrounding cancer cells, thereby inducing alterations that influence tumor progression, including immunoevasion, immunesuppression, auto- and paracrine signaling, and angiogenesis.

PHYSIOLOGICAL ROLES OF AUTOPHAGY-MEDIATED SECRETION

The first evidence for the involvement of autophagy-related proteins in protein secretion was obtained in yeast. Acyl coenzyme A-binding protein (Acbp) is secreted through an unconventional secretory pathway that depends on components of the autophagic process (Atg5, Atg7, Atg8, Atg12) (16). Importantly, this study also demonstrated that autophagy connects with compartments (multivesicular bodies) of endosomal trafficking and SNARE-dependent membrane fusion events are both required for the Acbp secretion. Since the discovery of autophagy-dependent secretion in yeast, also several mammalian factors have been identified to be dependent on autophagy-related proteins for their secretion.

Phosphoinositides are membrane lipids with specific recognition domains for the recruitment of cytosolic proteins and are involved in the regulation of membrane transport. So far, seven phosphoinositides have been described. They can be converted into each other by phosphoinositide kinases and phosphatases (17). PIKfyve is one of those converting kinases and has been implicated in membrane transport events. The substrate of PIKfyve, PI(3)P, plays a critical role in the initiation of autophagy and autophagosome maturation (18). Inhibition or depletion of PIKfyve by apilimod or siRNA resulted in decreased autophagic flux, probably caused by an impaired autophagosome/lysosome fusion. Interestingly, prostate cancer cells incubated with the PIKfyve inhibitor apilimod, secrete more autophagy-associated proteins (NBR1, P62, OPTN and CACO2, and membrane-bound LC3B); however, proteins involved in earlier stages of autophagy were not observed (ATG2A, ATG5, and ATG16L). Analysis of density gradients reveal that these autophagy-associated proteins are expressed on vesicles and appear as autophagosome subpopulations, suggesting induced secretory autophagy (19).

Cytokines and Inflammation

Autophagy is able to suppress inflammasome activation through maintaining mitochondrial homeostasis (20). In line, Zhang et al. observed increased inflammasome activation in macrophages after sepsis in Gamma (γ)-aminobutyric acid type A receptor (GABAAR)-associated protein (GABARAP) knockout mice (20). Secretion of cytokines is central in inflammasome activity. Well-documented cytokines that are associated with unconventional secretion in an autophagy-dependent manner are interleukin-1 beta (IL-1B) and IL-18. Dupont et al. demonstrated in bone marrow-derived macrophages during conditions of induced autophagy (starvation or Torkinib treatment) that secretion of IL-1B increased, which was reduced in the absence of ATG5. Consistent with data obtained from yeast Acbp, similar additional factors were required [as mammalian equivalents of yeast Golgi-associated protein (GRASP55) and small GTPase Rab8a], indicating a conserved system or cooperation (11).

Interestingly, three-dimensional STochastic Optical Reconstruction Microscopy (STORM) demonstrated that IL-1B was not only present in the inner vesicle of the autophagosome but was already recruited to the emerging phagophore. In this, IL-1B is actively translocated across the membrane and accumulated in the intermembrane space of the mature autophagosome (21). This, in contrast to the sequestration of degradable autophagosomal content which is located in the inner vesicle as a result of cytoplasmic engulfment and closure of the phagophore, novel mechanism indicates differences in cargo recruitment and indicates formation of different vesicles by the autophagy-related proteins that may guide the distinct fate of the produced vesicle.

Role of Autophagy-Dependent Secretion in Establishing a Barrier for Infection

Paneth cells are specialized epithelial cells present at the base of the crypts of Lieberkuhn. These cells control the intestinal microbiota through secretion of intracellular granules that contain antimicrobial peptides and lysozyme. Autophagy-proteins are essential for Paneth cell function. Knockdown of crucial autophagy genes [ATG16L1, ATG5, or ATG7 (22, 23)] result in alterations in Paneth cell granules, as illustrated by fewer and aberrant granules with diffuse lysozyme patterns and decreased presence of lysozyme in mucus. Due to its relevance in establishing a microbial barrier, the ATG16L1 gene was annotated as a Crohns disease (CD) risk allele (24) and the phenotype is similar to the abnormalities found in Paneth cells of CD patients.

In contrast to CD where the ileum and colon are often affected, ulcerative colitis is restricted to the colon only. Conditional knockdown of ATG7 in the colon (GlcNAc6ST-2-Cre) increased bacterial colonization in crypts. This phenotype was associated with a decreased release of mucin from goblet cells and reduced expression of antimicrobial and antiparasitic peptides (angiogenin 4, interlectin 1, and relmβ). Hence, colonic ATG7 knockdown results in increased susceptibility to the development of UC-like colitis (25).

In line with autophagy-dependent secretion by intestinal tissue, ATG5 and ATG16L1 deficiency results in reduced secretion of mucins (MUC5AC) by human tracheal epithelial cells (26).

Together, autophagy-dependent secretion is required to maintain effective antibacterial barriers by epithelial cells. In line, ATG16L1 conditional knockout mice (Villin-Cre) are more susceptible to *Salmonella typhimurium* infection (27). Although, beyond the contribution of establishing a barrier function, autophagy is required for limiting bacterial proliferation through ATG16L1- and LC3C-dependent degradation of cytosol-residing *Salmonella* (xenophagy) (28). Evidently, autophagy is a prerequisite for different cell types to maintain their function (e.g., mucus secretion) thereby and protection against inflammatory disorders.

Insulin Secretion

Pancreatic β-cells are specialized in secretion of insulin in response to high levels of blood glucose concentrations. Release of insulin

is mediated by the fusion of insulin-containing vesicles with the plasma membrane. These insulin-containing vesicles are transported toward the plasma membrane. GABARAP, one of seven mammalian variants of yeast Atg8, which is already known to be implicated in multiple cellular functions including fusion events in autophagy (29) and receptor trafficking (30), has recently been demonstrated to mediate insulin secretion. Herein, GABARAP binds insulin-containing vesicles in a PE conjugation-dependent mechanism (31). Together with its microtubules-binding feature [residues 10–22 (30)], GABARAP allows vesicle presentation to the motor protein KIF5B (located at microtubules) and facilitates vesicle mobility and insulin release (32).

NPY Secretion

In rat adrenal medullar pheochromocytoma derived cells, the neurites display ATG16L at dense-core vesicles. Although these vesicles do not co-localize with LC3, release of peptide hormone neuropeptide Y (NPY) is dependent on ATG16L1 functioning as a Rab33a (which regulates membrane trafficking events) effector. Interestingly, NPY secretion was not altered in cells that were deficient in autophagy by overexpressing ATG4B^{C74A} or knockdown of ATG13 or ULK1 and indicates a highly specialized role for ATG16L1 in hormone secretion that is independent of other canonical autophagy-associated proteins (33).

Autophagy-Mediated Secretion of Weibel–Palade Bodies in Vascular Endothelial Cells

Vascular injury stimulates endothelial cells to secrete factors to promote repair. Endothelial secretory granules [Weibel–Palade bodies (WPBs)] contain active molecules, including von Willebrand factor (vWF), P-selectin, interleukin-8, angiopoietin-2, and endothelin-1. The intracellular WPBs are characterized by striations parallel to its longitudinal axis and are delineated by a membrane. When secreted, the multimeric hemostatic vWF is tethered to the connective tissue to mediate platelet adhesion at sites of vascular injury. The processing of vWF to a mature form is important in the WPB formation. Treatment with chloroquine or bafilomycin A1, both raise lysosomal pH, and knockdown of ATG7 or ATG5 reduce the number of endothelial WPBs due to incorrect processing of vWF. In line, endothelial specific deletion of *Atg7* in mice led to reduced epinephrine-induced plasma vWF levels and increased bleeding time. Thus, autophagy in endothelial cells aids in hemostasis by proper maturation of WPBs (34).

Osteoclastic Bone Resorption

Bone remodeling is a lifelong process of bone degradation and formation important for bone healing after injury, bone restructuring, and sustaining bone homeostasis. Osteoclasts cooperate with osteoblasts in bone remodeling in which osteoclasts are responsible for bone degradation and reabsorption of mineralized bone matrix. The osteoclasts are large multinucleated cells that are characterized by their ruffled border by which contact area with the bone is increased. Fusion of secretory lysosomes with the ruffled border causes release of matrix-degrading factors including cathepsin K which aids bone matrix degradation. ATG5- or ATG7-deficient osteoclasts lack a normal ruffled border, impaired localization of secretory factors, including cathepsin K, and eventually have impaired bone resorption. Importantly, development of osteoclasts was not aberrant indicating that the autophagy deficiency led to functional impairment independent of sustaining cellular homeostasis. Moreover, secretory lysosome formation was unaffected, whereas Rab7 (key factor for lysosome fusion events) localization to ruffled border was ATG5 dependent. Together, these data indicated that autophagy-related proteins aid in secretory events at the osteoclast ruffled border by directing fusion of the secretory lysosome with the plasma membrane (35).

In conclusion, autophagy is important for secretory functions of various cell types. Important to note is that autophagy can have either direct or indirect contribution to protein secretion. For example, ATGs directly facilitate protein secretion by mediating cargo sequestration (as IL-1B) or vesicle trafficking (as insulin), but autophagy also maintains cellular homeostasis that is important to preserve the specialized function (IL-1B and vWF) and primes the plasma membrane for proper release of autophagy-independent secretory vesicle (as in osteoclasts). Furthermore, for sustaining a microbial barrier, autophagy's contribution is dual as both a canonical form of autophagy (xenophagy) and the regulation of important secretory factors as mucin contribute to prevent pathogen invasion.

SECRETORY AUTOPHAGY: WASTE DISPOSAL?

Damaged and aggregated proteins and aged organelles are typically degraded by autophagy. Substrates for autophagy are ubiquitylated and recognized by autophagy receptors and degraded. Recent work indicates that defective or saturated autophagy, i.e., by defective autophagosome/lysosome fusion results in cargo secretion into the extracellular environment.

Lysosomal dysfunction is associated with the secretion of aggregation prone proteins that are associated with neurodegenerative diseases as Parkinson's and Alzheimer's disease. α-Synuclein is a presynaptic neuronal protein that is genetically and neuropathologically linked to Parkinson's disease. Wild-type α-synuclein is typically degraded by the autophagy and the proteasome (36). Interestingly, tubulin polymerization-promoting protein/p25a, expressed in the CNS, sorts α-synuclein into autophagsomes but simultaneously prevents its degradation through inhibition of autophagosome/lysosome fusion. Instead p25a controls α-synuclein clearance by its release in the extracellular environment in an autophagy-dependent manner (37).

Alzheimers' disease is characterized by the accumulation of intracellular Amyloid beta (AB) peptide and tau aggregates and extracellular AB plaques. In normal conditions, intracellular proteins are cleared by autophagy and autophagosomes are resolved in the process. However, during Alzheimer disease, autophagosomes accumulate, indicative of impaired autophagy. Autophagy deficiency (ATG7 knockout) in excitatory neurons results in intracellular AB accumulation, confirming its role in clearance of AB aggregates by autophagy. Although AB increased intracellularly, extracellular AB plaque formation was drastically

reduced. Reconstitution of ATG7 expression by lentiviral transduction, rescued the secretory phenotype. In parallel, pharmacologic modulation by either induction or inhibition of autophagy, by rapamycin or spautin-1, increased and reduced extracellular AB release, respectively. Thus, autophagy influences intracellular transport and secretion of AB (38).

Mitochondria, the energy producing centers of the cell, generate ROS as a byproduct of oxidative phosphorylation. In many cancers, ROS production is increased due to mutations in mitochondrial DNA, hypoxia, or disturbed metabolism, leading to cancer progression (39). The homeostasis of mitochondrial ROS plays an important role in the regulation of autophagy. Depolarized and ROS leaking mitochondria are typically degraded by a selective form of autophagy, mitophagy. In depolarized mitochondria, PINK recruits Parkin to mediate selective removal of the organelle in a degrative autophagy-dependent manner. However, a recent report shows that there is an alternative way to maintain mitochondrial homeostasis in the cell. Mesenchymal stem cells (MSC) pack depolarized mitochondria in microvesicles and release them in the extracellular environment to outsource mitophagy where they are recognized by and transferred to macrophages. These released microvesicles are highly enriched in LC3 and ATG12 compared to whole-cell extracts. This mitochondrial transfer probably serves to increase MSC survival (40). Similarly, lipopolysaccharide (LPS)-stimulated rat hepatocytes secrete mitochondrial proteins CPS1 and COXIV, a component of the mitochondrial respiratory chain and associated with the inner mitochondrial membrane, and mitophagy-related proteins PARK2, and PINK1 and LC3B-II. These effects are inhibited by the autophagy inhibitor 3methyladenine or after *Atg5* knockout, suggesting a role for autophagy in the secretion of mitochondria after LPS stimulation (41).

Also endothelial cells are able to release vesicles with autophagosome characteristics. During apoptosis, endothelial cells release in addition to apoptotic bodies, vesicles in an unconventional manner (13). Ultrastructural analysis by electron microscopy showed single membrane vesicles up to 10 μm which contained structures of mitochondria, multivesicular bodies, and autophagosomes. Further proteomic analysis revealed the release of autophagy-associated proteins ATG16L1, LAMP2, and LC3B. The biological function is of this phenomenon remains to be elucidated (12).

The previous section lists the evidence that cells are able to release autophagic vesicles into the extracellular environment. Vesicle release during defects in the autophagic process, specifically during autophagosome/lysosome fusion, suggests alternative mechanisms in waste removal.

EFFECTS ON THE TUMOR MICROENVIRONMENT

Regardless of the clinical advances in the past decades that have improved cancer patient outcome, cancer is still one of the leading causes of death in the world. Importantly, the efficacy of treatment strategies is heavily influenced by cancer cell autonomous features but also by the tumor microenvironment (TME). Solid tumors consist of a variety of cell types, including the cancer cells, endothelial cells, immune cells, and fibroblasts and contain well- and poorly perfused areas that results in inefficient nutrient and oxygen supply (42, 43). Normal (non-transformed) cells in the TME are reprogrammed by the cancer cells to their benefit. This is exemplified by growth supporting angiogenesis and the suppression of anti tumor immunity. Importantly, an existing connection between autophagy and tumorigenesis has already been established. For example, deletion of a single *BECN1* allele [Beclin1 protein important regulator of autophagy (44)] predisposes mice to spontaneous tumor development (45, 46). Further, depletion of FIP200 (important for autophagy initiation) in mammary cancer cells inhibits tumor initiation and progression including metastases (47). Autophagy in cancer cells supports their survival (by aiding the high energy demand) and abets resistance to metabolic and oxidative stresses (e.g., hypoxia) (4, 48–52). Although this role is well established, the contribution of autophagy-related intercellular communication that influences tumor progression through evasion of immunosurveillance, immunogenic cell death (ICD), angiogenesis, and an invasive phenotype is an emerging field with great interest. For example, in cancer, the RAS genes HRAS and KRAS are frequently mutated. Although the exact role of autophagy in tumor progression of RAS-mutated tumors is still under debate, autophagy seems to be dispensable for the growth and survival of KRAS-mutated cancer cell lines derived from human tumors (53). However, when non-RAS-mutated cells are transformed with oncogenic RAS, these cells are highly dependent on autophagy for tumorigenic events (54). In addition, the invasive phenotype of HRASV12-transformed breast cancer cells is reduced in ATG7 knockdown cells. This invasive phenotype could be rescued by incubating these autophagy-deficient cells with conditioned medium of autophagy-proficient cells. This supports an autophagy-dependent secretory system that supports tumor progression (55).

In the next section, the current knowledge on proteins secreted through autophagy-mediated processes that influence tumor progression is discussed (**Table 2**).

Influencing Immunogenic Cell Death to Evade Immunosurveillance

Under normal circumstances, immune cells [including dendritic cells (DCs), natural killer (NK) cells, and T cells] recognize and eliminate newly formed neoplastic cells due to their high immunogenic nature as a result of their mutational burden (immunosurveillance). Cancer cells that have obtained an immune evasive phenotype are able to circumvent recognition and subsequent elimination by the cooperative immune cells. These cancer cells are then selected for characteristics that circumvent local immunosurveillance and contribute to the growth of the lesion. With recent advances, the immune evasive feature is a topic of interest for the development of therapeutic strategies. Ideally, the elicited cancer therapy-induced cell death should provoke an immunogenic chain reaction that includes boosting the immune system to tilt the balance toward recognition rather than evasion, called ICD. ICD invokes the release of immunomodulatory proteins [damage-associated molecular patterns (DAMPs)] that

TABLE 2 | Effects on the tumor microenvironment.

Protein(s)	Mechanism	Effect	Reference
Influencing immunogenic cell death to evade immune surveillance			
ATP	ATG5 knockdown	After radiotherapy and MTX exposure "eat me signal" for immune cells. Stimulus for DC recruitment, IFNγ-producing CD4 and CD8 T cells	(60–62)
HMGB1	$ATG5^{fl/fl}$	Promote processing and presentation of tumor antigens by DCs, enhanced immuno surveillance	(11)
Cytokine release and influence on the tumor microenvironment			
IL1-B, IL-6, IL2	GABARAP Knockout mice	Increased secretion by macrophages	(78)
IFNγ	GABARAP Knockout mice	Increased secretion by lymphocytes	(78)
CXCL9, CXCL10, and CXCL11	FIP200 conditional knockout	Enhanced secretion, leading to improved immuno surveillance	(47)
Prometastatic: driving an invasive behavior of cancer cells			
LIF, FAMC3, DKK3, IL-8	ATG7 knockdown	These factor promote metastasis *via* MMP2 upregulation (IL-8), epithelial to mesenchymal transition (FAM3C, DKK3, LIF), and promotion of angiogenesis (IL-8 and DKK3)	(80)
	ATG7, ATG12, ATG3 knockdown chloroquine or bafilomycin A1	Autophagy-deficient $HRAS^{v12}$-transformed breast cancer cell lines display reduced invasive protrusions. Conditioned medium of autophagy-proficient cells rescued the invasive phenotype	(79)
IL6, CCL-2, CCL-20, VEGFA, MMP2	3-MA, ATG5, and ATG7 knockdown	TLR3 and TLR4 activation leads to autophagy-dependent secretion of these factors associated with a migratory and invasive phenotype of lung cancer cells	(82)
IL6	ATG7 or beclin knockdown	Autophagy deficiency lead to an increase or decrease in low or high autophagic breast cancer cells, respectively. Autophagy-dependent secretion of IL-6 are able to promote mammosphere formation and may be important in CSC maintenance	(83, 84)
Prometastatic: proper Weibel–Palade body formation in vascular endothelium to facilitate metastasis			
WPB proteins		Autophagy is important to sustain secretion of WPBs containing proteins that influence tumor progression	(85)
Chemoresistantance			
HMGB1		HMGB1 causes doxorubicin resistance in neighboring breast cancer cells	(88)

incite antitumor immunity (56). ICD can be induced by selective chemotherapeutics, including mitoxantrone (MTX) and oxaliplatin (OXA) and radiotherapy [reviewed in Ref. (57)]. Cancer cells undergoing ICD stimulate and activate the innate immune cells. Subsequently, this can result in the cross-priming of the adaptive immune system for the antigens of dying cancer cells, thereby leading to an effective activation of antitumor immunity. This can elicit a long-term therapeutic effect (even after therapy has stopped) and is fundamental to observed abscopal effects. In line, clinical studies have demonstrated that lymphopenia negatively affects chemotherapeutic response of solid tumors (58) and that ICD-associated DAMPs can be used as predictive and prognostic biomarkers (59).

Important immunogenic DAMPs that are displayed by cells undergoing ICD are secretion of ATP, surface exposure of calreticulin (CRT), release of heat shock proteins, and high mobility group box 1 (HMGB1). Interestingly, ATG5 knockdown in colon cancer cells reduces ATP release after radiotherapy (60) and MTX exposure (61), which was associated with a decreased effect on tumor growth inhibition. Interestingly, in autophagy-deficient cells, no differences in CRT surface exposure or HMGB1 release were observed (61). The relevance of autophagy-dependent radiotherapy-induced ATP release was further supported by the observation that treatment with an inhibitor of ecto-ATPase only increased radiosensitivity in immunocompetent but not immunodeficient mice. Here, a partial rescue of lymphocyte infiltration indicates that the autophagy-dependent radiotherapy-induced ATP release enhances antitumor immunity (60). Similarly, for MTX treatment of osteosarcoma cells, it was also shown that ATG5 is required for ATP secretion. Interestingly, on a more mechanistic level, Martins et al. demonstrated that ATP (stored in lysosomes) is released upon MTX and OXA treatment and is associated with LAMP1 (lysosomal marker) translocation to the plasma membrane. Nonetheless, a role for autophagy seems to be maintaining an intracellular ATP pool (may even be cargo sequestration) required for (LAMP1+) lysosome-dependent ATP release. Accordingly, the replenishment of ATP to lysosomes was reduced when autophagy genes were knocked down (62). ATP release conveys an important "eat me" signal for immune cells. Once it is released, ATP may attract innate effector cells of the immune system into the tumor bed. Consistently, it was demonstrated that autophagy-dependent ATP release from MTX-treated colon cancers was a stimulus for DC recruitment, IFNγ-producing CD4 and CD8 T cells that had favorable effects on MTX sensitivity (61). Correspondingly, ATG7 in a genetically induced melanoma mouse model was required for MTX-dependent growth inhibition that was reliant on functional CD4 and CD8 T cells (63). Further, caloric restriction or treatment with caloric restriction mimetics, that increase autophagy activity, enhance autophagy-dependent ATP release and improve MTX-induced tumor growth delay in a T cell dependent fashion (64).

Oppositely, a different study using a different ICD inducer (photo-oxidative ER stress inducer hypericin) has demonstrated

autophagy-independent ATP release, but observed enhanced surface CRT exposure when autophagy was attenuated. The enhanced DC maturation and IL-6 secretion further promote IFNγ-producing T lymphocytes (65). Interestingly, surface CRT exposure after MTX or hypericin treatment could be ablated in cells lacking lysosome-associated LAMP2A, an essential gene for a chaperone-mediated autophagy (66). Moreover, regarding ATP, it was demonstrated that extracellular residing ATP does not relay an immunogenic response *per se* (67) and further illustrates the context dependence of effects resulting in immunogenicity.

The involvement of autophagy-related proteins in the release of the DAMP HMGB1 has been demonstrated using ATG5$^{fl/fl}$ Cre^{+} bone marrow-derived macrophages (11) and dying glioma cancer cells [in which HMGB1 was found in a subset of autophagosomes before release (68)]. The released HMGB1 by dying cancer cells can bind Toll-like receptor (TLR-) 4 and promote the processing and presentation of tumor antigens by DCs. This leads to cross-priming of T-cells and enhances immunosurveillance (69). Furthermore, endothelial cell exposure to HMGB1 triggers pro-angiogenic effects (70), including endothelial cell migration, sprouting and induction of an autocrine signaling cascade that results in elevated expression of leukocyte adhesion molecules ICAM-1, VCAM-1, and E-selectin. Moreover, HMGB1 induced expression of VEGF-A, VEGFR1, VEGFR2, and neuropilin-1 (71) and stimulation of angiogenesis (72).

In conclusion, ICD is an important pillar of therapy-induced antitumor immunity as it relays important signaling to the immune system, including DCs. DC stimulation may be important to induce tumor cure as demonstrated by ICD-based DC-vaccines in high grade glioma-bearing mice (73). Nevertheless, the influence of autophagy on the display of DAMPs may be ICD-inducer dependent and requires further understanding for effective use.

Cytokine Release and Influence on the Tumor Microenvironment

ATP can bind the P2RX7 receptor and activate the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in DCs and macrophages (74). This inflammasome activation can be suppressed by autophagy (20), which would be favorable as inflammasome activation and subsequent IL-1B release by, e.g., macrophages have pro-tumorigenic effects. However, the P2RX7 receptor impairs autophagy by blocking lysosomal function and stimulates release of vesicles with autophagolysosome characteristics (75). This suggests that activation of the P2RX7 receptor leads to a secretory phenotype, *via* inhibition of autophagy. Depletion of IL-1B arrests growth in melanoma (76), and macrophage-derived IL-1B-induced IL-17 expression from γδ T cells resulted in expansion of tumor-associated neutrophils that suppress cytotoxic T cells in breast cancer, resulting in increased number of pulmonary and lymph node metastases (77). Alternatively, in a murine colon carcinoma model, autophagy-dependent ATP release after MTX treatment promoted recruitment of IFNγ-producing CD8+ T cells into the tumor in an IL-1B-mediated fashion (61).

Lipopolysaccharide/LPS + ATP stimulated GABARAP knockout macrophages to secrete more IL-1B and IL-6. In addition, GABARAP knockout lymphocytes produced more IL-2 and interferon-γ. In this model, GABARAP KO was associated with reduced tumor incidence. These effects were validated on tumor control in a melanoma tumor cell-inoculated model (78) and indicated that GABARAP in non-cancerous cells is sufficient to sustain pro-tumorigenic effects potentially due to control of cytokine secretion.

A study using MMTV-PyMT mouse model of breast cancer bearing a conditional deletion of autophagy gene *FIP200* shows that these tumor cells have a different chemokine secretion profile than FIP200-proficient cells. The TME polarizes toward an improved immunosurveillance as enhanced secretion of chemokines, including CXCL9, CXCL10, and CXCL11, leads to increased infiltration of IFNγ-producing CD8+ and CD4+ T cells (47).

In conclusion, autophagy-related secretion is important in controlling the cytokine profile of different cell types.

Prometastatic: Driving an Invasive Behavior of Cancer Cells

Oncogenic mutations in RAS are highly prevalent in cancers and drive different pro-tumorigenic features, including proliferation, survival, and invasion. Autophagy-deficient HRAS^{V12}-transformed breast cancer cell lines display reduced invasive protrusions in genetic knockdown models (including ATG7, ATG12, and ATG3) and after pharmacological inhibition (chloroquine or bafilomycin A1). Addition of conditioned medium of autophagy-proficient cells rescues the invasive phenotype, indicating a role for autophagy-dependent secretion in triggering cellular migration. Correspondingly, the pulmonary metastatic potential of HRAS^{V12} tumors is reduced in autophagy-deficient cells, effects dependent on autophagy-related secretion (55). Despite these results, in this model, the role of IL-6 in tumor progression is ambiguous in literature as both pro-tumorigenic (metastasis, angiogenesis, immune suppression) and antitumorigenic (CD8+ T cell trafficking to lymph nodes and tumors) effects on the TME are described (79).

Kraya et al. observed a cytokine profile that differed between melanoma cells with high and low autophagy activation that could be mimicked by introducing ATG7 knockdown in an autophagy high cell line. The main secretory factors the authors focused on, which were dependent on autophagy(-protein), are leukemia inhibitory factor (LIF), family with sequence similarity 3 member C (FAM3C), dickkopf WNT signaling pathway inhibitor 3 (DKK3), and IL-8. These factors are able to promote metastasis *via* mechanisms that include MMP2 upregulation (IL-8), epithelial to mesenchymal transition (FAM3C, DKK3, LIF), and promotion of angiogenesis (IL-8 and DKK3) (80).

Toll-like receptor 3 and TLR4, which are expressed on immune cells, including macrophages and DCs, but also a variety of cancer cell, can activate the release of an array of cytokines (81). Recently, it has been demonstrated that TLR3 and TLR4 activation [in an LPS- or poly(I:C)-induced model] in lung cancer cell lines (A549 and H460) causes (1) Lys63-linked ubiquitynilation of TNF receptor-associated factor 6 (TRAF6) and (2) induced autophagy. Herein, autophagy was required

for TRAF6 ubiquitinylation that leads to downstream activation of NFκB and MAPK signaling and subsequent cytokine production. As a result, autophagy deficiency in these lung cancer cells reduced release of IL-6, C–C motif chemokine ligand (CCL)-2, and CCL-20. CCL-2 secretion is associated with cell migration and CCL-20 with a metastatic phenotype. Accordingly, autophagy deficiency impaired migratory capacity. IL-6 can induce VEGFA and MMP release that are associated with an invasive phenotype. Indeed, the invasive phenotype was dependent on autophagy and IL-6 and associated with VEGFA and MMP2 release (82).

In breast cancer, autophagy inhibition through ATG7 or Beclin1 knockdown altered IL-6 secretion. Interestingly, autophagy deficiency increased IL-6 secretion by MCF7 (low autophagy-dependent survival) and decreased IL-6 secretion by MDA-MB-468 (high autophagy-dependent survival) cells. IL-6 secretion is important for cancer stem cell (CSC) maintenance and is sufficient to induce CD44+:CD24low/− phenotype in breast cancer cells (83). In line, autophagy deficiency decreased mammosphere formation capacity of MDA-MB-468 cells. Rescue experiments illustrated that mammosphere formation was improved by IL-6 treatment and conditioned media from autophagy-proficient MDA-MB-468 cells. Autophagy-dependent secretion of IL-6, but also other factors, are able to promote mammosphere formation and may be important in CSC maintenance (84).

Prometastatic: Weibel–Palade Body Formation in Vascular Endothelium to Facilitate Metastasis

Aberrant signaling in tumor-associated endothelial cells contributes to excessive neovascularization that is a feature of solid tumors [reviewed in Ref. (72)]. As discussed above, autophagy is important to sustain the secretion of protein-containing WPB by proper vWF maturation. In tumor endothelial cells (TECs), these WPBs contain important secretory factors that can influence tumor progression. In line, P-selectin is sorted into WPBs as a result of its ability to interact with vWF and is translocated to the cell membrane upon stimulation (85). Once localized at the luminal side of the endothelial cell, it facilitates metastasis formation by promoting adhesion of circulating tumor cells (86). Impairment of autophagy may, therefore, reduce development of metastases. In addition to vWF and P-selectin, WPBs can contain other secretory proteins as angiopoietin 2 which is positively associated with tumor progression (due to its angiogenic potential) and interleukin 8 which is important in tumor progression and metastasis (due to its angiogenic and immune response modulating potential). Collectively, autophagy-dependent WPB formation may facilitate tumor progression, although these aspects require further investigation.

Therapy Resistance

Autophagy has been implicated in promoting chemo- and radioresistance. Although often presumed to be caused by its degradative feature, we demonstrated in irradiated cancer cells that knockdown of ATG7 or LC3B, but not treatment with lysosomal inhibitor chloroquine, sensitizes cancer cells to radiation (87). This further supports a role of autophagy-related proteins to promote radioresistance through an alternative process such as secretion. For example, HMGB1 is secreted through autophagy-dependent mechanisms during ICD (11, 68). Although this factor is an important DAMP that can increase immunogenic responses, HMGB1 increases doxorubicin resistance in neighboring breast cancer cells (88). In line, the interaction of HMGB1 with the receptor for advanced glycation end products (RAGE) that is expressed on various cell lines in the tumor increases chemo resistance by inducing pro-survival autophagy (89).

In addition to increasing angiogenesis and the prometastatic potential by DKK3, DKK3 expression is associated with docetaxel chemo sensitivity in lung cancer cells through decreasing expression of the drug efflux pump P-glycoprotein (90). Furthermore, DKK3 overexpression in an esophageal adenocarcinoma cell line was associated with increased 5-FU and cisplatin resistance, invasion, and activation of the TGF-B signaling (91).

In short, autophagy-dependent secretion is involved in antitumor effects through enhancing immunosurveillance, but is also important in tumor progression through stimulation of angiogenesis, changing drug resistance, triggering EMT, and increasing metastases development. Manipulation of the secreted arsenal of proteins, and tilting the balance more toward an antitumor strategy may be an attractive novel approach in cancer treatment.

AUTOPHAGY-DEPENDENT RECEPTOR TRAFFICKING IN TUMOR PROGRESSION

Autophagy execution requires cargo recognition, packaging, vesicle transport, vesicle fusion, and degradation. In addition to the catabolic function of autophagy, the autophagy machinery is utilized for more purposes, including intracellular trafficking and endocytic signaling. In addition to these roles in secretion, autophagy mediates the retro- and anterograde trafficking of membrane-bound receptors that may influence tumor progression.

For example, the GABARAP protein family mediates membrane of cell- surface expression of receptors like the GABA (A) receptor (GABAAR) (92), the human kappa opioid receptor (hKOPR) (93), transient receptor potential cation channel subfamily V member 1 (TRPV1) (94), the angiotensin II receptors (AGTR) (95, 96), and the epidermal growth factor receptor (EGFR) (51).

Epidermal growth factor receptor controls cell proliferation, migration and differentiation and is frequently overactivated in several cancer types due to amplification or mutation (97). EGFR expressing tumors depend on autophagy for their survival and proliferation. Inhibition of autophagy by the administration of chloroquine abrogated the radioresistant phenotype of these tumors (52) [reviewed in Ref. (98)]. Interestingly, during hypoxia,

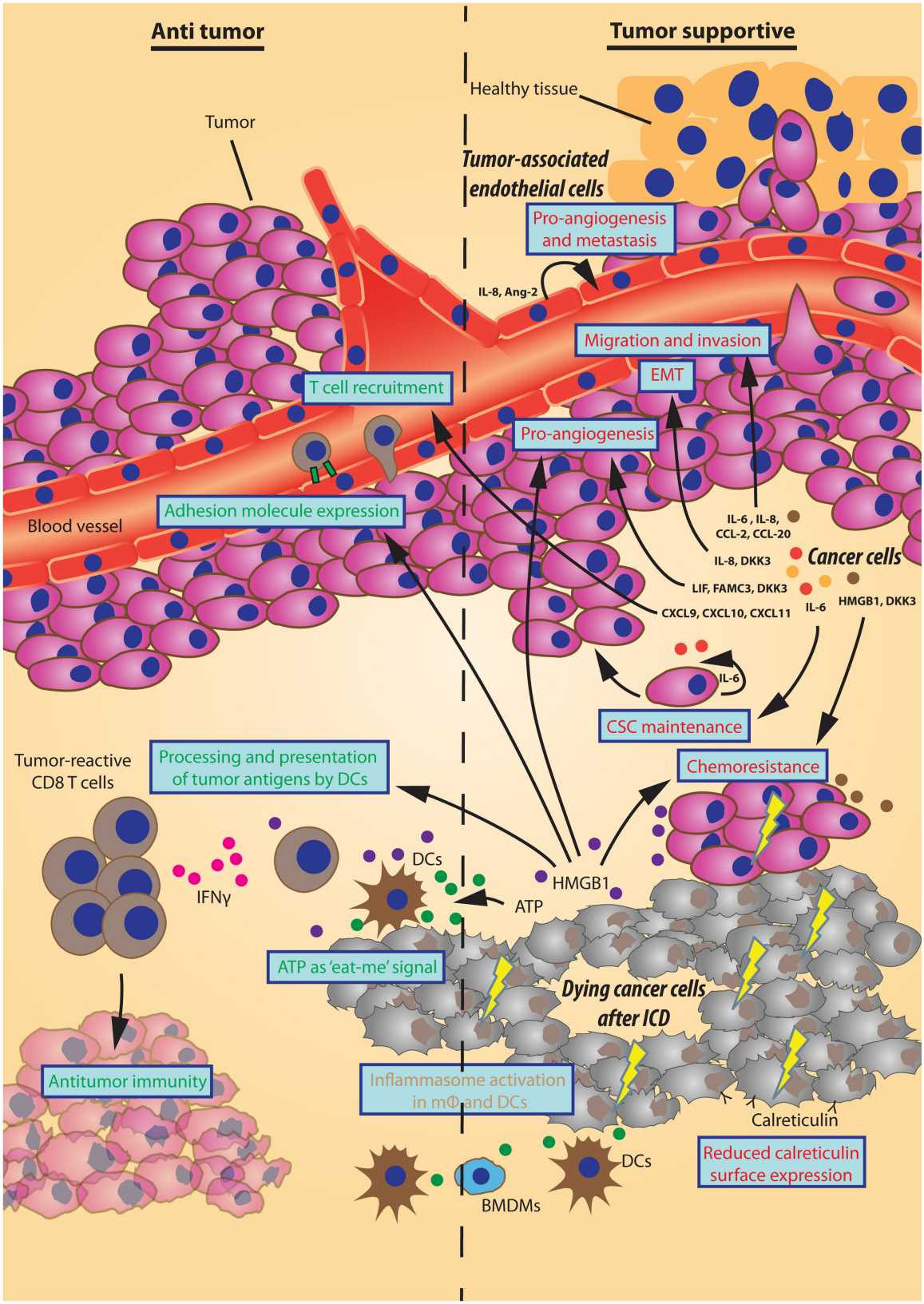

FIGURE 1 | **Effects of autophagy-dependent secretion on tumor progression**.

(*Continued*)

FIGURE 1 | Continued
Schematic representation of autophagy-mediated secretory events that either inhibit or support tumor progression displayed here on the left ("Antitumor") and right side ("Tumor supportive"), respectively. The different sources of autophagy-dependent secretory factors (*bold italic*) establish multiple effects on the tumor microenvironment (in blue boxes) by the designated factors. As such, factors that promote angiogenesis, invasion, a migratory phenotype, cancer stem cell (CSC) maintenance, or chemoresistance support tumor progression. Also, a reduced surface expression of calreticulin by cancer cells undergoing immunogenic cell death (ICD) hinders an immunogenic response. Oppositely, some factors have counteractive effects on tumor progression by improving immune cell adhesion or recruitment. Moreover, the "eat-me" signal ATP together with HMGB1/TLR4-mediated improved processing and presentation of tumor antigens by dendritic cells (DCs) promote interferon gamma (IFNγ)-producing T cells to aid antitumor immunity. The ATP/P2RX7-mediated activation of the inflammasome in macrophages (mφ) and DCs can have an array of effects of which the final inhibition/support of tumor progression may be context-dependent.

translocation of EGFR to the plasma membrane is controlled by GABARAPL1 (51). Upon hypoxia exposure, GABARAPL1 colocalizes with EGFR at the cytoplasmic site of the plasma membrane. Moreover, knockdown of GABARAPL1 resulted in a decrease in EGFR membrane expression, but not in overall EGFR expression, suggesting a role for GABARAPL1 in anterograde transport of EGFR.

The KOR and GABAAR are involved in neurological processes and play a role in a variety of processes like pain sensation, consciousness, and mood.

The GABA(A) receptor (GABAAR) is well known for its inhibitory role on active neurons and is expressed on the postsynaptic throughout the whole body, although mainly expressed in the mammalian brain. Surprisingly, overexpression of the GABAAR leads to several types of cancer including breast, liver, lung, and pancreatic cancers and contributes to migration of breast cancer cells through activation of extracellular-regulated kinase 1/2 (ERK1/2) (99–104). GABARAP and GABARAPL1 are both involved in GABAAR trafficking toward the plasma membrane. In this role, GABARAP probably serves as a cargo-receptor which mediates GABAAR incorporation in the transport vesicle by a direct interaction with the γ2 subunit of the receptor. GABARAP-knockdown mice show no defects in GABAAR expression, suggesting that GABARAP is redundant and other molecules, like its homolog GABARAPL1, can take over its function (105).

In contrast to the pro-tumorigenic effects of EGFR and GABAAR signaling, membrane expression mediated through autophagy-related proteins also results in the expression of receptors that may inhibit tumor progression, for example through KOR signaling. The KOR is well characterized for its analgesic role. However, the KOR also acts as a negative regulator of cell proliferation in breast, lung, and prostate cancers (106, 107). Opioid receptors belong to the GPCR family, and activation of the receptors modulates the MAPK pathway and inhibits prosurvival PI3K/AKT signaling molecules and may antagonize EGFR signaling (106). Both GABARAP and GABARAPL1 are required for anterograde transport of the KOR receptor. Interestingly, because of its stronger interaction, GABARAPL1 does not need C-terminal modification in contrast to GABARAP, which requires membrane association to transport the KOR to the plasma membrane (93).

Taken together, the GABARAP family proteins mediates trafficking and surface expression of receptors with both tumor promoting (EGFR, GABAAR) and tumor inhibitory characteristics (KOR). This suggests that the GABARAP family contributes to cancer progression in a context-dependent manner, being in a tumor-promoting or -inhibitory role.

CONCLUDING REMARKS

Autophagy has been considered as an important tumor suppressive process for cellular homeostasis by effectuating lysosomal degradation of the cells' toxic constituents. Importantly, autophagy mediates an additional cellular feature, the trafficking, and release of specific proteins. These effects are important during physiological conditions (e.g., maintaining a barrier for infection by mucus and lysozyme secretion and waste secretion), but also mediate important effects in tumor progression (**Figure 1**). The autophagy-mediated secreted factors may stimulate cellular proliferation *via* auto- and paracrine signaling and establish a communicative tool between cells that can either stimulate or limit tumor progression. The autophagy-mediated release of DAMPs seems to be ICD inducer-dependent and polarize the TME toward a less immunesuppressive phenotype. Alternatively, tumors are characterized by promoting an immunosuppressed TME by cytokine signaling. Furthermore, autophagy-mediated secretory signaling promotes an invasive phenotype. An important note is that autophagy may convey direct or indirect effects on secretory events through formation of the mobilized vesicle, facilitation of anterograde trafficking or alterations in homeostasis, and/or autonomous cell signaling.

In conclusion, autophagy (or autophagy-related proteins) is an important cellular process that is more elaborate than solemnly a degradative pathway. It facilitates multiple secretory events that can promote tumor progression by limiting immunosurveillance and stimulating invasiveness and angiogenesis.

AUTHOR CONTRIBUTIONS

TK, MS, and KR reviewed literature, wrote the paper, drafted the outline, and approved content.

FUNDING

This work was financially supported by the Dutch Cancer Society (KWF Grants UM 2012-5506 and 2015-7735 to KR), worldwide cancer research award 16-0265 (to KR), and the FWO Postdoctoral Fellowship program (12P0817N to MS).

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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.

frontiers in Oncology

REVIEW
published: 18 April 2017
doi: 10.3389/fonc.2017.00065

Autophagy-Regulating microRNAs and Cancer

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Macroautophagy (autophagy herein) is a cellular stress response and a survival pathway that is responsible for the degradation of long-lived proteins, protein aggregates, as well as damaged organelles in order to maintain cellular homeostasis. Consequently, abnormalities of autophagy are associated with a number of diseases, including Alzheimers's disease, Parkinson's disease, and cancer. According to the current view, autophagy seems to serve as a tumor suppressor in the early phases of cancer formation, yet in later phases, autophagy may support and/or facilitate tumor growth, spread, and contribute to treatment resistance. Therefore, autophagy is considered as a stage-dependent dual player in cancer. microRNAs (miRNAs) are endogenous non-coding small RNAs that negatively regulate gene expression at a post-transcriptional level. miRNAs control several fundamental biological processes, and autophagy is no exception. Furthermore, accumulating data in the literature indicate that dysregulation of miRNA expression contribute to the mechanisms of cancer formation, invasion, metastasis, and affect responses to chemotherapy or radiotherapy. Therefore, considering the importance of autophagy for cancer biology, study of autophagy-regulating miRNA in cancer will allow a better understanding of malignancies and lead to the development of novel disease markers and therapeutic strategies. The potential to provide study of some of these cancer-related miRNAs were also implicated in autophagy regulation. In this review, we will focus on autophagy, miRNA, and cancer connection, and discuss its implications for cancer biology and cancer treatment.

Keywords: autophagy, microRNA, post-transcriptional control, cancer growth, metastasis, chemotherapy, radiotherapy, biomarker

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *30 December 2016*
Accepted: *21 March 2017*
Published: *18 April 2017*

Citation:
Gozuacik D, Akkoc Y, Ozturk DG and Kocak M (2017) Autophagy-Regulating microRNAs and Cancer. Front. Oncol. 7:65. doi: 10.3389/fonc.2017.00065

INTRODUCTION

MicroRNAs (miRNAs) are small RNAs that play a key role in the regulation of gene expression. miRNAs do not code for proteins, but they control stability and translation of messenger RNAs (mRNAs) of protein-coding genes, and change abundance of proteins that are encoded by them. By this way, miRNAs modulate and orchestrate cellular pathways, including cell growth, differentiation, apoptosis, and migration pathways (1–3). Around 2,000 unique miRNAs were discovered in man and their numbers are growing.

Dysregulation of miRNA expression often correlates with human diseases. Up- or downregulation of miRNAs was reported in several cancer types as well. miRNA abnormalities contribute to various stages of cancer formation and progression, and even determine resistance to cancer treatment. Differential expression of miRNAs between tumors and their corresponding normal tissues led them

to be introduced as potent cancer markers. Changes in specific miRNA levels were reported in almost all types of malignancies, including lung cancer, colon cancer, pancreatic cancer, breast cancer, and leukemia (4–8).

Autophagy is a highly conserved cellular mechanism that allows digestion and recycling of long-lived proteins, protein aggregates, intracellular pathogens, and even whole organelles, such as mitochondria. Active at a basal level in all cell types, autophagy is rapidly upregulated under stress conditions. Being a key guardian of cellular homeostasis, abnormalities of autophagy almost invariably lead to health problems, including cancer (9–13). A growing number of studies that were published in the last couple of years underline the importance of miRNAs in autophagy regulation. In this review article, we will briefly summarize miRNA and autophagy pathways and analyze emerging connections and correlations between autophagy, miRNAs, and cancer.

MicroRNAs

MicroRNAs constitute an evolutionary conserved family of single-stranded, non-coding RNA molecules. These small RNAs are 17–25 nt in length. They control biological events through post-transcriptional gene silencing (14). miRNAs are found in a wide range of living organisms, e.g., from plants to mammals, providing evidence that gene expression control by miRNAs is an ancient mechanism (15). Computational predictions revealed that more than 60% of all human genes contain potential miRNA-binding sites; hence, all these genes might be subject to regulation by these small RNAs (16).

In the genome, miRNA genes and gene clusters can be found in both intergenic and intronic regions (6, 17). miRNAs residing in the same cluster might share the same transcriptional regulatory units. Hence, miRNAs may be expressed as polycistronic transcripts, allowing a coordinated expression pattern for functionally related miRNAs (18). Cellular levels of intronic miRNAs usually depend on the expression of the host protein-coding gene. Isolated miRNA genes exist as well; these genes possess their own promoters and can be expressed independently (18).

Long primary miRNAs or pri-miRNAs are 60–70 nt length RNA transcripts that are generally transcribed from miRNA genes in an RNA polymerase II (pol II)-dependent manner (19). However, transcription of some miRNA types may depend on RNA polymerase III (pol III) (20). Like protein-coding mRNAs, primary miRNA transcripts also contain a 5′ cap and a 3′ poly-A tail. miRNA may also be subject to splicing. Pri-miRNAs transcribed by RNA pol II may sometimes generate more than one functional miRNAs from a single pri-miRNA transcript (19).

Following transcription, a number of consecutive RNAse-dependent reactions are required in order to process intermediary RNA oligonucleotides and produce mature and functional miRNAs. They are then processed in the nucleus by a core ribonuclease complex including Drosha and its regulatory subunit DGCR8 to generate hairpin-structured premature-miRNAs (pre-miRNAs) of 60–70 nt. After cleavage they can be recognized by exportin-5 and transport from nucleus to the cytoplasm. In cytoplasm, DICER protein further cleaves the hairpin structure of pre-miRNAs which leads to the formation of ~21–22 nt long miRNA duplexes. Then these duplexes loaded onto a complex called RNA-induced silencing complex (RISC). Argonaute (AGO) proteins are important components of the RISC complex which they guide single-stranded mature miRNAs to their target mRNAs. The fate of the mRNA determined the degree of complementarity between mature miRNA seed sequences (~8 nt in the core region of the miRNA) and "miRNA response elements (MRE)" on target mRNA sequences. In the slicer-dependent mechanism, base pairing with the guide miRNA results in an endonuclease-dependent cleavage of the target mRNA. miRNA-directed de-capping and/orde-adenylation of the target mRNA may proceed the degradation process whereas a partial complementarity may block the translation machinery (17, 21) (**Figure 1**).

Autophagy

Macroautophagy (autophagy herein) is an evolutionary conserved cellular recycling pathway during which cargos, including long-lived proteins, protein aggregates, and damaged organelles (such as mitochondria and peroxisomes) are eliminated through lysosomal degradation. During this biological process, cargo molecules in the cytosol are sequestered by vesicles (autophagosomes) that are bound by double or multiple membrane bilayers (**Figure 2**) (34). Autophagosomes eventually fuse with lysosomes and form "autolysosomes," leading to degradation of autophagic components and their cargos by the action of lysosomal hydrolases. Eventually following breakdown, degradation products are recycled back to cytosol, allowing their reuse by the cell. Basal autophagy is active in all eukaryotic cells and can be upregulated under a variety of cellular stress conditions, including starvation, growth factor deprivation, disease-related aggregate-prone protein accumulation, hypoxia, pathogens, etc.

Under stress, autophagy acts as a survival mechanism. It exerts a cytoprotective effect by providing building blocks and energy resources to cells, and by eliminating reactive oxygen generating damaged organelles and protein aggregates. These responses ensure adaptation to stress, promote energy homeostasis, and hence survival of the cell. On the other hand, under some conditions, uncontrolled autophagy was shown to lead to a caspase-independent, necrotic-like cell death that was called "the autophagic cell death."

MECHANISMS OF AUTOPHAGY

Autophagy is regulated by a set of evolutionarily conserved ATG gene products (35). In addition to ATGs proteins, several other proteins were implicated in autophagy regulation. miRNAs have been shown to regulate autophagy through their effects on various autophagy regulatory proteins that function at different stages of the pathway, namely induction, vesicle nucleation, vesicle elongation, retrieval, and fusion stages. Here, we will briefly overview autophagosome formation stages and introduce major proteins and complexes involved in the process (**Figure 2**). For a more detailed analysis, the readers are referred to the following excellent review articles (35, 36).

Induction

Target of rapamycin (TOR) kinase-containing protein complexes are key regulators of the autophagy pathway. Mammalian TOR

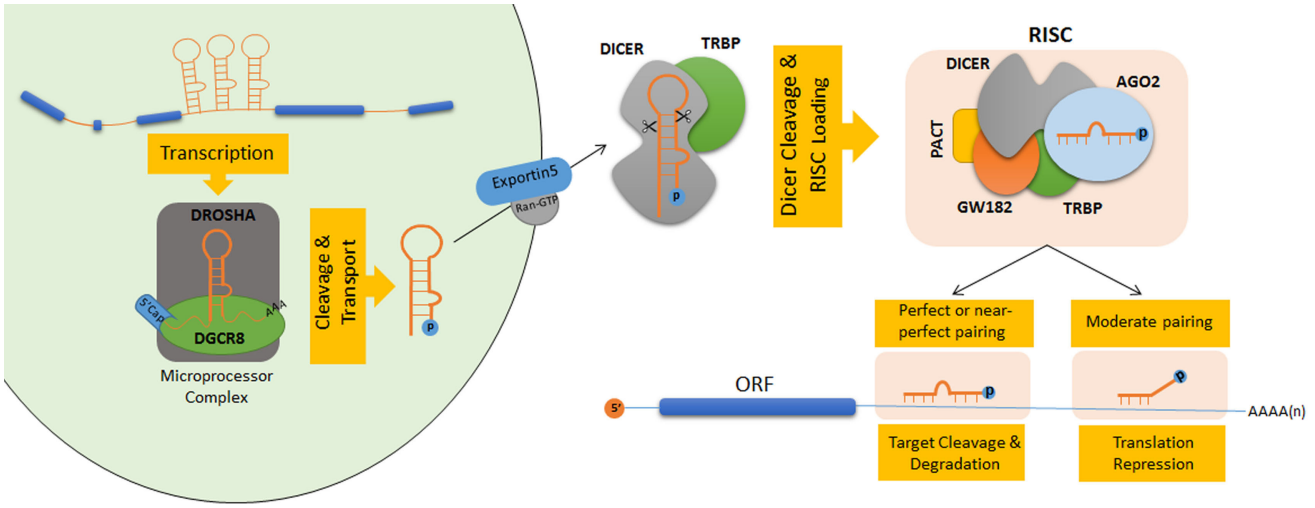

FIGURE 1 | Schematic model of microRNA (miRNA) biogenesis and maturation in human. Nuclear cleavage events performed by protein complexes showing ribonuclease III activity lead to the processing of pri-miRNAs into small hairpin-shaped pre-miRNAs (22, 23). The core ribonuclease complex (the microprocessor complex) consists of a heterotetramer of Drosha and DGCR8 (DiGeorge syndrome critical region gene 8 or Pasha) proteins. During this reaction, flanking ssRNA–dsRNA junctions in pri-miRNAs are recognized by DGCR8 which guides Drosha to specific cleavage sites around 11 bp away from the stem–ssRNA junctions (19, 24). Pre-miRNAs that are released after Drosha cleavage exhibit characteristic features of RNase III endonuclease products having 5′ phosphate groups and 2 nt overhangs at their 3′ sequences (25). After cleavage by Drosha, 3′ overhangs are recognized by exportin-5 (XPO5) complexes in the nucleus (26). Pre-miRNAs are then transferred to the cytosol by canonical Ran-GTP-dependent transport mechanisms (27). In the cytosol, another RNase III-type endonuclease, called the Dicer, cleaves pre-miRNAs near their terminal loops, and leads to their conversion to double-stranded 20–22 nt miRNA duplexes (28–30). Terminal loop of pre-miRNAs are recognized through the N-terminal helicase domain of Dicer. Its PAZ domain interacts with 2 nt 3′ overhangs at the termini of pre-miRNAs and directs them to its catalytic RNase III domain for cleavage (30). RNA-induced silencing complex (RISC) captures the cleavage product through its Argonaute (AGO) protein component (31). ATP-dependent chaperone activity of Hsc70/Hsp90 proteins is important for small RNA duplex loading onto Ago proteins. Following passenger-strand degradation or ejection, AGO proteins remain in complex with a single-strand guide miRNA (32). In humans, among the four AGO proteins (AGO1–4), only the AGO2 protein has the ability to slice target mRNAs (33).

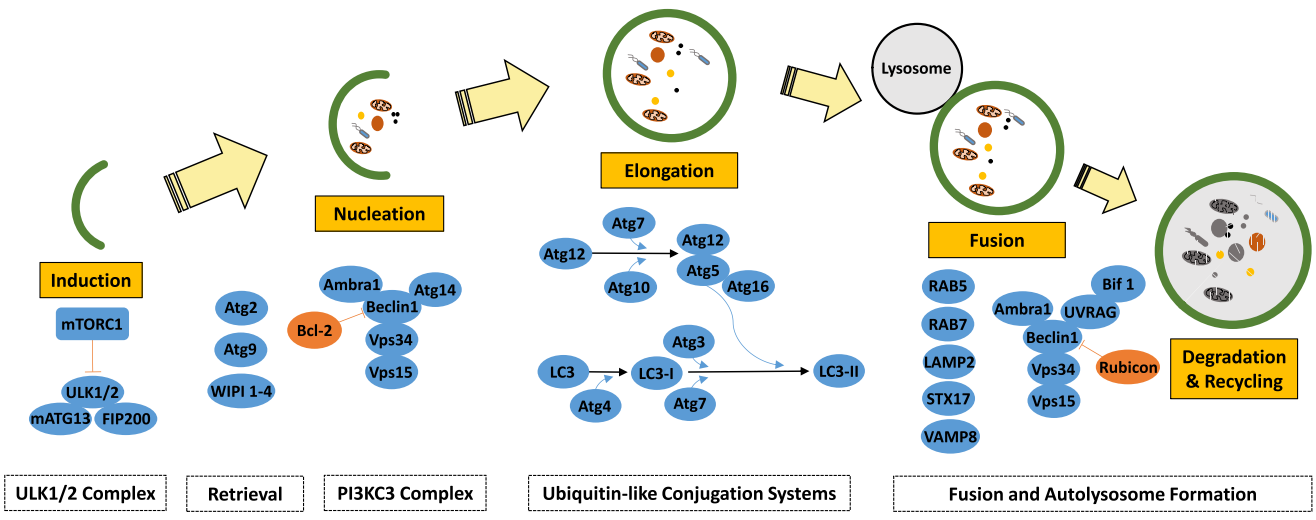

FIGURE 2 | Schematic representation of the autophagy pathway and core autophagy proteins.

(mTOR) kinase forms two autophagy-related protein complexes: mTORC1 and mTORC2 complexes. In addition to mTOR, mTORC1 is composed of RAPTOR, GβL, and PRAS40 proteins. On the other hand, mTORC2 include mTOR, RICTOR, GβL, SIN1, and PROTOR proteins.

Various stress-causing signals, including amino acid starvation, growth factor deprivation, and low ATP levels, that are conveyed by RAG proteins, the AKT pathway, and the AMPK pathway, converge at mTORC1, that strictly coordinates cell growth-related events, including initiation of translation, ribosome biogenesis,

protein synthesis, and cell size in the light of these inputs. On the other hand, mTORC2 is mainly involved in cytoskeletal reorganization and cell migration. The mTORC1 complex is also a major regulator of autophagy, yet mTORC2 also contributes to the control of autophagy through AKT pathway regulation.

mTORC1 is a central regulator of autophagy. Under normal conditions, mTORC1 keeps autophagy under control through direct inactivation of the ULK1/2 protein complex that is composed of ULK1/2, ATG13, ATG101, and FIP200 proteins (35). Stress triggers inactivation of the mTORC1 complex. ULK1/2 then can autophosphorylate and phosphorylate ATG13 and FIP200 proteins, and turn on the autophagosome initiation and nucleation machinery (37).

Vesicle Nucleation

The next step in the canonical autophagy machinery is vesicle nucleation. It is initiated by the Class III phosphatidylinositol 3-kinase (PI3K) complex, consisting of the PI3K protein VPS34 and VPS30, ATG14/Barkor, VPS15, and ATG6/*BECN1* (Beclin 1) proteins. Lipid kinase activity of the PI3K complex is responsible from the accumulation of phosphatidylinositol 3-phosphate (PI3P) molecules on membranes, including the outer leaflet of the endoplasmic reticulum (ER). PI3P molecules serve as landing pads for autophagy-related proteins such as WIPI1–4 and DFCP1, marking sites of autophagosome formation and leading to omegasome/cradle development. Other regulators of the complex include BCL-2 family proteins, AMBRA1, and RUBICON (38).

Elongation

Two ubiquitylation-like conjugation systems, namely the ATG12–ATG5–ATG16 and ATG8 (MAP1LC3, or briefly LC3 in mammals) systems, regulate autophagic membrane elongation and completion (35). The first system involves conjugation of the ATG12 protein to ATG5 through action of the E1-like enzyme ATG7 and the E2-like enzyme ATG10. The ATG12–ATG5 conjugate forms a larger multimeric complex of around 800 kDa in mammals with the addition of the ATG16L1 protein. The second system involves conjugation of the LC3/ATG8 protein to a lipid molecule, generally to a phosphatidylethanolamine (PE). LC3 should be cleaved at its carboxy terminus by ATG4 cysteine proteases in order to generate the cytosolic free LC3-I form that is capable of lipid conjugation. ATG7 (E1-like) and ATG3 (E2-like) proteins, as well as the ATG12–ATG5–ATG16L1 complex (E3-like activity) are required for the conjugation of PE to free LC3-I proteins, giving rise to autophagic membrane-bound LC3-II form. By this way, LC3 proteins ensure elongation and expansion of autophagic membranes and their closure.

ATG9-Dependent Vesicle Retrieval

ATG9 (mammalian homolog: ATG9L1) is a multi-spanning transmembrane protein that localizes not only to PAS but also to endosomes and to the trans-Golgi network (39).

Cycling between these compartments, ATG9 is necessary for lipid delivery to autophagosomes and recycling of some proteins. ATG9 trafficking is regulated by RAB proteins (e.g., RAB1 and RAB11), TRAPP protein complexes, ATG2 and ATG18 (mammalian WIPI1–4) proteins (40, 41).

Lysosomal Fusion

At the final stage, outer membrane of mature autophagosomes fuses with lysosomal membranes to form autolysosomes (42). Autophagosome–lysosome fusion machinery involves SNARE complexes (e.g., VAMP8, STX17), integral lysosomal proteins (e.g., LAMP2), and RAB proteins (e.g., RAB5 and RAB7). Moreover, dyneins are necessary for the transport of autophagosomes along microtubules, allowing them to meet late endosomes and lysosomes. BIF1 and UVRAG proteins that play a role in the regulation of membrane curvature formation and endosomal trafficking contribute to the formation and maturation of autophagosomes through their interaction with the *BECN1*/Beclin 1 protein (43, 44). Following fusion, cargos that are carried by autophagosomes are digested by lysosomal acid hydrolases, including cathepsins, and they are broken down to their building blocks (e.g., proteins into amino acids). Recycling of digested molecules is achieved following their transport from lysosome lumen into the cytosol.

Autophagy Receptors

Historically, autophagy described as a non-selective important cellular homeostasis phenomenon (45). However, identification of different autophagy receptors that are able to recognize different cargos pointed out to the selectivity of the autophagy (46, 47). A number of autophagy receptors have been discovered including SQSTM1/p62 (48), NBR1 (49), NDP52 (also known as a CALCOCO2) (50), OPTN (51), and NIX (also known as BNIP3L) (52). These receptors share motifs that allow bridging between LC3 on the autophagosomes [LC3-interacting region (LIR)] and generally ubiquitylated targets [ubiquitin-binding domain (UBD)]. Since they are also delivered to autolysosomes together with the cargo, autophagy receptor degradation is commonly used as a marker of autophagic degradation activity.

INTERPLAY BETWEEN miRNAs AND AUTOPHAGY

Regulation of Autophagy by miRNAs

Studies in recent years introduced miRNAs as new players in the regulation of autophagy. Indeed, miRNAs were shown to change levels of several key proteins that are playing a role at various steps of the autophagy pathway, from upstream signaling pathways to later stages of autolysosomal degradation.

Regulation of Induction by miRNAs

As major upstream regulators of the autophagy pathway, mTOR-containing protein complexes and other proteins in the pathway were shown to be direct or indirect targets of a number of miRNAs. For example, five different components of the mTOR pathway, namely p70S6K, eukaryotic translation initiation factor 4E (eIF4E), Mknk1, Mknk2, and Mapkap1, were identified as direct targets of miR-7.

In hepatocellular carcinoma (HCC) cells, miR-7 was also introduced as a key regulator of the PI3K/Akt pathway, and shown to target mTOR, p70S6K, and PIK3CD (53). miR-199a and miR-101 were other miRNAs that could directly target mTOR in different cell types (54–57).

ULK1/2 complex components were also direct targets of miRNAs. In squamous cell carcinoma cells, cisplatin-induced miR-885-3p directly targeted ULK2 and contributed to the regulation of autophagy (58).

miR-26b targeted ULK2 as well, inhibiting autophagy in prostate cancer cells (59). Direct interactions between *MIR7* cluster members miR-20A and miR-106b and ULK1 could lead to the inhibition of leucine deprivation-induced autophagy in C2C12 myoblast cells, while blockage of endogenous miR-20a and miR-106b could restore normal autophagic activity (60). In another study, miR-25 was introduced as a novel regulator of autophagy and cell death through its direct effects on ULK1 expression (61). miR-17-5p, an miRNA that was upregulated upon BCG infection of macrophages, also regulated ULK1. By this way, miR-17-5p inhibited host cell autophagy that could normally eliminate intracellular BCG (62). In another study, Chen and coworkers proposed that ULK1 could inhibit p70S6K in starvation-induced autophagy of neuroblastoma SH-SY5Y cells and further identified that miR-4487 and miR-595 were novel ULK1-targeting miRNAs (63).

Regulation of Vesicle Nucleation by miRNAs

miR-30a was among the first miRNAs to be implicated in autophagy regulation. Zhu et al. showed that miR-30a inhibited rapamycin-induced autophagy in MCF-7 cells by directly targeting *BECN1*/Beclin 1 (64). Autophagy regulation by miR-30a was confirmed in subsequent studies. miR-30a sensitized HeLa cells to chemotherapy, through attenuation of cisplatin-induced autophagy in a *BECN1*/Beclin 1-dependent manner. Moreover, combined treatment with imatinib and miR-30a increased drug sensitivity in chronic myeloid leukemia cells through regulation of ATG5 and *BECN1*/Beclin 1 (65). In line with these data, a recent study showed that miR-30a levels were significantly reduced in chemoresistant osteosarcoma cells (66).

In a functional unbiased miRNA screen, we have found that *BECN1*/Beclin 1 could be targeted by the members of the *MIR376* family. miR-376a and miR-376b regulated starvation- and rapamycin-induced autophagy in breast and liver cancer cells by directly targeting 3′-UTR sequences of *BECN1*/Beclin 1 and ATG4C (67, 68). Moreover, these studies led us to propose "a gas and break model" of autophagy regulation under stress conditions: According to this model that was supported by our experimental data, autophagy activating stress signals trigger sequential upregulation of autophagy inhibitory miRNAs, and miRNA-mediated limitation of the autophagic activity prevents hyperactivation of autophagic degradation and ensures survival during prolonged stress conditions (68, 69).

In addition to *MIR30A* and *MIR376* family members, *BECN1*/Beclin 1 was identified as a target of miR-519a as well. In squamous cell carcinoma cells, miR-519a was shown to block autophagy that was activated by cisplatin (58). On the other hand, irradiation-induced autophagy in breast cancer cells was controlled by miR-199-5p that downregulated both *BECN1*/Beclin 1 and DRAM1 (70). In another study, high-fat diet upregulated miR-384-5p in a mouse model of atherosclerosis, and this miRNA impaired macrophage autophagy through direct targeting of *BECN1*/Beclin 1. In this context, autophagy deficiency in macrophages further promoted development of atherosclerosis (71). In colon cancer cells, oxaliplatin-induced autophagy was inhibited by miR-409-3p (72). Here, miR-409-3p-targeted *BECN1*/Beclin 1 and sensitized tumor cells to chemotherapy. Another miRNA that suppressed *BECN1*/Beclin 1 was miR-216a. Irradiation-mediated autophagy was blocked, and apoptosis was activated in radioresistant pancreatic cancer cells through action of this miRNA (73).

Other regulators of the *BECN1*/Beclin 1-VPS34 complex were also modulated by miRNAs. AMBRA1 was identified as an miR-23a target in dermal human fibroblasts which were exposed to either UVB or PUVA irradiation, and miR-23A-specific antagomirs increased autophagy (74).

Regulation of Elongation by miRNAs

Components of autophagy-related ubiquitination-like conjugation were also shown to be controlled by miRNAs. Independent studies showed that miR-181a, miR-30a, miR-374a, and miR-224-3p could directly target ATG5, miR-30d, miR-630, and miR-200b suppressed ATG12 while miR-20a and miR-885-3p targeted Atg16L1, and miR-519A could affect levels of both ATG16 and ATG10 (58, 65, 75–80).

A number of studies in the literature provided evidence that ATG7 levels were regulated by several different miRNAs. Suppression of autophagy through targeting of ATG7 by miR-375 was shown to reduce viability of HCC cells during hypoxia (81). ATG7 was also a target of miR-20a that also affected ATG16L1 levels (82). Another miRNA that was shown to target ATG7 was miR-17. The miRNA could modulate autophagy by negatively regulating ATG7 expression in human glioblastoma cells (83). Moreover, miR-137, which takes part in neuronal maturation and neurogenesis, suppressed starvation-induced autophagy by targeting ATG7 in glioblastoma cells (84). Another study revealed that under hypoxia stress, miR-96 played a dual role in autophagy regulation in prostate cancer cells. miR-96 could fine tune autophagy by targeting ATG7 and mTOR (85). In another study, Wang et al. showed that miR-188-3p could specifically participate in the regulation of ATG7 expression and impair autophagy in the heart (86). Ectopic expression of miR-199a-5p decreased ATG7 protein levels and suppressed autophagy in HCC cells (87).

Both LC3 and LC3 processor ATG4 family members were regulated by miRNAs. Another study showed an indirect correlation between miR-204 and LC3 levels. Upregulation of miR-204 levels upon myocardial ischemia-reperfusion caused an increase in LC3 protein levels in adult rat models (88). In addition to *BECN1*/Beclin 1, miR-376 family members miR-376a and miR-376b could negatively regulate ATG4C in breast and liver cancer cells (67, 68). In a luciferase-based functional miRNA screen, another member of ATG4 family, ATG4D, was identified as a target of miR-101. The same miRNA was introduced as an inhibitor of basal as well as rapamycin- and etoposide-induced autophagy (89). *SQSTM1/p62* encoding for a selective autophagy receptor was reported to be directly targeted by the *MIR17/20/93/106* family of miRNAs (90).

Regulation of ATG9-Dependent Retrieval by miRNAs

miR-34a was shown to regulate ATG9A levels during angiotensin II-induced myocardial hypertrophy (5) as well as during neural stem cell differentiation (91). Another protein in the same pathway, the ATG2B was identified as a direct target of miR-130a, an miRNA that inhibited autophagy and promoted cell death in chronic lymphocytic leukemia cells (92). ATG2 was also among the targets of miR-30D, an miRNA that was shown to target multiple core proteins in the autophagy pathway (78).

Regulation of Autophagosome Maturation and Lysosome Fusion by miRNAs

A number of miRNAs were reported to control the autophagosome–lysosome fusion machinery as well. RAB proteins regulating endocytic pathways namely RAB1B, RAB22A, RAB14 were shown to be targeted by miR-502, miR-373, and miR-451, respectively (93, 94). Furthermore, miR-205 could downregulate lysosomal proteins RAB27A and LAMP3 in a prostate cancer cell model (95). In a study involving ischemic injury and spontaneous recovery, LAMP2 was identified as a direct target of miR-207 (96). miR-487-5p could target LAMP2 as well (97). UVRAG that also plays a role in endosomal trafficking and autophagosome maturation was shown to be a target of miR-374, miR-630, miR-125, and miR-351 that have and inhibitory effect on autophagy (76, 98).

Control of miRNAs and miRNA Generation Pathways by Autophagy

A complex interplay between the autophagy machinery and miRNA biogenesis and maturation systems exists. miRNA-processing enzymes, DICER1, and the RISC component AGO2 were described as direct targets of autophagolysosomal degradation. In fact, DICER1 and AGO2 were found to associate with the autophagy receptor NDP52 in a GEMIN3/4-dependent manner, and receptor–target complexes were degraded upon autophagy activation (99). On the other hand, downregulation of DICER1 expression attenuated autophagy induction during acute promyelocytic leukemia differentiation (100). Similarly, targeting of ATG2B and DICER1 by miR-130A inhibited autophagy in chronic lymphocytic leukemia cells, and knockdown of DICER1 alone was sufficient to block autophagy in this context (92). In line with these findings, AGO2 accumulation was observed in ATG5$^{-/-}$ and ATG16$^{-/-}$ mouse embryonic fibroblasts and ATG7$^{-/-}$ intestinal organoids (101). Although it was suggested that autophagy degraded only small RNA-free DICER1 and AGO2 proteins, degradation of a number of miRNAs including miR-224 was reported to be dependent on the autophagic activity (102). All these data indicate that miRNA-autophagy connections work both ways. Therefore, regulation of autophagy by miRNAs might have cellular consequences beyond mere autophagy suppression or activation, potentially having repercussions on miRNA control pathways and global miRNA landscape in cells.

AUTOPHAGY AND CANCER

Autophagy As a Tumor Suppressor

Studies in the literature draw a complex picture about the involvement of autophagy in cancer formation and progression. The role of autophagy seems to be context- and tumor type-dependent, i.e., early versus late stage disease, fast versus slow growing tumors show different degrees of autophagy dependence.

Studies focusing on early stages of cancer formation indicate a tumor-suppressor role of autophagy during malignant transformation. For example, haploinsufficiency of *Becn*1/*Beclin* 1 in genetically modified mice resulted in tumor formation in various systems, including lung adenocarcinomas, HCCs, and heamatological malignancies (103–107). Similarly, *Atg*5 and *Atg*7 deletions in the liver resulted in the formation of liver adenomas (108). *Atg*4C-deficient mice were prone to develop fibrosarcomas that were induced by chemical carcinogens (109). In line with these results, *UVRAG* expression suppressed and *Bif*1 deletion enhanced tumor formation in mice (43, 44). Analysis of a series of human tumors confirmed these experimental results. For example, monoallelic deletions and lower *BECN1* protein levels were found in human prostate, breast, and ovarian cancers tissues that were analyzed (103, 104). Similarly, *ATG*5 expression was lost in human gastric, colorectal, and HCC specimen, and monoallelic mutations of UVRAG were reported to be frequent in human colon cancers (110–112). Mechanisms of cancer suppression by autophagy were studied as well. Autophagy is responsible for the degradation of abnormally folded and/or mutant proteins and damaged organelles (e.g., mitochondria) that in fact constitute a major source of reactive oxygen species (ROS). Consequently, elimination of these sources of ROS by autophagy was shown to alleviate DNA damage accumulation and prevent genomic instability (11). Targeted elimination of some cancer-related proteins by autophagy was also reported. Autophagy-dependent selective degradation of oncogenic SQSTM1 (P62), PML-RARA, mutant p53, and BCR-ABL1 proteins may be cited as prominent examples (113–116). Autophagic degradation of hypoxia-inducible and proangiogenic HIF2α protein in a constitutive manner was also reported to suppresses kidney tumorigenesis (117).

Moreover, while autophagy mainly acts as a prosurvival mechanism and a stress response, autophagy activation under certain conditions was connected to cell death (118–120). Hence at least in some contexts, autophagic cell death might also contribute to tumor suppressive functions. In line with this view, blockage of autophagy in some contexts prevented death of cancer cells [e.g., Ref. (121, 122)]. Furthermore, several tumor suppressor and death-related proteins, including DAPK, DRP1, ZIPk, and a p19ARF form (smARF) triggered a non-apoptotic and autophagy-dependent cell death in cancer cells (123–126). Oncogene-induced senescence that eventually leads to cell death was also shown to depend on autophagy (127). On the other hand, same hostile conditions (e.g., starvation and low nutrient supplies) that trigger autophagy may also activate phenomena such as entosis (cell-in-cell) where cancer cells cannibalize each other [and the references therein; (128, 129)].

The role of autophagy in immune responses and inflammation was also suggested to be important for its cancer-related effects.

For instance, anticancer immunosurveillance that involves recognition and elimination of nascent cancerous cells by the immune system may be dually regulated by autophagy in different cell types (e.g., development and maturation of immune system components versus hijacking of the immune system by tumor autophagy) (130, 131). Additionally, autophagy was shown to limit inflammation that, in especially in a chronic form, is a major trigger form of some types of cancer (e.g., HCC). Elimination of inflammasomes and limitation of pro-inflammatory interleukins (132, 133) and NF-kB signaling (134) as well as inhibition of pro-inflammatory signals controlled by pattern recognition receptors (135, 136) and prevention metabolic stress and inflammatory cell infiltration to tissues (137) all depended on intact autophagy function.

Autophagy As a Tumor Promoter

In established and especially fast-growing tumors, survival-related role of autophagy predominates. Cancer cells face with unfavorable conditions that challenge their endurance to various types of stress. Abnormal and insufficient tumor vascularization leads to hypoxia, changes in local pH, scarce nutrient, growth factor, and hormone supply, while energy and oxygen demands increase due to fast proliferation. Therefore, the tumor environment imposes high levels of metabolic stress upon malignant cells. Autophagy supports tumor cell survival and growth under these harsh conditions. For example, in oncogenic RAS- or RAF-driven fast-growing tumors, autophagy ensured tumor cell proliferation and survival, mitochondrial quality control and maintenance of energy levels, building block (e.g., aminoacid) abundance. These autophagy-dependent conditions were crucial factors supporting metabolic activities of cancer cells (9, 138). Elevation of basal autophagy levels was especially indispensable for the survival of tumor cells that were found in the less vascularized regions of solid tumors (137).

Cells from invasive and metastatic tumors are subject to extreme stress that originates from detachment from neighboring cells and from the basal lamina in their tissue of origin, evasion from the primary sites, shear forces and immune system attack in the blood stream, invasion and spread in a "foreign" secondary site (139). Under these conditions, autophagy was shown to provide resistance to metabolic stress conditions and anoikis (detachment-induced cell death) supporting cancer cell survival (9, 140–142). Autophagic capacity of tumor cells was reported as a determining factor during epithelial–mesenchymal transition (EMT), metastasis, and dormancy of tumor cells in some contexts (143, 144). Yet in HCC cells, EMT and migration properties were not affected, but anoikis resistance and distant metastasis capacity were reduced when autophagy was suppressed (145). In another study, knockdown of ATG5 in melanoma cells decreased cells' capacity to survive metabolic stress and to colonize lungs in mice following intravenous injection (146). Similarly, depletion of ATG12 decreased the invasive capacity of glioma cells (147).

Furthermore, motility, invasion, and metastatic capacity of oncogenic RAS-transformed tumor cells depended on their autophagy competence and autophagy-dependent production of secreted factors (141). Establishment of dormancy state and survival of dormant cancer cells depended on their autophagy competence. For example, induction of autophagy by ARH-I/DIRAS3 was essential for dormancy of ovarian cancer cell micro metastases in xenograft models (148).

Autophagy plays a critical role in endothelial cell biology as well as tumor vascularization. Although endothelium-specific deletion of the key autophagy gene Atg7 in mice did not result in any prominent vascular abnormality or vascular density change, there were abnormalities of endothelial cell function (e.g., defect in the maturation and secretion of von Willebrand factor) (149). In a cancer context, selective degradation of angiogenesis regulators such as gastrin-releasing peptide or HIF2α by autophagy affected tumor vasculature and limited tumor growth (117, 150). In line with these observations, *BECN1*/Beclin 1 heterozygous mice had higher levels of circulating erythropoietin and HIF2α, increased angiogenesis under hypoxia, and enhanced tumor growth compared to wild-type mice (151). ATG5 knockdown in B16-F10 melanoma cells increased tumor vessel tortuosity; on the other hand, endothelial cell-specific deletion of ATG5 led to the formation of smaller and less mature tumor vasculature with endothelial cell lining and perfusion defects (146). Therefore, autophagic activity is important for angiogenesis under physiological and pathological conditions.

Cancer metabolism found to be distinct from that of normal healthy cells. High metabolic demands drive cancer cells to evolve different strategies such as usage of glycolysis and other alternative metabolic pathways (e.g., salvage pathways) as sources of energy. These metabolic conditions in combination with the hypoxic environment that accompanies rapid tumor growth and poor vascularization usually result in acidosis. Acidic tumor microenvironment has also been found to alter autophagy cancer cells as an adaptation mechanism to rough environmental conditions (152–154).

Autophagy and Cancer Treatment

An important response of cancer cells to treatment with anticancer agents and radiation is autophagy activation (155). In most cases, autophagy confers resistance to anticancer therapy, yet in some tumor types, activation of autophagy was reported to have lethal effects on cancer cells. In any case, strategies aiming at modulation of autophagy bear the potential of improving responses to classical anticancer agents. Choice of the best strategy seems to depend on tumor type as well as tumor stage and treatment type. Additionally, autophagy manipulation renders otherwise resistant cancer types sensitive to therapeutic agents, and combination of autophagy drugs with conventional treatments might overcome drug resistance (156).

Sensitization to chemotherapy is one of the most studied topics in the autophagy field. In the scientific literature, beneficial effects of the combination of autophagy modulators with chemotherapy or radiotherapy were extensively studied. In many cancer types, inclusion of PI3K inhibitors (e.g., 3-MA or LY294002) in experimental treatments enhanced the efficacy of various chemotherapeutic agents and radiation through their autophagy blocking effects. For example, treatment with 3-MA sensitized esophageal squamous carcinoma cells to radiation therapy (157). Similarly, administration of 3-MA enhanced the efficacy of 5-Fluorouracil and cisplatin and promoted apoptosis in colon and lung

cancer cells (158, 159). On the other hand, lysosomotropic agents [e.g., Chloroquine (CQ) or hydroychloroquine (HQ)] that neutralize the pH of lysosomes and that prevent autolysosome formation were shown to exert anticancer effects and/or enhance the efficacy of antineoplastic treatments in numerous publications [e.g., Ref. (160–162)]. For instance, in non-small-cell lung cancer bevacizumab plus CQ combination was found to increase the efficacy of cancer treatment (161).

Concomitantly, CQ and HCQ potentiated cytotoxic effects of p53 and alkylating agents in a mouse model of lymphoma (163). siRNA-based depletion of autophagy modulators was also able to sensitize carcinoma cells from different origins to chemotherapy and radiation treatment (164).

AUTOPHAGY, miRNAs, AND CANCER

Among autophagy-related miRNAs, many of them were involved in different stages of cancer formation and progression. These miRNAs were shown to influence cancer growth, cancer cell metabolism, hypoxia responses and neovascularization, cancer cell migration, and metastasis, and even response to drugs and radiotherapy. Moreover, some autophagy-related miRNAs were tested as anticancer agents or cancer biomarkers. In many studies, it was suggested that the effects of miRNAs on autophagy genes and proteins were critical for cancer-related outcomes, but in others data were correlative. Conversely in some other cases, targeting of miRNAs or miRNA-related components by autophagic degradation systems were decisive in the control of cancer progression. In this section, we will summarize existing literature that mainly implicates autophagy-related roles of these miRNAs in cancer biology and clinical outcomes (see **Table 1** for a complete list of miRNAs).

Cancer Cell Survival and Growth

As discussed above, autophagy competence is important for the growth and survival of cancer cells. A number of miRNAs were shown to regulate autophagy and control tumor cell growth and proliferation.

Expression of a number of miRNAs with autophagy-related targets resulted in growth inhibition in different cancer cell types: For example, overexpression of miR-143 inhibited proliferation of H1299 non-small lung cancer cells, and ATG2b was identified as an autophagy-related direct target of the miRNA (209). Overexpression of miR-9-3p in medullary thyroid cancer cell lines (TT and MZ-CRC-1 cells) decreased cellular levels of several autophagy-related proteins, including ATG5, PIK3C3, mTOR, and LAMP1, and inhibited autophagy, leading to G2 arrest and cell death (167). In another study, miR-502 inhibited autophagy through RAB1B and p53 targeting, and its overexpression suppressed colon cancer cell cycle progression and cell growth *in vitro* and in a tumor xenograft model (94). Von Hippel–Lindau (VHL) tumor suppressor is lost in the majority of renal cancers. A VHL-regulated miRNA, miR-204, blocked autophagy through miRNA-mediated downregulation of LC3B and suppressed growth of renal clear cell carcinoma (RCC) both in *in vitro* tests and *in vivo* in mice (220). VHL also repressed another protein involved in RCC growth, namely transient receptor potential melastatin 3 (TRPM3) through direct targeting by miR-204 (219). In fact, TRPM3 is a non-selective channel that is permeable to calcium and other cations. miR-204 directly targeted another TRPM3 regulator, CAV1, as well. On the other hand, overexpression of TRPM3 in RCC cells caused a Ca^{2+} influx that activated the calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) and AMPK, which in turn activated ULK1 and triggered autophagy. TRPM3-mediated cation fluxes inhibited miR-214, an miRNA that directly targets LC3A and LC3B and inhibits autophagy. Therefore, an interplay between VHL and TRPM3 involving two miRNAs, namely miR-204 and miR-214, controls autophagy activation and renal cell carcinoma growth (219). Another miRNA-related autophagy control mechanism involves Yin Yang 1 (YY1), a transcription factor and an epigenetic regulator that is upregulated in various cancer types. miR-372, which was subject to epigenetic regulation by YY1, was found to target the autophagy receptor SQSTM1/p62 in a direct manner (229).

Under nutrient starvation condition, YY1 suppressed miR-372 expression, leading to SQSTM1/p62 expression and subsequent autophagy in breast cancer cell lines. Overexpression of miR-372 blocked autophagy activation and inhibited breast cancer xenograft growth *in vivo,* underlining the importance of YY1-mediated miR-372 suppression and autophagy for cancer cell proliferation (229). Other intricate connections also exist between autophagy-related miRNAs and cancer. Long non-coding RNA (lncRNA) PTENP1 is a pseudogene of the tumor-suppressor PTEN gene. Both PTENP1 and PTEN are downregulated in HCC cells. Interestingly, PTENP1 serves as a decoy for PTEN-targeting miRNAs, including miR-17, miR-19b, and miR-20a. These miRNAs also targeted PHLPP (a negative AKT regulator) and autophagy genes ULK1, ATG7, and p62. Overexpression of PTENP1 in HCC cells elevated the levels of PTENP1 and PTEN and suppressed growth-stimulating and autophagy-inhibiting PI3K/AKT pathway, as well as it suppressed cell proliferation and invasion and migration. Under these conditions, autophagy and apoptosis were induced. Mice experiments supported these findings: Vector-mediated introduction of PTENP1 into mice-mitigated HCC growth, attenuated cell proliferation, and triggered autophagy and apoptosis (171). Autophagy-mediated degradation of oncogenic or tumor suppressive molecules may also be manipulated by autophagy controlling miRNAs. One such example involves miR-125b1, an miRNA that is highly expressed in acute promyelocytic leukemia. miR-125b1 blocked proteolysis of the PML-RARA oncogenic protein by the autolysosomal system and contributed to the inhibition of leukemia differentiation (203). In this study, DNA damage-regulated autophagy modulator 2 (DRAM2), a critical regulator of autophagy, was described as a novel autophagy-related target of miR-125b1 (203). In another report, authors provided evidence that the oncomir miR-224 that promoted hepatoma cell migration and tumor formation was selectively recruited to autophagosomes, and the miRNA itself was degraded by autophagy (102). miR-224 affected tumor formation through silencing of Smad4. Importantly, impaired autophagy correlated with miR-224 accumulation and poor overall survival rate in HCC patients (102). Another recent study introduced miR-18A and an RNA-binding protein, hnRNP A1,

TABLE 1 | Autophagy-related microRNAs (miRNAs) in cancer.

miRNAs	miRNA status in cancer	Effect on autophagy	Autophagy-related targets	Type of target interaction	Tested cell line (tissue origin)	Reference
miR-let7f1	N.D.	Inhibition	HMGB1	Direct	UW228, D425 (medullablastoma)	(165)
miR-7	N.D.	Activation	EGFR	Indirect	H1299, A549 (lung cancer) T.Tn (esophageous)	(166)
miR-7	N.D.	N.D.	PIK3CD, mammalian TOR (mTOR) p70S6K	Direct	QGY-7703 (hepatacellular carcinoma)	(53)
miR-9-3p	N.D.	Inhibition	ATG5	Direct	TT and MZ-CRC-1 (medullary thyroid carcinoma)	(167)
miR-10a	Upregulated	Inhibition	Bim, TFAP2C, p16, and p21	Direct	U251, LN-308, and U373 (glioblastoma)	(168)
miR-15a/16	N.D.	Activation	RICTOR	Direct	HeLa (cervical cancer)	(169)
miR-16	Downregulated	Inhibition	BCL-2	Direct	A549-T24 (lung cancer)	(170)
miR-17						
miR-17, miR-19b miR-20a	N.D.	Activation	PTEN, PTENP1	Indirect	Mahlavu (hepatacellular carcinoma)	(171)
miR-17	Upregulated	Inhibition	ATG7	Direct	T98G and U373-MG (glioblastoma)	(83)
miR-17-5p	Downregulated	Inhibition	BECN1	Direct	A549-T24 (lung cancer)	(172)
miR-18a	N.D.	N.D.	hnRNPA1	Indirect	SW620 and HCT116 (colorectal cancer)	(173)
miR-20a	Upregulated	N.D.	ATG7	Direct	SiHa (cervical cancer)	(82)
miR-20a	Downregulated	Inhibition	FIP200	Direct	MCF-7, MDA-MB-231 (breast cancer)	(174)
miR-20a						
miR-21	Upregulated	Inhibition	N.D.	Indirect	U373, U87 (glioblastoma)	(175)
miR-21	N.D.	Inhibition	PTEN	Direct	SiHa, HeLa (cervical cancer)	(176)
miR-21	Upregulated	Inhibition	PTEN	Indirect	SiHa, HeLa (cervical cancer)	(177)
miR-21	Upregulated	Inhibition	PTEN	Indirect	Huh7, HepG2 (liver cancer)	(178)
miR-21	N.D.	Inhibition	PTEN	Indirect	MCF-7 (breast cancer)	(179)
miR-21	Upregulated	N.D.	PDCD4	Indirect	Tca8113 (tongue squamous cell carcinoma)	(180)
miR-22	N.D.	Inhibition	HMGB1	Direct	MG-63 (osteosarcoma)	(181)
miR-22	N.D.	Inhibition	BTG1	Direct	SW620, RKO (colorectal cancer)	(182)
miR-23a	Upregulated	N.D.	TOP2B	Indirect	Tca8113 (tongue squamous cell carcinoma)	(183)
miR-23a	Downregulated	Inhibition	ATG12	Direct	BxPC3 (pancreas)	(184)
miR-23B-3p	Downregulated	Inhibition	HMGB2, ATG12	Direct	SGC7901/VCR (gastric cancer)	(185)
miR-24-3p	Downregulated	Inhibition	ATG4A	Direct	H446/EP (lung cancer)	(80)
miR-25	N.D.	Inhibition	ULK1	Direct	MCF-7 (breast cancer)	(61)
miR-26a	Downregulated	Inhibition	ULK2	Direct	PC3, C4-2 (prostate cancer)	(59)
miR-29a	N.D.	N.D.	HDAC4	Direct	KMS11, SKMM1, and NCI-H929 (myeloma)	(186)
miR-29b	Downregulated	Inhibition	PSME4	Direct	AMCL1, AMCL2 (myeloma)	(187)
miR-30a	N.D.	Inhibition	BECN1	Direct	MDA-MB-468, MCF-7 (breast cancer) H1299 (lung cancer) T98G (glioblastoma)	(64)
miR-30a	N.D.	Inhibition	BECN1, ATG5	Direct	K562 (CML)	(65)
miR-30a	N.D.	Inhibition	BECN1	Indirect	HeLa (cervical cancer)	(188)
miR-30a	Downregulated	Inhibition	BECN1	Indirect	786-0, A489 (renal carcinoma)	(189)
miR-30a	Downregulated	Inhibition	BECN1	Direct	MG-63 (osteosarcoma)	(66)
miR-30a	Downregulated	Inhibition	BECN1	Indirect	SH-SY5Y (neuroblastoma)	(190)
miR-30d	N.D.	Inhibition	ATG2B, ATG12, ATG5, BNIP3L	Direct	A2780, OVCAR10 and 2008 (ovarian cancer), T47D and MCF-7 (breast cancer)	(78)
miR-30d	N.D.	Inhibition	BECN1	Direct	SW1736, 8305 C (anaplastic tyroid carcinoma)	(191)
miR-32	N.D.	Inhibition	DAB2IP	Direct	PC3, DU145 (prostate)	(192)
miR-34a	N.D.	Inhibition	HMGB1	Direct	Y79, Weri-RB1 (retinoblastoma)	(193)
miR-34-5p	Upregulated	N.D.	BECN1	Direct	A172, T98G (glioblastoma)	(194)
miR-5195-3p						
miR-93/106b	N.D.	Activation	p21	Indirect	SaOS-2 and MNNG/HOS (osteosarcoma)	(195)
miR-96	N.D.	Bi-phasic regulation	mTOR, ATG7	Direct	LNCaP, 22Rv1, and LAPC4 (prostate cancer)	(85)
miR-100	Downregulated	Activation	IGFR1, mTOR	Direct	HepG2, Huh7 (liver cancer)	(196)
miR-101	N.D.	Inhibition	STMN1, ATG4D, RAB5A	Direct	MCF-7 (breast cancer)	(89)
miR-101	N.D.	Inhibition	STMN1, ATG4D, RAB5A, mTOR	Direct	HepG2 (liver cancer)	(197)
miR-101	Downregulated	Inhibition	EZH2	Direct	HepG2 (liver cancer)	(198)
miR-101	Downregulated	Inhibited	STMN1	Direct	CNE-2, 5–8 F, and 6-10B (nasopharyngeal carcinoma)	(199)
miR-106	N.D.	Inhibition	ATG16L1	Direct	HCT116 (colorectal cancer)	(200)
miR-93						
miR-124	Downregulated	Activation	PTB1	Direct	DLD-1, WiDr (colorectal cancer)	(201)
miR-124	Downregulated	Inhibition	PIM1	Direct	DU145 and PC3 (prostate cancer)	(202)
miR-144						

(*Continued*)

TABLE 1 | **Continued**

miRNAs	miRNA status in cancer	Effect on autophagy	Autophagy-related targets	Type of target interaction	Tested cell line (tissue origin)	Reference
miR-125b1	Upregulated	Inhibition	DRAM2, ATG4D UVRAG	Direct	NB4 (acute promyelocytic leukemia)	(203)
miR-126	Downregulated	Activation	IRS1	Indirect	Met5a, H28, and IstMes2 (malignant mesothelmia)	(204)
miR-129	N.D.	Activation	NOTCH1	Direct	U87, U231 (glioblastoma)	(205)
miR-130a	Downregulated	Inhibition	ATG2B, DICER1	Direct	MEC-1 (leukemia)	(92)
miR-137	N.D.	Inhibition	ATG7	Indirect	U87 (glioblastoma)	(84)
miR-138	N.D.	Activation	BIM	Direct	LN-308, ZH-305 (glioblastoma)	(206)
miR-140-5p	Downregulated	Inhibition	SMAD2	Direct	HCT116, RKO, and SW480 (colorectal cancer)	(207)
miR-143	Downregulated	Inhibition	GABARAPL1	Direct	AGS and MKN28 (gastric cancer)	(208)
miR-143	N.D.	Inhibition	ATG2B	Direct	H1299 (lung cancer)	(209)
miR-144	Downregulated	Activation	TIGAR	Direct	A549, H460 (lung cancer)	(210)
miR-152	Downregulated	Inhibition	ATG14	Direct	A2780/CP70, SKOV3/DDP (ovarian cancer)	(211)
miR-155	N.D.	Activation	RHEB, RICTOR RPS6KB2	Direct	NSE (nasopharyngeal cancer) and HeLa (cervical cancer)	(212)
miR-155-3p	N.D.	Activation	CREBRF	Direct	U251 and T98G (glioblastoma)	(213)
miR-181a	N.D.	Inhibition	ATG5	Direct	MCF-7 (breast cancer) Huh7 (liver cancer) K562 (chronic myelogenous leukemia)	(75)
miR-181a	N.D.	Inhibition	ATG5	Indirect	SGC7901/CDDP (gastric cancer)	(214)
miR-183	Upregulated	Inhibition	UVRAG	Indirect	HCT116 and HT29 (colorectal cancer)	(215)
miR-193b	Upregulated	Activation	STMN1	Indirect	KYSE450 (esophageal cancer)	(216)
miR-199a	N.D.	N.D.	mTOR	Direct	Huh7, HepG2, SNU475 (liver cancer)	(55)
miR-199A-5p	Downregulated	Inhibition	ATG7	Direct	Huh7, HepG2 (liver cancer)	(87)
miR-199A-5p	N.D.	Inhibition	DRAM1, BECN1	Direct	MCF-7, MDA-MB-231 (breast cancer)	(70)
miR-200b	N.D.	Inhibition	ATG12	Direct	SPC-A1/DTX, H1299/DTX (lung cancer)	(217)
miR-200c	N.D.	Activation	UBQLN1	Direct	MDA-MB-231 (breast cancer)	(218)
miR-204	N.D.	Inhibition	Transient receptor potential melastatin 3 (TRPM3)	Direct	786-O, A498, and Caki-1 (kidney cancer)	(219)
miR-204	N.D.	Inhibition	LC3	Direct	786-O, A498, and Caki-1 (kidney cancer)	(220)
miR-205	Downregulated	Inhibition	RAB27A, LAMP3	Indirect	DU145, PC3 (prostate cancer)	(95)
miR-205	N.D.	Inhibition	TP53INP1	Direct	DU145, LNCaP (prostate cancer)	(221)
miR-212	Downregulated	Inhibition	SIRT1	Direct	LnCap, PC3 (prostate cancer)	(222)
miR-214	Downregulated	Inhibition	UCP2	Direct	MCF-7/LCC9 (breast cancer)	(223)
miR-214	N.D.	Inhibition	LC3A, LC3B	Direct	786-O, A498, and Caki-1 (kidney cancer)	(219)
miR-216a	Upregulated	Inhibition	BECN1	Direct	PANC-1 (pancreas cancer)	(73)
miR-216b	Downregulated	Inhibition	BECN1	Direct	A549, Calu-3 (lung cancer)	(224)
miR-218	Downregulated	Inhibition	HMGB1	Direct	RL95-2 (endometrial carcinoma)	(225)
miR-224	Upregulated	Inhibition	SMAD4	Direct	Hep3B, Hbx transgenic mice (liver cancer)	(102)
miR-224-3p	Downregulated	Inhibition	ATG5, FIP200	Direct	U251 and U87 (glioblastoma)	(77)
miR-224-3p	Upregulated	Inhibition	FIP200	Direct	HeLa, SiHa, C33A (cervical cancer)	(226)
miR-290-295	N.D.	Inhibition	ULK1, ATG7	Direct	B16F1, R2L (melanoma)	(227)
miR-340	Downregulated	Inhibition	ROCK1	Direct	U373, U87 (glioblastoma)	(228)
miR-372	N.D.	Inhibition	SQSTM1	Direct	MCF-7, MCF10A (breast cancer)	(229)
miR-373	Downregulated	N.D.	RAB22A	Direct	SKOV3 (ovarian cancer)	(230)
miR-374a	N.D.	Inhibition	UVRAG, ATG5	Direct	JHU-029 (squamous cell carcinoma)	(76)
miR-375	Downregulated	Inhibition	ATG7	Direct	Huh7, Hep3B (liver cancer)	(81)
miR-376a	N.D.	Inhibition	BECN1,ATG4C	Direct	MCF-7 (breast cancer) Huh7 (liver cancer)	(68)
miR-376b	N.D.	Inhibition	BECN1, ATG4C	Direct	MCF-7 (breast cancer) Huh7 (liver cancer)	(67)
miR-409-3p	Downregulated	Inhibition	BECN1	Direct	Lovo Oxa R (colorectal cancer)	(72)
miR-451	Downregulated	N.D.	RAB14	Direct	A549, SPC-A1, and NCI-H520 (lung cancer)	(93)
miR-451a	N.D.	Inhibition	N.D.	N.D	MCF-7, LCC2 (breast cancer)	(231)
miR-487b-5p	Upregulated	Inhibition	LAMP2	Direct	A549, H1299 (lung cancer)	(97)
miR-502	Downregulated	Inhibition	RAB1B	Direct	HCT116 (colorectal cancer)	(94)
miR-519a	N.D.	Inhibition	BECN1, ATG10 ATG16L1	Direct	JHU-029 (squamous cell carcinoma)	(76)
miR-630	N.D.	Inhibition	ATG12, UVRAG	Direct	JHU-029 (squamous cell carcinoma)	(76)
miR-634	N.D.	Inhibition	XIAP, BIRC5, APIP, OPA1, TFAM, LAMP2	Direct	KYSE850 (esophageal squamous cell carcinoma)	(232)
miR-638	Upregulated	Inhibition	TP53INP2	Direct	SK-Mel-28 and SK-Mel-147 (melanoma)	(233)
miR-885-3p	N.D.	Inhibition	ULK2,AKT1,BCL-2 ATG16L2	Direct	JHU-029 (squamous cell carcinoma)	(58)
miR-4487	N.D.	Inhibition	ULK1	Indirect	SH-SY5Y (neuroblastoma)	(63)
miR-595						

as a target of autophagic degradation. Tumor-suppressor miR-18a is an apoptosis inducer in colon cancer cells, and this effect depended on the presence of hnRNP A1. The ribonucleoprotein was responsible for the stabilization of cyclin D1 and CTGF [or insulin-like growth factor-binding protein 8 (IGFBP-8)] mRNAs, and spared cancer cells from apoptosis. In order to limit tumor growth and promote cell death, miR-18a directly bound to hnRNP A1 and made it available for degradation

by the autophagic machinery (173). Ge et al. reported that miR-100 overexpression resulted in death of HCC cells. Cell death depended on the activation of ATG7-dependent but *BECN1*-independent autophagy by the miRNA. For autophagy induction, miR-100 suppressed the expression of *mTOR and IGF-1R* by binding to their 3′-untranslated regions. Consistently, mice xenograft experiments revealed that miR-100 inhibited *in vivo* growth of HCC cells. Moreover, a correlation between miR-100 downregulation and upregulation of the autophagy receptor and target SQSTM1/p62 protein was observed in human HCC tissue samples compared to controls (196). Conversely, downregulation of miR-10a activated autophagy, apoptosis, and cell death in glioma cells (168). While expressed in low levels in the normal brain, the miRNA was found to be upregulated in glioma tissues and cells. miRNA upregulation correlated with poor prognosis. Inhibition of the miRNA led to cell cycle arrest, senescence, autophagy, apoptosis cell death, and reduced glioma growth in a mouse model *in vivo* (168). BCL-2L11/Bim, TFAP2C/AP-2γ, CDKN1A/p21, and CDKN2A/p16 were identified as relevant and direct targets of the miRNA in this context. Moreover, especially in glioma cells that were apoptosis-defective but still dying upon miRNA inhibition, strong autophagy activation was observed.

Since CDKN2A/p16 downregulation should lead to the suppression of the alternative reading frame products of the same gene, namely p14/p19ARF and mitochondrial smARF, authors suggested that these proteins might be instrumental in autophagic cell death activation following miRNA inhibition (234). Indeed, loss of the p16 and ARF-encoding CDKN2A gene was observed in around half of all gliomas, possibly contributing to autophagic cell death aversion during growth of the tumor (234).

Cancer Cell Metabolism

Several studies in the literature implicated autophagy-related miRNAs in the regulation of metabolism and metabolic stress responses of cancer cells.

For example, *MIR290–295* cluster members (miR-291-3p, miR-291-5p, miR-292-3p, miR-292-5p, miR-294, and miR-295) targeted ATG7 and ULK1 on their 3′-UTR sequences, and reduced their protein levels in melanoma cells (227). Glucose starvation-induced cell death of metastatic B16F1 melanoma cells depended on their autophagic activity, and autophagy inhibition by miRNAs conferred resistance to death. Therefore, resistance to metabolic stress-induced death by *MIR290–295* cluster was a result of autophagy inhibition by these miRNAs (227). Another miRNA that had an impact on cellular metabolism was miR-124. This miRNA was mainly downregulated in colorectal adenoma and cancer specimen. miR-124 targeted polypyrimidine tract-binding protein 1 (PTB1), a protein that controls splicing of pyruvate kinase muscles to isoform 1 or isoform 2 (*PKM1* and *PKM2*) (201). PKM1 is mostly expressed in normal cells and tissues, where it stimulates oxidative phosphorylation. On the other hand, PKM2 is largely expressed in proliferating cells, including cancer cells, and it promotes glycolysis even under oxygen-rich conditions, supporting cancer cell metabolism and growth. Through suppression of PTB1, miR-124 induced a switch between PKM isoforms, from isoform PKM2 to PKM1, and increased oxidative phosphorylation and reactive oxygen accumulation in cancer cells. Consequently, ectopic expression of the miRNA or knockdown of PTB1 induced autophagy and apoptosis in colon cancer cells in *in vitro* and mice (201). miR-126 was downregulated in malignant mesothelioma tissues, and its expression was shown suppress tumor growth, possibly due to its effects on cancer cell metabolism. miR-126 suppressed IRS1, decreased glucose uptake, and caused energy deprivation that in turn switched on AMPK, leading to the activation of ULK1 (204). Moreover, miR-126 affected levels of other metabolism-related proteins, such as pyruvate dehydrogenase kinase and acetyl-CoA-citrate. These signals and metabolic changes that were triggered by the miRNA led to autophagy activation and inhibition of cancer growth both in *in vitro* cell culture and *in vivo* tests (204). Expression of another metabolism-related miRNA, miR-144 was found lower in lung cancer cell lines A549 and H460. Overexpression of the miRNA in these tumor cells was sufficient to block their proliferation and to promote autophagy and apoptosis (210). The authors identified TIGAR, a p53-induced regulator of glycolysis and apoptosis, as a direct target of the miRNA. TIGAR was shown to be important for rewiring of tumor cell energy metabolism and reduction of oxidative burden in cancer cells. Indeed, knockdown of TIGAR phenocopied the effects of the miRNA on cell growth, autophagy, and apoptosis. These results suggest that downregulation of miR-144 might be the result of a positive selection for TIGAR expression in lung cancer cells (210).

Hypoxia Responses

Tumor cells face hypoxia as a result of abnormal vascularization and irregular blood supply. Under these circumstances, hypoxic tumor cells rely on autophagy for survival. A number of miRNAs were reported to control hypoxia-induced responses, including those that regulated autophagy in this context.

Upon hypoxia treatment, miR-124 and miR-144 were downregulated in DU145 and PC3 prostate cancer cell lines (202). Overexpression of these miRNAs reduced hypoxia-induced autophagy and enhanced radiation-induced cell death in prostate cancer cells (202). Authors claimed that suppression of the oncogene PIM1 was important for the observed effects. Another miRNA that was induced by hypoxia was miR-96. Expression of miR-96 in prostate cancer cells to moderate levels induced autophagy through direct suppression of mTOR. Yet, higher levels of the miRNA could also block ATG7 expression, therefore to explain these observations, authors proposed a miRNA level-dependent autophagy regulation model that prevented of autophagy hyperactivation during hypoxia. Indeed, in a series of prostate cancer tissues, miR-96 expression inversely correlated with mTOR and ATG7 (85). In Huh7 and Hep3B HCC cell lines, miR-375 expression was decreased following hypoxia treatment, and miR-375 levels were lower in HCC specimen compared to normal liver tissues (81). Interestingly, miR-375 suppressed pro-survival autophagy under hypoxia condition through targeting of ATG7 3′-UTR.

As stated above, autophagy is the main cellular clearance mechanism that eliminates damaged mitochondria in cells. As a consequence, overexpression of the miRNA blocked mitochondrial autophagy and sensitized HCC cells to hypoxia-induced

mitochondrial cell death. Likewise, hypoxia led to the downregulation of miR-224-3p in glioma tissues. In cellular systems, expression of miR-224-3p abolished hypoxia-induced autophagy, whereas knocking down endogenous miR-224-3p increased autophagic activity under normoxia (77). miR-224-3p was shown to block autophagy by directly suppressing ATG5 and FIP200. Furthermore, the study showed that upregulation of the miRNA potentiated hypoxia-related cell death *in vitro* and inhibited glioblastoma tumor growth *in vivo*. In support of this observation, miR-224-3p levels inversely correlated with ATG5 and FIP200 expression in human glioma tissues (77). Another outcome of hypoxia in glioma cells was the stimulation of IL6 production and cytokine-mediated autophagy activation (213). In line with this, the amount of IL6 correlated with HIF1A levels and tumor grade in glioma tissues. In glioma cellular models, IL6-STAT3 axis led to the upregulation of an miRNA, miR-155-3p, and stimulated autophagy through a rather indirect manner. miR-155-3p directly targeted and decreased the levels of CREB3 inhibitor protein, CREBRF. Downregulation of CREBRF resulted in a CREB3-dependent increase in ATG5 transcription and autophagy stimulation was the end result. The role of the signaling pathway involving miR-155-3p in glioma cell survival was confirmed *in vitro* in cells, as well as *in vivo* in a tumor xenograft model. Blocking of IL6, hence autophagy inhibition, by antibody drugs alone or in combination with temozolomide (a first-line drug for glioma treatment) decreased cancer cell survival and the tumor burden. In contrast with miR-155-3p, complementary strand of the mature miR-155, namely miR-155-5p, was reported to block autophagy through downregulation of mTOR pathway components RHEB, RICTOR, and RPS6KB2, conducting cells to cycle arrest (212). Therefore, control of stability of either miR-155-3p *or* -5p strands of the same miRNA duplex might determine the final autophagy-related outcome under hypoxia. Alternatively, the competition between the two strands might determine whether autophagy will be inhibited or activated under hypoxia stress.

Angiogenesis

Considering the importance of the autophagic activity for endothelial cell function and angiogenesis, one of the roles of hypoxia-induced autophagy in the cancer context is related to tumor neovascularization. Obviously, some of the autophagy-regulating miRNAs were shown to control the contribution of autophagy on the survival, growth, and spread of endothelial cells, having a direct impact on tumor vascularization.

For example, inhibition of an miRNA, miR-195, that is capable of targeting the autophagy protein GABARAPL1, stimulated autophagy in endothelial progenitor cells, promoted cell proliferation, migration, and angiogenesis under hypoxia (235). Addition of 3-MA was able to block all these cellular outcomes, pointing out to their autophagy dependence (235). miR-212, an miRNA that was downregulated in prostate cancer, inhibited autophagy through its direct effects on autophagy activator SIRT1 (222). Under these conditions, angiogenesis was suppressed and cancer cells were driven to senescence (222). On the other hand, inhibition of miR-130a correlated with autophagy induction through an RUNX3-*BECN1*/Beclin 1 axis and potentiated death of endothelial progenitor cells (236). High or fluctuating glucose levels had a similar effect on endothelial cells. Glucose level fluctuation led to an increase in miR-1273g-3p levels, which then, induced endothelial cell autophagy and blocked proliferation and migration of cells (237).

Above-mentioned studies give hints about the role of miRNA-autophagy connections in the regulation of endothelial cell homeostasis and angiogenesis *in vitro*. Further controlled *in vivo* studies are required to strengthen the link and establish their relevance to tumor vascularization.

Cancer Cell Migration and Metastasis

Connections that exist between autophagy pathways and cellular migration also affect cell motility, invasion, and metastatic spread of cancer cells. Some of the miRNAs that regulate autophagy also had an influence on cancer cell migration and metastasis. Unfortunately, in most of these studies, a direct role for autophagy on migration was not established, yet there are hints in the current literature about an autophagy connection.

In some studies, miRNAs that attenuated migration and metastasis also targeted autophagy. For example, in colorectal cancer tissues, miR-140-5p levels inversely correlated with tumor progression toward invasion and metastasis. miR-140-5p directly targeted Smad2 that is involved in cancer stem cell maintenance and EMT and the autophagy protein ATG12 (207). The end result was suppression of autophagy, blockage of colon cancer cell proliferation and invasion *in vitro*, and inhibition of tumor formation and metastasis *in vivo* (207). In osteosarcoma cells, miR-22 downregulated cisplatin and doxorubicin-induced autophagy, and HMGB1 was identified as an autophagy-related target of the miRNA (181). Under these conditions, miRNA overexpression inhibited cellular proliferation, colony formation, *in vitro* migration, and transwell invasion capacity of cancer cells (181). These studies suggest that inhibition of autophagic activity by miRNAs may contribute to their anti-metastatic effects.

On the other hand, in some other contexts, prevention of migration and metastasis correlated with autophagy activation. For instance, miR-638, an miRNA that is an overexpressed miRNA in metastatic melanomas, increased proliferation and colony formation capacity of melanoma cells (233). Additionally, miR-638 overexpressing cells performed better in *in vitro* migration and invasion tests and in *in vivo* metastasis experiments. Aggressive behavior of melanoma cells depended on the suppressive effects of miR-638 on its target gene TP53INP2, a TP53-inducible nuclear protein that serves as a scaffold for autophagosome formation (233, 238). Antagomir-mediated neutralization of the miRNA led to the upregulation of its target genes and triggered p53-dependent autophagy and apoptosis (233) Therefore, miR-638 protected melanoma cells from autophagy and apoptosis to promote invasion and metastasis. While investigating the factors regulating ovarian cancer cell migration, Ferraresi et al. discovered that several miRNAs that were deregulated in response to IL6 and resveratrol (a polyphenolic compound inducer of autophagy) treatments (239). Six miRNAs that were regulated in an opposite manner by IL6 and resveratrol, namely miR-1305, miR-1260a, miR-141-3p, miR-424-5p, miR-15a-5p, and miR-7-5p, had as a common target, ARH-I (DIRAS3).

The protein encoded by this gene is a Ras homolog GTPase and a tumor suppressor in ovarian cancer, and it was shown to inhibit cell migration and stimulate autophagy and dormancy in this cancer type through its interaction with Beclin 1 (240). In this setting, IL6 treatment prevented LC3-positive vacuole accumulation and promoted cellular motility, while resveratrol had the opposite effect on both autophagy and cell migration (239). In some contexts, autophagy was shown to be responsible for direct elimination of miRNAs that promoted migration (102). These results point out to an anti-metastatic role of autophagy under certain circumstances.

miRNAs As Cancer Biomarkers

Among the miRNAs that are involved in autophagy regulation, some of them were introduced as potential tumor biomarkers. For example, miR-221/222 was evaluated as a prognosis predictive biomarker in the plasma of patients with breast cancer that have been treated with a neoadjuvant chemotherapy (241). On the other hand, miR-205 and miR-342 levels were found to be significantly low in triple-negative breast cancer tissues (242). Again in triple-negative breast cancers, miR-155, miR-493, miR-30e, and miR-27a were tested as prognostic biomarkers, and upregulation of miR-155 and miR-493 was associated with a better patient outcome, while suppression of miR-30e and miR-27a correlated with a worse outcome (243). In ovarian cancers, a decrease in miR-152 levels was associated with cisplatin resistance (211) and miR-29b expression correlated with better prognosis (244). On the other hand, in prostate tumors, a decrease in miR-212 expression in tumor tissues and sera of patients indicated a diagnostic potential for this miRNA (222). There are several other studies implicating autophagy-related miRNAs in cancer diagnosis and in some cases reporting their prognosis prediction potential. See **Table 2** for some examples of autophagy-related miRNAs with biomarker potential.

Although the contribution of autophagy competence and activity was not studied in all biomarker studies, it is possible that autophagy-related effects of the miRNAs might be contributing to the tumor behavior and disease prognosis. Correlative analyses that combine molecular and cellular data on autophagy are required to establish and confirm the relationship between autophagic capacity of tumors and diagnostic/prognostic value of autophagy-related miRNAs.

TABLE 2 | Autophagy-related microRNAs (miRNAs) as biomarkers.

miRNAs	miRNA status in cancer	Prognostic or diagnostic marker	Tissue	Reference
miR-16	Decreased	Prognosis	Melanoma	(245)
miR-16	Decreased	Prognosis	Childhood ALL	(246)
miR-17-5p	Increased	Diagnosis	Gastric cancer	(247)
miR-17-5p	Increased	Diagnosis	Nasopharyngeal cancer	(248)
miR-21	Increased	Diagnosis	Diffuse large B cell lymphoma	(249)
miR-26b	Decreased	Prognosis	Cervical cancer	(250)
miR-29b	Decreased	Prognosis	Ovarian cancer	(244)
miR-30d	Increased	Diagnosis	Low-grade serous ovarian cancer	(251)
miR-34a	Decreased	Diagnosis	Diffuse large B cell lymphoma	(252)
miR-140-5p	Decreased	Prognosis	Colorectal cancer	(207)
miR-143	Decreased	Diagnosis	Pancreas cancer	(253)
miR-155	Increased	Diagnosis	Diffuse large B cell lymphoma	(252)
miR-155	Increased	Diagnosis	Diffuse large B cell lymphoma	(249)
miR-155	Decreased	Diagnosis	Pancreas cancer	(253)
miR-183, miR-375	Increased	Prognosis	Sporadic medullary thyroid cancer	(254)
miR-205	Decreased	Diagnosis	Triple negative breast cancer	(242)
miR-210	Increased	Prognosis	Melanoma	(255)
miR-210	Increased	Diagnosis	Diffuse large B cell lymphoma	(249)
miR-212	Decreased	Diagnosis	Prostate cancer	(222)
miR-216a	Decreased	Diagnosis	Pancreas cancer	(253)
miR-221/222	Increased	Diagnosis	HR-negative breast cancer	(241)
miR-224-3p	Increased	Diagnosis	HPV-positive cervical cancer	(226)
miR-340	Decreased	Prognosis	Glioblastoma	(228)
miR-409-3p	Decreased	Prognosis	Gastric cancer	(256)

Autophagy-Related miRNAs and Response to Cancer Treatment

Response to Radiotherapy

Radiation treatment is one of the standard treatment modalities for many cancer types. Radiotherapy involves the use of ionizing radiation at doses that damage cancer cells. Since normal cells in the surrounding tissues may also be affected, dose adjustments and focused applications are important issues to be considered to obtain effective treatment protocols with minimal side effects. Mechanism of action of radiation in cancer cells include generation of oxygen radicals, damage to organelles such as mitochondria and ER, and direct and oxidative damage to DNA and other cellular components (257) All these insults trigger autophagy responses as well. Indeed, autophagy emerges as one of the factors that can influence the efficacy of radiation treatment of cancer (257, 258). Evidently, autophagy-regulating miRNAs have the capacity to modify responses of cancer cells to radiation treatment.

For example, miR-23b was shown to target ATG12 and inhibit autophagy, and overexpression of the miRNA sensitized pancreas cancer cells to radiation (184). In another study, miR-216a downregulation correlated with autophagy activation in radiation-resistant prostate cancer cells through depression of *BECN1*/Beclin 1, and forced expression of the miRNA led to radiosensitivity and cell death (73).

On the other hand, miR-32 was shown to induce autophagy through suppression of autophagy inhibitor DAB2IP, enhancing prostate cancer cell survival following radiation treatment (192). Strikingly in some contexts and tumor types, autophagy seems to confer resistance to radiation-induced cancer cell death. For instance, inhibition of miR-17 that targeted ATG7 activated autophagy and sensitized U373-MG glioma cells to low-dose ionizing radiation treatment, affecting their long-term

viability (83). In another study, miR-199a-5p increased basal and radiation-induced autophagy breast cancer cells, and autophagy activation by this miRNA in MCF-7 cells correlated with sensitivity to radiation (70).

The effects of autophagy-related miRNAs on radiation responses were summarized in **Table 3**. Whether discrepancies between these observations are a result of a switch between protective and prosurvival autophagy and its autophagic, apoptotic, or necrotic cell death promoting role is not clear to date and further molecular studies are required. Nevertheless, altogether these studies underline the fact that autophagy manipulation in a context-dependent manner might potentiate responses of cancer cells to radiotherapy and improve treatment outcomes.

Response to Chemotherapy

Most of the tested chemotherapy agents have been shown to induce autophagy in cancer cells, and miRNAs that control autophagic activity were reported to affect susceptibility of cancer cells to cancer drugs.

For example, miR-101 and miR-199a-5p that both had inhibitory effects on autophagy potentiated liver cancer cell death by cisplatin (87, 197). Similarly, in liver cancer cells, toxic effects of another chemotherapy agent, doxorubicin, were increased when miR-101 was overexpressed (198). In lung cancer cells, miR-24-3p increased sensitivity to etoposide and cisplatin, miR-200b to docetaxel, miR-216B to paclitaxel, and miR-487-5p to temozolomide (97, 217, 224). All of the above miRNAs were shown to block autophagy in the lung cancer context. On the other hand, miR-17 and miR-16 increased paclitaxel sensitivity of lung cancer cells through simultaneous downregulation of autophagy and activation of apoptosis following *BECN1*/Beclin 1 and BCL-2 suppression, respectively (170). Similar combined effects of miRNAs and chemotherapy agents were observed in medullablastomas, multiple myelomas, chronic myeloid leukemias, gliomas, cervix, ovary, breast, prostate and head and neck cancers, esophageal, gastric, colorectal, and thyroid carcinomas. Importantly, same miRNAs were able to show chemotherapy potentiation effects in more than one cancer type in independent studies. For example, miR-21 augmented chemotherapy responses of various cancer drugs on liver, breast, and head and neck cancers, indicating that observed effects may well be independent of cancer type (178–180, 183). **Table 4** summarizes the effects of autophagy-regulating miRNAs on chemotherapy responses.

CONCLUSION

Studies that are published so far about the regulation of autophagy by miRNAs start to reveal a general picture about this emerging field. In human cells, there are around 2,000 miRNAs and 25,000 protein-coding genes. Approximately 60% of all these protein-coding genes are predicted to be controlled by miRNAs (16). Yet, accumulating data in the literature indicate that genes of almost all proteins that are involved in autophagosome formation and maturation as well as components of autophagy-related signaling pathways (e.g., AMPK, AKT, and mTOR pathways) are strictly controlled by several miRNAs (**Table 1**). Some autophagy genes may even be targeted by more than one miRNA that have divergent responses to stress stimuli [e.g., Ref. (64, 67)].

Since autophagy is an evolutionarily well-preserved pathway in all organisms from yeast to man, and it is essential for cellular and organismal homeostasis and survival, strict control of autophagy at every level should be an expected outcome.

Upregulation or downregulation of miRNAs was observed in almost all types of cancer, indicating that proper functioning of miRNA networks ensure normal growth and behavior of cells. A single miRNA is able to control dozens of genes, changing thresholds and responsivity or even function of signaling pathways and signal-related events. Modulation of cellular stress and death responses and regulation of cell growth, cell-extracellular matrix, and cell-to-cell interactions as well as cellular migration capacities are all subject to control by miRNAs. Therefore, deregulation of miRNA networks might directly contribute to cancer cell formation, EMT, neovascularization, tissue invasion, and metastasis. Even some miRNAs were classified as oncogenes (oncomirs) and others as tumor suppressors. A number of miRNAs that were shown to play a role in cancer biology were also involved in autophagy regulation, and many of them were reported to directly target autophagy-related genes.

Autophagy abnormalities are generally either the cause or an exacerbating factor in a large majority of diseases in man and in other organisms. And cancer is no exception. Autophagy plays

TABLE 3 | Effect of autophagy-related microRNAs (miRNAs) on radiotherapy.

miRNAs	miRNA status in cancer	Effect on autophagy	Autophagy-related target	Effect on radiotherapy	Tested cell line (tissue origin)	Reference
miR-17	Upregulated	Inhibition	ATG7	Radioresistance	U373 (glioblastoma)	(83)
miR-21	Upregulated	Inhibition	N.D.	Radioresistance	U373, U87 (glioblastoma)	(175)
miR-21	Upregulated	Inhibition	PTEN	Radioresistance	HeLa, siHa (cervical)	(177)
miR-23b	Downregulated	Inhibition	ATG12	Radiosensitivity	BxPC3 (pancreas)	(184)
miR-30b	Downregulated	Activation	BECN1	N.D.	SH-SY5Y (neuroblastoma)	(190)
miR-32	N.D.	Inhibition	DAB2IP	Radioresistance	PC3, DU145 (prostate)	(192)
miR-101	Upregulated	Inhibition	STMN1	Radiosensitivity	CNE-2, 5–8 F (nasopharyngeal carcinoma)	(199)
miR-199a-5p	N.D.	Inhibition	BECN1, DRAM1	Radiosensitivity	MDA-MB-231 (breast)	(70)
miR-200c	N.D.	Activation	UBQLN1	Radiosensitivity	MDA-MB-231 (breast)	(218)
miR-205	N.D.	Inhibition	TP53INP1	Radiosensitivity	DU145, LNCaP (prostate)	(221)
miR-216a	Upregulated	Inhibition	BECN1	Radiosensitivity	PANC-1 (pancreas)	(80)

TABLE 4 | Effect of autophagy-related microRNAs (miRNAs) on chemotherapy.

miRNAs	miRNA status in cancer	Effect on autophagy	Autophagy-related target	miRNA effect on chemotherapy	Chemotherapeutic agent	Tested cell line (tissue origin)	Reference
miR-let7f1	N.D.	Inhibition	HMGB1	Chemosensitivity	Cisplatin	D425, UW228 (medullablastoma)	(165)
miR-15a/16	N.D.	Activation	RICTOR	Chemosensitivity	Camptothecin	HeLa (cervical cancer)	(169)
miR-16 miR-17	Downregulated	Activation	BCL-2	Chemoresistance	Paclitaxel	A549-T24 (lung cancer)	(170)
miR-17	Upregulated	Inhibition	ATG7	Chemosensitivity	Temozolomide	U373 (glioma)	(83)
miR-17-5p	Downregulated	Inhibition	BECN1	Chemosensitivity	Paclitaxel	A549-T24 (lung cancer)	(172)
miR-21	Upregulated	Activation	PTEN	Chemosensitivity	Sorafenib	Huh7, HepG2 (liver cancer)	(178)
miR-21	N.D.	Inhibition	PTEN	Chemoresistance	Tamoxifen Fulvestrant	MCF-7 (breast cancer)	(179)
miR-21	Upregulated	N.D.	PDCD4	Chemosensitivity	Cisplatin	Tca8113 (tongue squamous cell carcinoma)	(180)
miR-21	Downregulated	N.D.	N.D.	Chemosensitivity	Cisplatin	Tca8113 (tongue squamous cell carcinoma)	(183)
miR-22	N.D.	Inhibition	BTG1	Chemosensitivity	5-FU	SW620, RKO (colorectal cancer)	(182)
miR-23a	Upregulated	N.D.	TOP2B	Chemoresistance	Cisplatin	Tca8113 (tongue squamous cell carcinoma)	(183)
miR-23b-3p	Downregulated	Inhibition	ATG12, HMGB2	Chemosensitivity	5-FU, Cisplatin	SGC7901/VCR (gastric cancer)	(185)
miR-24-3p	Downregulated	Inhibition	ATG4A	Chemosensitivity	Etoposide Cisplatin	H446/EP (lung cancer)	(80)
miR-25	N.D.	Inhibition	ULK1	Chemosensitivity	Isoliquiritigenin	MCF-7 (breast cancer)	(61)
miR-29b	Downregulated	Inhibition	PSME4	Chemosensitivity	Bortezomib	AMCL1, AMCL2 (multiple myeloma)	(187)
miR-30b	N.D.	Inhibition	BECN1	Chemosensitivity	Imatinib	K562 (CML)	(65)
miR-30a	Downregulated	Inhibition	BECN1	Chemosensitivity	Cisplatin	HeLa (cervical cancer)	(188)
miR-30d	N.D.	Inhibition	BECN1	Chemosensitivity	Cisplatin	SW1736, 8305 C (anaplastic tyroid carcinoma)	(191)
miR-30a	N.D.	Inhibition	BECN1	Chemosensitivity	Sorafenib	786-0, A489 (renal carcinoma)	(189)
miR-30a	Downregulated	Inhibition	BECN1	Chemosensitivity	Doxorubicin	MG-63 (osteosarcoma)	(66)
miR-101	N.D.	Inhibition	STMN1 RAB5A Atg4D mammalian TOR (mTOR)	Chemosensitivity	Cisplatin	HepG2 (liver cancer)	(197)
miR-101	N.D.	Inhibition	STMN1 RAB5A Atg4D	Chemosensitivity	Etoposide	MCF-7 (breast cancer)	(89)
miR-101	Downregulated	Inhibition	EZH2	Chemosensitivity	Doxorubicin	HepG2 (liver cancer)	(198)
miR-138	N.D.	Activation	BIM	Chemosensitivity	Temozolomide	LN-308, ZH-305 (glioblastoma)	(206)
miR-143	Downregulated	Inhibition	GABARAPL1	Chemosensitivity	Qercetin	AGS, MKN28 (gastric cancer)	(208)
miR-143	Downregulated	Inhibition	ATG2B	Chemosensitivity	Doxorubicin	SAOS-2-Dox, U2OS-Dox (osteosarcoma)	(259)
miR-152	Downregulated	Inhibition	ATG14	Chemosensitivity	Cisplatin Doxorubicin	A2780/CP70, SKOV3/DDP (ovarian cancer)	(211)
miR-181a	N.D.	Inhibition	ATG5	Chemosensitivity	Cisplatin	MCF-7 (breast cancer)	(75)
miR-181a	N.D.	Inhibition	ATG5	Chemosensitivity	Cisplatin	SGC7901/CDDP (gastric cancer)	(214)
miR-193b	Upregulated	Activation	STMN1	Chemosensitivity	5-FU	KYSE450 (esophageal cancer)	(216)
miR-199a-5p	Downregulated	Inhibition	ATG7	Chemosensitivity	Cisplatin	Huh7, HepG2 (liver cancer)	(87)
miR-205	N.D.	Inhibition	RAB27A, LAMP3	Chemosensitivity	Cisplatin	DU145 (prostate cancer)	(95)
miR-200b	N.D.	Inhibition	ATG12	Chemosensitivity	Docetaxel	SPC-A1/DTX, H1299/DTX (lung cancer)	(217)
miR-214	Downregulated	Inhibition	UCP2	Chemosensitivity	Tamoxifen Fulvestrant	MCF-7/LCC9 (breast cancer)	(223)
miR-214	Upregulated	N.D.	N.D.	Chemoresistance	Cisplatin	Tca8113 (tongue squamous cell carcinoma)	(183)
miR-216b	Downregulated	Inhibition	BECN1	Chemosensitivity	Paclitaxel	A549, Calu-3 (lung cancer)	(224)
miR-218	Downregulated	Inhibition	HMGB1	Chemosensitivity	Paclitaxel	RL95-2 (endometrial carcinoma)	(225)
miR-409-3p	Downregulated	Inhibition	BECN1	Chemosensitivity	Oxaliplatin	Lovo Oxa R (colorectal cancer)	(72)
miR-451a	N.D.	Inhibition	N.D.	Chemosensitivity	Tamoxifen	MCF-7/LCC2 (breast cancer)	(231)
miR-487-5p	Upregulated	Inhibition	LAMP2	Chemoresistance	Temozolomide	A549, H1299 (lung cancer)	(97)

a role in various steps of cancer formation, growth, and spread. Not surprisingly, there is a growing literature about deregulation of autophagy-related miRNAs in cancer. Direct contribution of autophagy abnormalities to phenotypes that were observed following miRNA deregulations was not established in some cancer-related publications, and it is possible that contribution of autophagy is somewhat indirect in some of these studies. Nevertheless, it is unimaginable that autophagy-related effects of these miRNAs will be of no consequence to cancer cell behavior. Moreover, there is a rapidly growing literature about autophagy-related miRNA levels and sensitivity to chemotherapy or radiation treatment. In line with the prosurvival role of autophagy, in most of these studies, autophagy suppression by miRNAs was shown to sensitize cancer cells to therapy. In contrast, in some cases, aberrant activation of autophagy that correlated with changes in miRNA levels was itself detrimental for cancer cells and led to apoptotic, autophagic, or necrotic cell death. Therefore, miRNA manipulations through using mimics or antagomirs, or other strategies, might potentially be used as adjuvant therapies for cancer treatment. Advances in gene therapy

protocols and improvement of gene delivery vehicles (e.g., new generation gene therapy viruses, liposomes, nanoparticles, etc.) might allow the use of miRNA manipulation strategies in cancer gene therapy trials.

Another important and more immediate use of miRNAs in oncology involves exploitation of their disease marker potential. In addition to allowing early and accurate diagnosis of cancer, miRNAs may be used to follow patient responses to therapy and relapses. In addition to tumor biopsy materials, miRNAs can potentially be detected in any bodily fluid, including blood, urine, saliva, etc. Autophagy-related miRNAs were also found to be up- or downregulated in many cancers, and several studies point out to their potential use as biomarkers (**Table 2**).

In conclusion, autophagy-related miRNAs constitute a very important control layer on top of all other autophagy-regulatory mechanisms that were described so far. In the last few years, there is an exponential increase in the number of articles studying miRNA-autophagy connection. These efforts will eventually result in the construction of a detailed and functional map of autophagy-related miRNA networks. Accumulation of knowledge on miRNA-mediated control of autophagy under physiological and pathological conditions might lead to the development of new approaches that can be used for the diagnosis, treatment, and follow-up of serious health problems involving autophagy abnormalities, including cancer.

AUTHOR CONTRIBUTIONS

DG designed the structure of the review, wrote, and edited the review article. YA and DO wrote the review article prepared the tables. MK contributed to the preparation of the tables and prepared the figures.

ACKNOWLEDGMENTS

This work was supported by the European Union COST Action Transautophagy (CA15138) and The Scientific and Technological Research Council of Turkey (TUBITAK) 1001 project numbers 114Z241 and 114Z982. YA is supported by a TUBITAK-BIDEB 2211 scholarship for Ph.D. studies.

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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.

frontiers in Oncology

REVIEW
published: 18 November 2016
doi: 10.3389/fonc.2016.00246

Hijacker of the Antitumor Immune Response: Autophagy Is Showing Its Worst Facet

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Macroautophagy (hereafter referred to as autophagy) is a housekeeping process constitutively executed at basal level in all cells to promote cellular homeostasis by regulating organelle and protein turnover. However, autophagy deregulation caused by several stress factors, such as hypoxia, is prevalent in many cancers. It is now well established that autophagy can act as tumor suppressor or tumor promoter depending on tumor type, stage, and genetic context. In developed tumors, autophagy promotes the survival of cancer cells and therefore operates as a cell resistance mechanism. Emerging evidence point to the prominent role of autophagy in disabling the antitumor immune response by multiple overlapping mechanisms leading to tumor escape from immune cell attack mediated by both natural killer cells and cytotoxic T-lymphocytes. Such a role has inspired significant interest in applying anti-autophagy therapies as an entirely new approach to overcome tumor escape from immune surveillance, which constitutes so far a major challenge in developing more effective cancer immunotherapies. In this review, we will summarize recent reports describing how tumor cells, by activating autophagy, manage to hijack the immune system. In particular, we will focus on the emerging role of hypoxia-induced autophagy in shaping the antitumor immune response and in allowing tumor cells to outmaneuver an effective immune response and escape immunosurveillance. In keeping with this, we strongly believe that autophagy represents an attractive future therapeutic target to develop innovative and effective cancer immunotherapeutic approaches.

Keywords: autophagy, hypoxia, antitumor immune response, tumor microenvironment, Natural Killer cells, Cytotoxic T Lymphocytes, chloroquine and immunological checkpoint-based immunotherapy

OPEN ACCESS

Edited by:
Patrizia Agostinis, State University of Leuven, Belgium

Reviewed by:
Gabriele Multhoff, Technische Universität München, Germany
Eric Chevet, INSERM, France

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *21 September 2016*
Accepted: *04 November 2016*
Published: *18 November 2016*

Citation:
Viry E, Noman MZ, Arakelian T, Lequeux A, Chouaib S, Berchem G, Moussay E, Paggetti J and Janji B (2016) Hijacker of the Antitumor Immune Response: Autophagy Is Showing Its Worst Facet. Front. Oncol. 6:246. doi: 10.3389/fonc.2016.00246

INTRODUCTION

Autophagy and Hypoxia

Under physiological conditions, autophagy is executed at low level to degrade damaged proteins and/or organelles in order to sustain metabolism and cell homeostasis. The level of basal autophagy varies depending on the tissue type and some tissues are particularly dependent on autophagy (e.g., brain, liver, and muscle) (1). It is now generally appreciated that autophagy is deregulated in some pathological conditions including cancer and it seems that the role of autophagy in cancer is

context and circumstance-dependent (2). Thus, autophagy suppresses tumor progression by limiting chromosomal instability (3); however, established tumors appear to utilize autophagy in order to survive periods of metabolic or hypoxic stress (3). In line with its role in sustaining viability and conferring stress tolerance, targeting autophagy has inspired significant interest for mitigating tumor growth and/or restoring response to anticancer therapies. Consistent with the part of autophagy in promoting tumor progression, it has been shown that targeting autophagy reduces cell migration and invasion *in vitro* and attenuates metastasis *in vivo* in a breast cancer mouse model by promoting focal adhesion disassembly through targeted degradation of paxillin (4). Furthermore, several new studies have reported that autophagy activation plays also a major role in tumor immunity. Autophagy enhances tumor antigens processing and presentation thereby promoting adaptive antitumor immunity. In antigen-presenting cells (APC), autophagy promotes antigen presentations by major histocompatibility complexes (MHC). Such antigens presented by MHC class II and I are recognized by CD4+ and CD8+ T cells, respectively, in order to induce specific cytotoxic T-lymphocyte (CTL)-mediated immune response. Thus, autophagy allows the traffic of engulfed antigens to endosomes, where they are digested by cathepsins in order to be loaded onto MHC class II molecules and translocate to the plasma membrane to be finally presented to CD4+ T cells (5). Based on the role of autophagy in antigen presentation and processing, it has been proposed that autophagy plays a beneficial role for the induction of antitumor immunity. In this context, hypoxia has been reported to increases tumor cell shedding of MHC class I resulting into increased resistance to natural killer (NK)-mediated lysis (6) *via* increased expression of ADAM10 (7). The role of autophagy in antigen processing and presentation was reported in several comprehensive reviews (8–12) and will not be the subject of this review. However, emerging evidence strongly suggest that autophagy shows its worst facet when induced within the hypoxic tumor microenvironment (13). Indeed, autophagy impairs the antitumor immune responses mediated by CTL and NK cells and has been reported to enhance the immunosuppressive properties of myeloid-derived suppressor cells (MDSCs). These issues will be discussed in more detail in the present review.

It is now well established that hypoxia develops due to a mismatch between tumor growth and neovascularization. Cellular responses to hypoxia are mediated by hypoxia-inducible factor (HIF) family of transcription factors. Both HIF-1 and HIF-2 are composed of two subunits: an O_2 regulated subunit (HIF1-α and HIF2-α) and a constitutively expressed subunit (HIF1-β and HIF2-β) (14). In the presence of oxygen, HIF-1α is hydroxylated by prolyl hydroxylase domain protein 2 (PHD2) on a proline residue leading to an interaction with the Von Hippel-Lindau (VHL) protein. Therefore, HIF-1α is polyubiquitylated and consequently targeted for degradation by the ubiquitin proteasome system. Under hypoxic conditions, HIF-1α is stabilized, accumulated in the cytoplasm, and then translocated to the nucleus where it can form a heterodimer complex with HIF-1β. Finally, HIF-1 binds to hypoxia response elements (HREs) on the chromatin in order to induce the transcription of more than 300 genes, involved in many biological processes, including angiogenesis, cell survival, metastasis, stem cell-like phenotype, and immune escape (15).

Three major pathways have been reported to induce autophagy under hypoxia. Briefly, HIF-1α-mediated induction of the expression of the BH3-only protein Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and the related protein, BNIP3L (16). BNIP3 and BNIP3L disrupt the autophagy inhibitory complex between Beclin1 (BECN1) and B-cell lymphoma 2 (Bcl-2) and activate autophagy in hypoxic cells, which operate as an adaptive survival response during prolonged hypoxia (**Figure 1**) (17). Autophagy can also be activated by endoplasmic reticulum (ER) stress in cancer cells. Indeed, ER stress stimulates the assembly of the pre-autophagosomal structures, the formation of autophagosomes, and the transport to the vacuoles in an autophagy-related gene (ATG) dependent manner (18). Autophagy can also be induced in hypoxic cells subjected to metabolic stress through the activated adenosine monophosphate-activated protein kinase (AMPK). This leads to the initiation of autophagy both directly and indirectly by inhibiting the mammalian target of rapamycin (mTOR) (19, 20).

Immune Checkpoints and Hypoxia

Immune checkpoint-based cancer immunotherapy has now emerged as a promising revolutionary treatment for many cancers including, but not limited to, melanoma, lung cancer, kidney cancer, bladder cancer, prostate cancer, and lymphoma (21). In the near future, immune checkpoint-based cancer immunotherapies will most probably join the ranks of surgery, radiation, chemotherapy, and targeted therapy as a pillar of classical anticancer therapies. Ipilimumab (anti-CTLA-4) was approved in 2011, and pembrolizumab and nivolumab (anti-PD-1) were approved in 2014 by U.S. Food and Drug Administration (FDA) for the treatment of melanoma. These antibodies will soon be approved for treatment of patients with lung cancer, kidney cancer, and many other tumor types. The fact that these antibodies do not target tumor cells, but rather remove inhibitory signals on antitumor T cells have led to durable clinical responses in some patients (21).

Programed death 1 (PD-1) is an inhibitory receptor expressed mostly on activated T cells as well as other immune cells, and its expression is associated with T cell exhaustion. PD-1 has two ligands, Programed death-ligand 1 and 2 (PD-L1 and PD-L2). The interaction between PD-1 and its ligands sends a negative signal to T cells to dampen the antitumor immune response. Antibody-based blockade of PD-1 was shown to enhance effector T cell responses and induce T cell–mediated tumor rejection in some mouse models (22). PD-1 or PD-L1 blockade has been proven to be successful in many cancers (21), and anti-PD-1 antibodies have recently been approved for use in the United States and Asia. Hypoxia-inducible factors were shown to enhance the effector responses (including both costimulatory and inhibitory molecules) of VHL deficient CD8+ T cells (with constitutive HIFs) to persistent viral antigen (23). We and others have reported that HIF-1α (24) and HIF-2α (25) regulate PD-L1 expression under hypoxia (**Figure 2**). Hypoxia through HIF-1α has been also shown to regulate functional CD137 (4-1BB) on

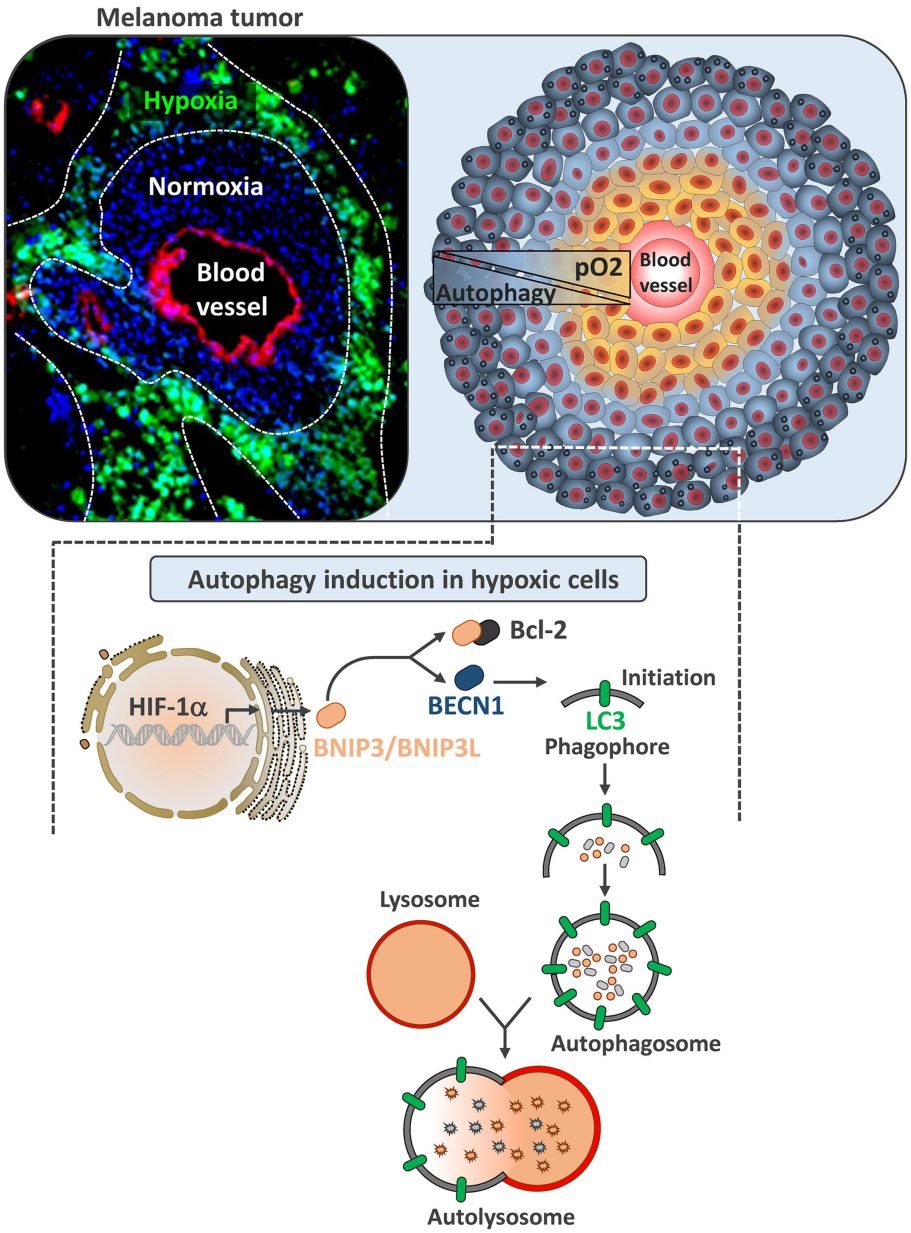

FIGURE 1 | **Autophagy induction in hypoxic melanoma cells**. Left image represents B16-F10 tumor section transplanted in C57BL/6 mouse. Tumor section was stained with Pimonidazole to illustrate hypoxic zones (in green). Blood vessels were stained with anti-CD31 (red) and nuclei with DAPI (blue). Right image is a schematic representation of tumor showing normoxic tumor cells close to the blood vessel (yellow) and hypoxic tumor cells (blue) at the periphery. In B16-F10 mouse melanoma, autophagy is selectively induced in hypoxic zones. Lower image represents the mechanism underlying the activation of autophagy by hypoxia. In hypoxic cells, hypoxia-dependent activation of HIF-1α upregulates BNIP3 and BNIP3L by binding to their promoter regions. Both of these BH3-only proteins (BNIP3 and BNIP3L) disrupt the complex between Beclin1 and Bcl-2, leading to the induction of autophagy.

tumor-infiltrating T lymphocyte (26) and more recently soluble CD137 in malignant tumor cells (27).

Indeed, we first found a differentially higher expression of PD-L1 on tumor-infiltrating MDSCs as compared to splenic MDSCs (24) and hypoxia dramatically and significantly increased the percentage of PD-L1 positive MDSCs isolated from spleen in different tumor-bearing mice. We further provided evidence that HIF-1α is a major regulator of PD-L1 mRNA and protein expression, and that HIF-1α regulates the expression of PD-L1 by binding directly to the HRE-4 in the PD-L1 proximal promoter. The immune suppressive function of MDSCs, enhanced under hypoxia, was abrogated following PD-L1 blockade and hypoxia-induced upregulation of interleukin-6 and 10 (IL-6 and IL-10) in MDSCs was significantly attenuated after PD-L1 block (24).

More recently, we showed that tumors from clear cell renal cell carcinoma (ccRCC) patients displaying VHL biallelic

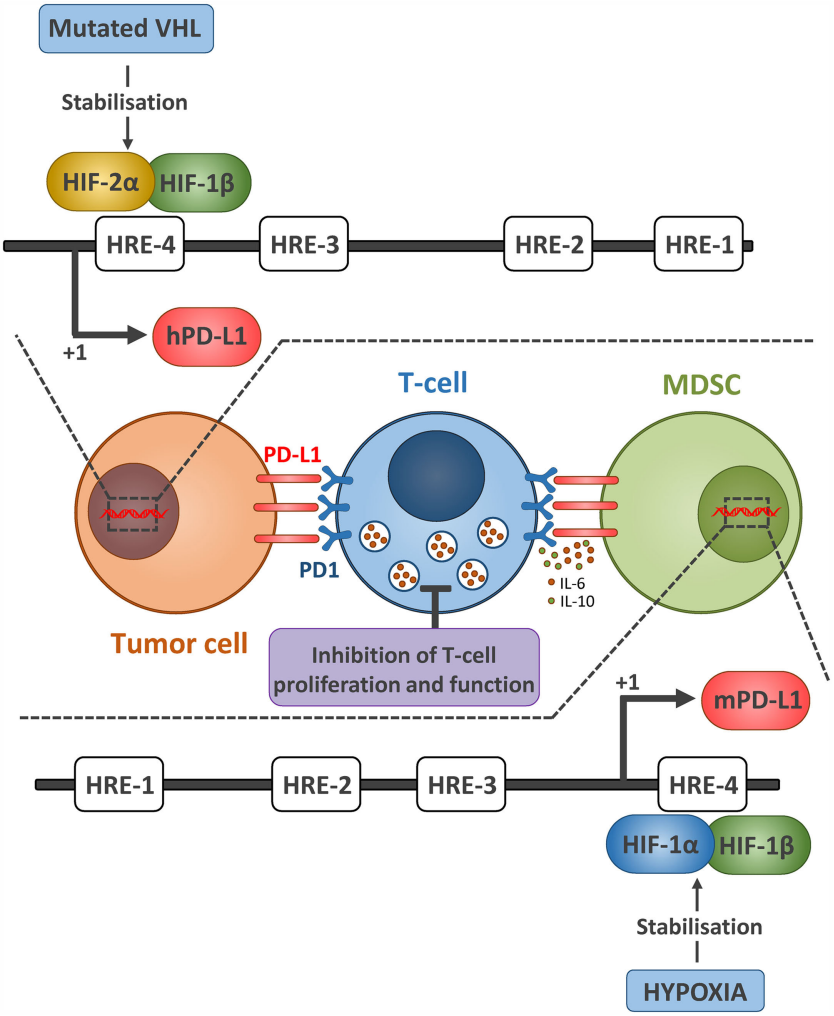

FIGURE 2 | Hypoxia *via* HIF-1α and HIF-2α regulates the expression of PD-L1 expression on tumor cell and MDSCs. The upper part represents the mechanism by which HIF-2α regulates the expression of PD-L1 in ccRCC tumor cells. Due to mutated VHL in ccRCC tumors, HIF-2α is constitutively stabilized and activated. HIF-2α translocates to the nucleus, binds to the HRE-4 in human PD-L1 promoter, and upregulates its expression. Whether this PD-L1 confers resistance to ccRCC sensitivity to antitumor effector cells remains to be investigated. The lower part represents the mechanism by which hypoxia *via* HIF-1α regulates the expression of PD-L1 in MDSCs. Similarly, stabilized HIF-1α in MDSCs isolated from tumors bound directly to the HRE-4 in the PD-L1 proximal promoter in MDSCs. The immune suppressive function of MDSCs, enhanced under hypoxia, was abrogated following PD-L1 blockade and hypoxia-mediated upregulation of IL-6 and IL-10 in MDSCs was significantly attenuated after PD-L1 blockade.

inactivation (i.e., loss of function) exhibit a significant increase in PD-L1 expression as compared to ccRCC tumors carrying one VHL wild-type allele. Using the inducible VHL 786-O-derived cell lines with varying HIF-2α stabilization levels, we showed that PD-L1 expression levels positively correlate with VHL mutation and HIF-2α expression. Targeting HIF-2α decreased PD-L1 while HIF-2α overexpression increased PD-L1 mRNA and protein levels in ccRCC cells. Interestingly, chromatin immunoprecipitation and luciferase assays revealed a direct binding of HIF-2α to a transcriptionally active HRE in the human PD-L1 proximal promoter in 786-O cells. In conclusion, VHL mutations positively correlate with PD-L1 expression in ccRCC and may influence the response to ccRCC patients to anti-PD-L1/PD-1 immunotherapy (25).

EFFECT OF AUTOPHAGY ON ANTITUMOR IMMUNE RESPONSES

The role of cancer cell-associated autophagy in the modulation of the antitumor immune response takes place at different levels. First, autophagy may act at early step of cancer development, thus regulating the immune surveillance in the context of tumorigenesis. Second, at later stage of cancer, the autophagy process was identified as an important modulator of the tumor cell proteome and secretome, allowing cancer cells to communicate with neighboring cells in the tumor microenvironment. Finally, cancer cell-associated autophagy may also acts as an intrinsic resistance mechanism evolved by tumor cells to overcome immune cell attack. Mounting evidence in the literature suggests

that depending on the context, the cancer model, and the type of stressor (e.g., hypoxia, chemotherapy), autophagy might either assist or prevent anticancer immunity.

Autophagy Modulates the Immune Surveillance during Tumorigenesis

Autophagy serves as an adaptive response during period of stress by maintaining cellular integrity and metabolic homeostasis. Extensive works coming from Eileen White's group have demonstrated the crucial role of autophagy in cancer tumorigenesis. Notably, autophagy process actively contributes to elimination of source of genotoxic stress in order to maintain genomic integrity (28–30). However, autophagy inhibition may halt tumorigenesis not only by affecting cancer cell metabolism and proliferation *per se* but also by enhancing the antitumor immunosurveillance. The recent study of Rao et al. illustrated the complex role of autophagy in either tumorigenesis or tumor progression depending on the stage of the disease (31). Inactivation of Atg5-dependent autophagy increased the number of tumor foci and favored the progression from hyperplasia to adenoma in a murine model of lung cancer (with KrasG12D mutation). However, in later stage of the disease, disabled autophagy reduced the progression from adenoma to adenocarcinoma. Immunoprofiling of early pulmonary lesions indicated that autophagy inactivation did not alter the global infiltration of the $CD3\varepsilon^+$ T cells, but the amount of infiltrating $Foxp3^+$ regulatory T cells (Tregs), which was increased in autophagy-deficient tumor. The authors clearly demonstrated that inactivation of autophagy in pneumocytes carrying a Kras mutation triggered a local expansion of Tregs cells, which control lung tumor initiation. This study established a cause-effect relationship between defective autophagy and infiltration of immunosuppressive Tregs cells. Other studies stated that autophagy inhibition may hamper tumorigenesis also by enhancing antitumor immunosurveillance. Recently, Levy et al. showed that conditional inactivation of the autophagy gene Atg7 in intestinal epithelial cells (IECs) suppresses the development of precancerous lesions in Apc mutant mice (32). The authors demonstrated that CD8+ T cells were essential effectors of the antitumor immune response mediated by the inhibition of autophagy. Autophagy inactivation in IECs favored the infiltration and the expansion of interferon (IFN)-producing CD8+ T cells in the intestinal mucosa and participated in their priming. Moreover, Atg7 deficiency endorsed a CD4+ Th1 signature, which is associated with a good outcome in patients with colorectal cancer (33). However, Tregs infiltration was also induced, but in contrast to most studies, Tregs did not show immunosuppressive properties in those experimental conditions. Interestingly, this study reported that autophagy deficiency in IECs influenced intestinal microbiota, which is a prerequisite for an efficient anticancer immune response (32). In addition, Wei et al. have reported that conditional deletion of the ULK1-associated protein FIP200 in a PyMT-driven breast cancer murine model reduced the initiation of mammary intraepithelial neoplastic lesions and altered tumor progression by enhancing antitumor immune surveillance. FIP200-deficient tumor cells showed an upregulated expression of genes implicated in IFN signaling and an increased production of the chemokine CXCL10 that may favor infiltration of CD8+ T lymphocytes. This study, once again, showed that CD8+ T cells are crucial effectors of the autophagy-induced immunomodulation, as depletion of CD8+ T cells with selective antibodies restored mammary tumor initiation and progression (34, 35).

The Dual Immunomodulatory Role of Autophagy Induction in Cancer Cells

Autophagy Influences the Adaptive Antitumor Immunity

The importance of autophagy was also highlighted during tumor progression to late stage of the disease. Autophagy acts as a pro-survival mechanism allowing cancer cells to overcome stress conditions. However, cells that fail to adapt stress will use autophagy to release specific signaling molecules allowing the immune clearance of damaged cells. Thus, the concept of immunologic cell death (ICD) has rapidly emerged as an important feature determining an effective antitumor immune response (36). ICD is characterized by the rapid surface exposure of calreticulin (ecto-CRT), the secretion of ATP, and the release of apoptotic proteins as high mobility group box 1 (HMGB1), which are excellent immunogenic signals (36, 37). *Bona fide* ICD leads to increased maturation and stimulation of dendritic cells (DCs), which are required for robust T cells response. An increasing amount of evidence suggests that autophagy plays a crucial role during ICD by modulating the cancer cell secretome and surface proteome following stress or cell death induction.

In response to chemotherapeutic stress, autophagy-deficient cells displayed ecto-CRT, released HMGB1 but secreted less ATP than autophagy-competent cells. Michaud et al. showed that autophagy contributes to immunogenic signaling *in vivo* through the secretion of ATP. Impairment of autophagy in mitoxantrone (MTX)-treated murine colorectal carcinoma cells decreased the intratumoral recruitment of DCs and T cells when injected in immunocompetent mice. Artificial increase of intratumoral ATP restored the immunogenicity of MTX-treated autophagy-deficient cells, confirming that activation of autophagy following chemotherapy-induced ICD facilitates ATP secretion and the intratumoral accumulation of DCs and T lymphocytes (38).

Another important immunostimulatory signal that requires autophagy is the exposure of CRT at the cancer cell surface. Ecto-CRT triggers the engulfment of the damaged cells by macrophages. It has been described by Garg et al. that exposure of CRT is abolished in cancer cells when chaperone-mediated autophagy is impaired (39). In contrast to expectations, the same authors demonstrated that ER stress-induced autophagy, following photodynamic therapy, negatively regulated CRT surface exposure without affecting ATP secretion. Inhibition of ER stress-induced autophagy, by knocking down Atg5 in cancer cells, improved the maturation of IL-6 secreting DCs, and thus triggered proliferation of IFNγ-producing CD4+ and CD8+ T cells. Moreover, inactivation of autophagy in untreated melanoma cells increased CRT surface exposure, suggesting that basal autophagy in cancer cells may support immune escape (39, 40).

The pro-inflammatory role of autophagy may also be related to the protein HMGB1. In one hand, the cytosolic form of HMGB1

is able to bind to BECN1, which further leads to the formation of autophagosomes. On the other hand, secreted HMGB1 can also trigger autophagy through the binding to the RAGE receptor (receptor for advanced glycation endproducts) (41, 42). Once released outside the cell, HMGB1 has been shown to own both immunosuppressive and immunostimulatory properties (43). Production of HMGB1 by colon cancer cells also decreases the differentiation of DCs. In line with these experimental results, high HMGB1 levels in primary tumor tissues correlate with low intratumoral CD205+ DCs (44). Conversely, tumor cell-derived HMGB1 was shown to suppress CD8+ T cells antitumor immunity through the induction of IL-10-producing Tregs (45). Recently, Ladoire et al. provided evidence that cytoplasmic microtubule-associated protein 1A/1B-light chain 3 (LC3II), and nuclear HMGB1 expression may influence the nature of the immune infiltrate in breast cancer. Thus, the absence of LC3II correlated with intratumoral, but not peritumoral, infiltration of Foxp3+ Tregs, CD68+ tumor-associated macrophages and less CD8+ T cytotoxic lymphocytes. Moreover, absence of HMGB1 expression in the nuclei was associated with the increase in both intratumoral and peritumoral infiltration by Foxp3+ and CD68+ cells. Taken together, these results suggest that autophagy blockade or HMGB1 loss in breast cancer cells have a negative impact on the anticancer immune surveillance (46).

Autophagy has also emerged as a critical pathway for tumor antigen cross-presentation, which is determinant for the initiation of an efficient adaptive immune response (47, 48). Autophagy actively participates in antigen sequestration and delivery to DCs for cross-priming of CD8+ T cells. Inhibition of autophagy in melanoma cells by knocking down BECN1 significantly reduced T cell proliferation *in vitro* and *in vivo* (47). Moreover, induction of autophagy by the vitamin E derivative α-tocopheryloxyacetic acid (α-TEA) in 3LL Lewis lung carcinoma and 4T1 mammary carcinoma cells led to the release of antigen-containing autophagosomes that increase CD8+ T cells activation (49). Interestingly, tumor antigens packaged into autophagosomes were more efficient for T cells activation than soluble antigens (47).

It is now clearly established that autophagy activation in cancer cells is an effective way to communicate with the tumor microenvironment. However, accumulating observations have highlighted that autophagy activation may also serve as an intrinsic mechanism of resistance evolved by cancer cells to overcome immune cell attack (**Figure 3**) (13). Noman et al. demonstrated that hypoxia-induced autophagy acts as a mechanism of resistance to cytotoxic T cells-mediated lysis through the activation of the signal transducer and activator of transcription 3 (STAT3) signaling (50, 51). Our group provided for the first time direct evidence between autophagy and the regulation of STAT3 signaling. Inactivation of autophagy, by specific silencing of BECN1 or ATG5, restored lung cancer cell sensitivity to T cells lysis, which was associated with a decrease in hypoxia-induced pSTAT3. Interestingly, *in vivo* administration of the autophagy inhibitor hydroxychloroquine (HCQ) with a tyrosinase-related protein-2 (TRP2) peptide-based vaccination strategy led to a significant reduction of melanoma growth compared to vaccine or HCQ treatment alone. Although the clear link between autophagy activation and induction of STAT3 signaling remains to be elucidated, it has been clearly established that STAT3 is an important mediator in the crosstalk between tumor and immune cells (**Figure 3**) (52). The aberrant STAT3 signaling in tumor cells can suppress the expression of pro-inflammatory danger signals (e.g., IFNγ, TNF, CXCL10), while induces the expression of immunosuppressive factors (e.g., VEGF, IL-10) that inhibit DCs maturation (53).

More recently, studies have reported that tumor cells undergoing epithelial-to-mesenchymal transition (EMT) escape from T cell-mediated lysis. During EMT, phenotypic changes occur and expression of certain tumor antigens is downregulated, thus restraining T cells specific recognition and killing (54). The consequence of EMT on immune system is likely to be dependent on the cancer cell origin. Indeed, depending on the tumor type, EMT may either avoid proliferation/stimulate apoptosis of NK, B, and T cells or promote expansion of Tregs (55). The relationship between autophagy and EMT is not clearly established so far. However, it has been reported that acquisition of mesenchymal phenotype in breast cancer cell was associated with modification of autophagy gene expression, indicating a concomitant autophagy activation in those cells (56). EMT induction through the overexpression of snail homolog 1 (SNAI1) in MCF7 breast cancer cell line led to autophagy induction through the upregulation of BECN1. These observations indicated that autophagy activation is a downstream target of EMT. Thus, targeting EMT-induced autophagy in mesenchymal cells was sufficient to restore T cell-mediated lysis without affecting cell morphology and the expression of EMT markers (**Figure 3**) (57, 58).

Autophagy Modulates the Innate Antitumor Immune Response

Our group also provided evidence that autophagy induction in hypoxic breast cancer cells influences the innate antitumor immunity mediated by NK cells (59, 60). We demonstrated *in vitro* that the NK-derived serine protease granzyme B (GZMB) was selectively degraded into autophagosomes in hypoxic breast cancer cells, in which autophagy is induced. Selective inhibition of autophagy, by silencing BECN1 or ATG5 in hypoxic cancer cells, restored the susceptibility to NK-mediated lysis. This concept was validated *in vivo* by using two syngeneic models of tumor transplantation. We showed that inhibition of autophagy in cancer cells contributed to a significant reduction of the tumor volume by improving elimination by NK cells (59). In line with this study, the role of autophagy in modulating the NK-mediated antitumor immune response was extended to ccRCC. The VHL-mutated ccRCC, exhibiting high autophagy rate, was less susceptible to NK-derived GZMB than VHL-corrected ccRCC. This resistance required the stabilization of HIF-2α and the subsequent overexpression of the autophagy sensor inositol 1, 4, 5-trisphosphate receptor, type 1 (ITPR1). Both inhibition of BECN1 or ITPR1 in VHL-mutated ccRCC cells restored NK-mediated killing (**Figure 3**) (61, 62).

Additionally, activation of autophagy in cancer cells was shown to be implicated in the destabilization of the interaction between target and effector cells. Hypoxia-induced autophagy was identified as a selective degradation pathway of the gap junction protein

connexin 43 (Cx43) that renders the melanoma cells resistant to NK cells-mediated attack. Expression of a non-degradable form of Cx43 or targeting autophagy in hypoxic cancer cells restored the accumulation of Cx43 at the immunological synapse and improved NK cells-mediated lysis (**Figure 3**) (63).

Finally, autophagy induction is known to be closely related to the pathway of exosome secretion (64). In addition, hypoxia was described to enhance exosome release by cancer cells (65). Exosomes are small extracellular vesicles of endosomal origin, are released by all cell types, and constitute a new component of cell–cell communication. They carry membrane proteins, enzymes, chaperone proteins, but also DNA and various types of RNA (mRNA, lncRNA, and miRNA). Microvesicles derived from hypoxic cells were shown to induce an invasive and pro-metastatic phenotype (66, 67) and to inhibit the immune response (68). In particular, we showed that vesicles produced by hypoxic cancer cells inhibit NK cell functions. Following their uptake, the vesicles transfer TGF-β1 to NK cells, decreasing expression of the activating receptor NKG2D on the cell surface. We also identified high levels of miR-23a in hypoxic vesicles that directly targets the expression of the marker of degranulation CD107a in NK cells. Those two immunosuppressive mechanisms inhibiting NK cell

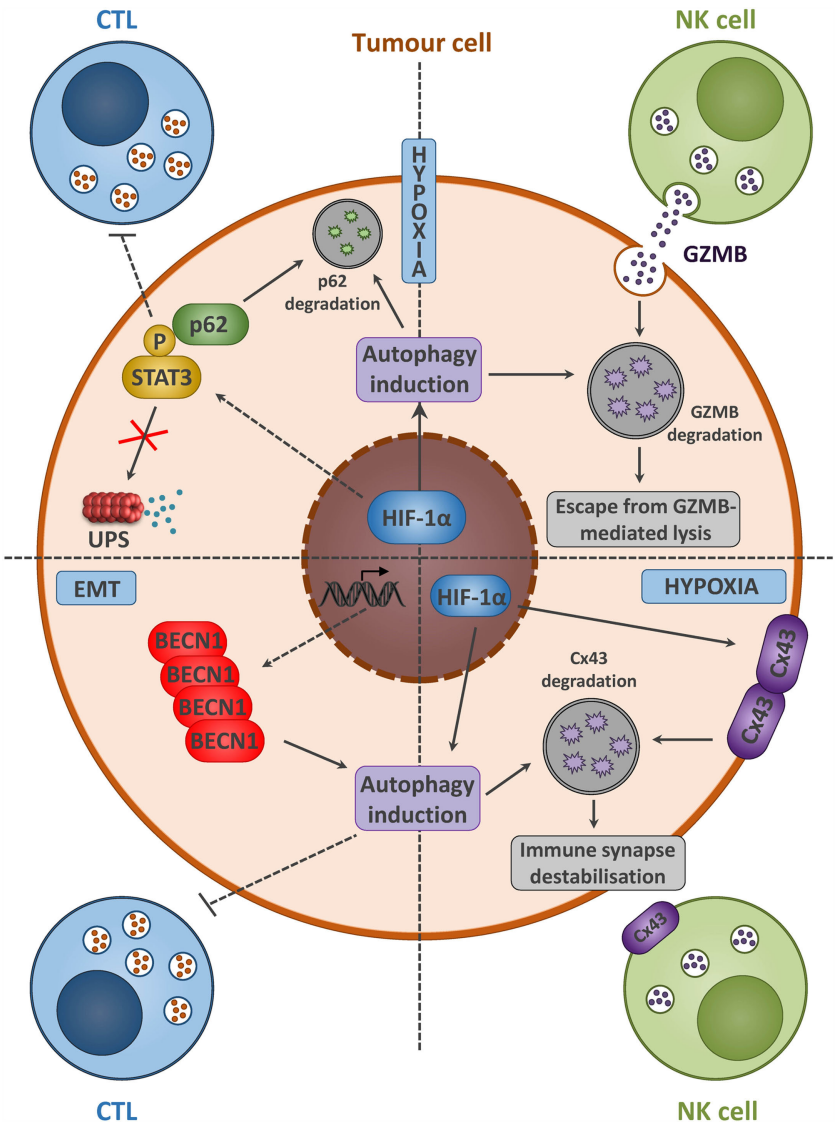

FIGURE 3 | Tumor cells with activated autophagy defend themselves from NK- and CTL-mediated killing by diverse mechanisms. Hypoxia and/or EMT activation induces autophagy in tumor cells. A summary of diverse mechanisms by which autophagy activation leads to the acquisition of tumor cell resistance to CTL and NK-mediated lysis. Hypoxia-induced autophagy in cancer cells acts as an intrinsic resistance mechanism to NK-mediated lysis through the selective degradation of the NK-derived serine protease granzyme B (GZMB) (upper right). Moreover, the selective degradation of the gap junction protein connexin 43 (Cx43) is also implicated in the destabilization of the interaction between tumor and NK cells (lower right). The activation of the signal transducer and activator of transcription 3 (STAT3) signaling along with autophagy activation in tumor cells leads to the acquisition of resistance to CTL-mediated lysis (upper left). Autophagy activation, following epithelial-to-mesenchymal (EMT) induction, leads to BECN1 overexpression, and is sufficient to overcome CTL-mediated attack (lower left).

functions strongly contribute to decrease the antitumor immune response (68).

AUTOPHAGY INHIBITION ALONG WITH NEW COMBINATORIAL IMMUNE CHECKPOINT BLOCKERS

Immune checkpoint-based immunotherapy revolution has just started, and new combination strategies with potent curative potential are currently emerging (22). In spite of outstanding and encouraging response, the majority of patients treated with anti-PD-1/PD-L1 monotherapies have partial tumor regressions and they fail to achieve higher objective responses. This suggests that in order to obtain durable tumor regressions, there is a need for combination therapies along with anti-PD-1/PD-L1 monotherapies. Several reports using various preclinical animal models have shown that combination therapies based on immune checkpoint blockers are strongly synergistic along with classical cancer therapies (including chemotherapy, tyrosine kinase inhibitors, radiotherapy, and vaccines) (21).

The combination of both anti-CTLA-4 and anti-PD1 has been shown to be twice as effective as either treatment alone in the rejection of B16-F10 melanoma tumors. This combination therapy increased T-effector to MDSC ratio and also increased IFN-γ/TNF-α double producing CD8+ T cells. Finally, PD-1 and CTLA-4 combination blockade expanded infiltrating T cells and reduced regulatory T and myeloid cells within B16 melanoma tumors (69). Similarly, in mouse models of colon carcinoma (CT26 cell line) and ovarian carcinoma (ID8-VEGF cell line), dual block (both anti-PD1 and anti-CTLA-4) combined with tumor vaccine leads to effective tumor rejection through the restoration of effector T cell function (70). More interestingly, it has been reported that the combination of anti-4-1BB and anti-PD-1 is more effective than that of anti-PD-1/anti-LAG-3 in suppressing B16-F10 melanoma tumor growth without adjuvant or vaccination (71). In a phase 1 clinical trial with advanced melanoma patients, the combination of anti-CTLA-4 and anti-PD-1 increased the response rate up to 53% patients with severe treatment-related adverse events (72). Autophagy inhibitor CQ is currently used in several clinical trials to sensitize tumor cells to radio and chemotherapy. We have previously shown that, by simultaneously boosting the immune system and inhibiting autophagy using CQ, we can significantly enhance the therapeutic efficacy of antigen-based cancer vaccines in B16 tumors (51). In this regard, a combination of autophagy inhibition and immune checkpoint-based immunotherapy would probably revert immune suppressive microenvironment and promote additional tumor regression. Although anti-CTLA-4 and anti-PD-1 are currently the focus of clinical attention, it is likely that blockade of additional checkpoints will result in even further clinical activity as multiple checkpoints appear to be co-expressed with PD-L1 and PD-1 on the same tumors (21, 22).

CONCLUDING REMARKS

Cancer immunotherapy based on immune checkpoint inhibitors marks a turning point in cancer treatment since it revolutionizing the way we treat cancer. Immune checkpoint-based immunotherapies have shown great promise for a subset of cancer patients. However, safe, robust, and attentive combination therapies are still needed to bring the benefit of cancer immunotherapy to a larger class of cancer patients. While searching for an optimal strategy of combinatorial immunotherapy, we have to keep in mind that different immune checkpoints are expressed simultaneously on both tumor cells, stromal cells, and different immune cells. Furthermore, there is a differential expression of both inhibitory and activating receptors on different immune cells within the tumor microenvironment. Autophagy remains to be a pretty interesting combination target while developing novel and cutting edge cancer immunotherapeutic approaches.

AUTHOR CONTRIBUTIONS

EV, MN, TA, AL, SC, GB, EM, JP, and BJ contributed to the writing of the manuscript and the conception of the figures.

FUNDING

This work was supported by grants from "Fondation Cancer" Luxembourg FC/2016/01; FNRS "Télévie" (7.4664.15; 7.4571.15; 7.6503.16; 7.4563.15) and Luxembourg Institute of Health (LECR-TM 2013 11 05 and LECR-TSI HEMATEXO).

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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.

frontiers in Oncology

REVIEW
published: 05 July 2017
doi: 10.3389/fonc.2017.00140

IP_3 Receptor-Mediated Calcium Signaling and Its Role in Autophagy in Cancer

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Calcium ions (Ca^{2+}) play a complex role in orchestrating diverse cellular processes, including cell death and survival. To trigger signaling cascades, intracellular Ca^{2+} is shuffled between the cytoplasm and the major Ca^{2+} stores, the endoplasmic reticulum (ER), the mitochondria, and the lysosomes. A key role in the control of Ca^{2+} signals is attributed to the inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs), the main Ca^{2+}-release channels in the ER. IP_3Rs can transfer Ca^{2+} to the mitochondria, thereby not only stimulating core metabolic pathways but also increasing apoptosis sensitivity and inhibiting basal autophagy. On the other hand, IP_3-induced Ca^{2+} release enhances autophagy flux by providing cytosolic Ca^{2+} required to execute autophagy upon various cellular stresses, including nutrient starvation, chemical mechanistic target of rapamycin inhibition, or drug treatment. Similarly, IP_3Rs are able to amplify Ca^{2+} signals from the lysosomes and, therefore, impact autophagic flux in response to lysosomal channels activation. Furthermore, indirect modulation of Ca^{2+} release through IP_3Rs may also be achieved by controlling the sarco/endoplasmic reticulum Ca^{2+} ATPases Ca^{2+} pumps of the ER. Considering the complex role of autophagy in cancer development and progression as well as in response to anticancer therapies, it becomes clear that it is important to fully understand the role of the IP_3R and its cellular context in this disease. In cancer cells addicted to ER–mitochondrial Ca^{2+} fueling, IP_3R inhibition leads to cancer cell death *via* mechanisms involving enhanced autophagy or mitotic catastrophe. Moreover, IP_3Rs are the targets of several oncogenes and tumor suppressors and the functional loss of these genes, as occurring in many cancer types, can result in modified Ca^{2+} transport to the mitochondria and in modulation of the level of autophagic flux. Similarly, IP_3R-mediated upregulation of autophagy can protect some cancer cells against natural killer cells-induced killing. The involvement of IP_3Rs in the regulation of both autophagy and apoptosis, therefore, directly impact cancer cell biology and contribute to the molecular basis of tumor pathology.

Keywords: Ca^{2+} signaling, inositol 1,4,5-trisphosphate, inositol 1,4,5-trisphosphate receptors, autophagy, apoptosis, cancer

OPEN ACCESS

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *07 April 2017*
Accepted: *19 June 2017*
Published: *05 July 2017*

Citation:
Kania E, Roest G, Vervliet T, Parys JB and Bultynck G (2017) IP_3 Receptor-Mediated Calcium Signaling and Its Role in Autophagy in Cancer. Front. Oncol. 7:140. doi: 10.3389/fonc.2017.00140

INTRACELLULAR Ca^{2+} SIGNALING: THE ENDOPLASMIC RETICULUM (ER), MITOCHONDRIA, AND LYSOSOMES

Intracellular Ca^{2+} signaling controls a plethora of cellular processes, including secretion, gene transcription, metabolism, and cell death, thereby impacting cell function and cell survival (1–3). Intracellular Ca^{2+} signals are characterized by their spatiotemporal properties. As a function of time, Ca^{2+} signals can occur as transient increases in [Ca^{2+}] (Ca^{2+} oscillations) (4) or as more

sustained increases in [Ca^{2+}] (global Ca^{2+} transients) (5). As a function of space, Ca^{2+} signals can occur in localized domains near the plasma membrane (PM) or organelles, such as the ER, mitochondria, lysosomes, Golgi, and nucleus (3, 6). Localized Ca^{2+} signaling is established in so-called microdomains due to the close apposition of different organellar compartments (7–9) or of organelles with the PM through molecular tethers (10–12).

The major intracellular Ca^{2+}-storage organelle is the ER, where most of the intracellular Ca^{2+} is accumulated *via* active Ca^{2+} transport mediated by sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA) followed by intraluminal Ca^{2+} buffering by calreticulin, calnexin, and other Ca^{2+}-binding proteins (13, 14). These mechanisms allow for an adequate Ca^{2+} filling of the ER, which is required for the activity of molecular chaperones and the folding of enzymes. Hence, a depletion of the ER Ca^{2+} stores leads to ER stress, a condition associated with impaired protein folding capacity (13, 15, 16). To cope with this, cells engage the unfolded protein response, a concerted program triggered through the three classical ER stress sensors: inositol-requiring enzyme1α, RNA-dependent protein kinase-like ER kinase, and activating transcription factor 6 (17). Mild or transient ER stress induces activity of chaperones, folding enzymes, reactive oxygen species (ROS) scavengers, and degradative pathways, such as autophagy, while severe or persistent ER stress induces cell death (18–20).

These Ca^{2+}-uptake mechanisms are counteracted by, on the one hand, Ca^{2+}-leak channels and, on the other hand, Ca^{2+}-release channels (21). Ca^{2+}-leak channels establish a constitutive, passive Ca^{2+} leak from the ER, preventing ER Ca^{2+} overload that would result in cell death. Different ER Ca^{2+}-leak channels have been identified, likely all contributing to this passive Ca^{2+} leak to some extent, although it is possible that some of these channels are restricted to certain cell types or systems (22–25). Presenilin 2, Ca^{2+} release-activated Ca^{2+} channel protein 2 (Orai2) and inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) isoform 1 (IP_3R1) have been proposed as major ER Ca^{2+}-leak channels, based on a systems biology approach using HeLa cells (26). However, the ER Ca^{2+}-leak rates in wild-type HEK293 versus HEK293 cells lacking all three IP_3R isoforms, directly measured by using a genetically encoded ER Ca^{2+} sensor, were very similar, indicating that at least in HEK cells and these experimental conditions, IP_3R does not contribute in an important way to the passive Ca^{2+} leak from the ER (27). These Ca^{2+}-leak channels impact the steady-state Ca^{2+} content of the ER, which determines the Ca^{2+} available for release upon agonist stimulation (28, 29). Ca^{2+} release from the ER occurs through IP_3Rs (30, 31) or ryanodine receptors (RyRs) (32–34). IP_3Rs are ubiquitously expressed and are activated in response to IP_3, which is produced from phosphatidylinositol 4,5-bisphosphate (PIP_2) upon hydrolysis mediated by phospholipase C (35). This typically happens in response to cellular stimulation with hormones, growth factors, neurotransmitters, or antibodies. However, it is clear that many cells even in basal, non-stimulated conditions, display a constitutively low level of IP_3-mediated signaling. The Ca^{2+} depletion of the ER, resulting from Ca^{2+} release, can trigger the activation of store-operated Ca^{2+} entry (SOCE) through stromal interaction molecule 1 (Stim1)-dependent activation of Ca^{2+} release-activated Ca^{2+} channel protein 1 (Orai1) channels (36–38).

Ca^{2+} release from the ER does not only result in a [Ca^{2+}] rise in the cytosol but also leads to [Ca^{2+}] increase in other organelles, including the mitochondria and the lysosomes (6, 39). This is due to contact sites between ER and mitochondria and between ER and lysosomes, decreasing the distance between these organelles and the ER (39–43). In addition to this, a highly negative potential of about −180 mV exists across the mitochondrial inner membrane, establishing a strong electrochemical driving force for Ca^{2+} uptake in the mitochondria (40). It is well known that IP_3R-mediated Ca^{2+} release from the ER can "quasi-synaptically" transfer into the mitochondria (7, 44). This occurs *via* the so-called mitochondria-associated ER membranes (MAMs) that also harbor the IP_3R and the voltage-dependent anion channel type 1 that permeates Ca^{2+} across the mitochondrial outer membrane (39, 41, 42). Once in the mitochondrial intermembrane space, Ca^{2+} is transported across the mitochondrial inner membrane *via* the mitochondrial Ca^{2+} uniporter (MCU) (45). The MCU has low inherent affinity for Ca^{2+} and is highly cooperative due to accessory proteins such as MICU1 (46). By comparison, Ca^{2+} uptake into the lysosomes is much less understood. Nevertheless, it is anticipated that a strong electrochemical gradient for H^+ is present resulting from a low lysosomal pH (~4–5) which can be used for lysosomal Ca^{2+} accumulation *via* the lysosomal H^+/Ca^{2+} exchanger (47, 48). Ca^{2+} can be released from these lysosomal Ca^{2+} stores *via* a variety of channels, including two-pore channels 1/2 (TPC1/2) and transient receptor potential superfamily channels such as TRPML1 (mucolipin1/MCOLN1)(47, 49–52). An important second messenger triggering lysosomal Ca^{2+} release through TPC1/2 is nicotinic acid adenine dinucleotide phosphate (NAADP) (53–55). TRPML1 present in endolysosomal vesicles can be activated by phospholipids such as phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$] (56, 57).

Ca^{2+} SIGNALING IN AUTOPHAGY

ER-Derived Ca^{2+} Signaling in Autophagy

It is well-established that Ca^{2+} signaling impacts autophagy initiation and progression (**Figure 1**). Ca^{2+} signaling modulates as well basal autophagic flux as mechanistic target of rapamycin (mTOR)-controlled autophagic flux, induced by nutrient starvation or rapamycin (58–60). Moreover, compounds that directly affect Ca^{2+} signaling, including agonists, Ca^{2+} ionophores, and SERCA inhibitors that indirectly cause ER Ca^{2+}-store depletion *via* the basal Ca^{2+} leak, can modulate the autophagic process (61). The ER, as the main intracellular Ca^{2+}-storage organelle, has been implicated in controlling basal autophagy. This is related to Ca^{2+}-dependent energizing of the mitochondria (62). Basal and constitutive Ca^{2+}-release events from the ER, mediated by IP_3R channels, have been involved in sustaining mitochondrial bioenergetics by driving NADH production and subsequent ATP synthesis by continuously providing Ca^{2+} to the mitochondria (62) (**Figure 1**). This is due to the presence of three mitochondrial Ca^{2+}-dependent tricarboxylic acid (TCA) cycle enzymes, which activities are enhanced by mitochondrial Ca^{2+} (40). Abrogating these Ca^{2+} signals through pharmacological inhibition or genetic knock down of IP_3Rs resulted in an increased autophagic flux

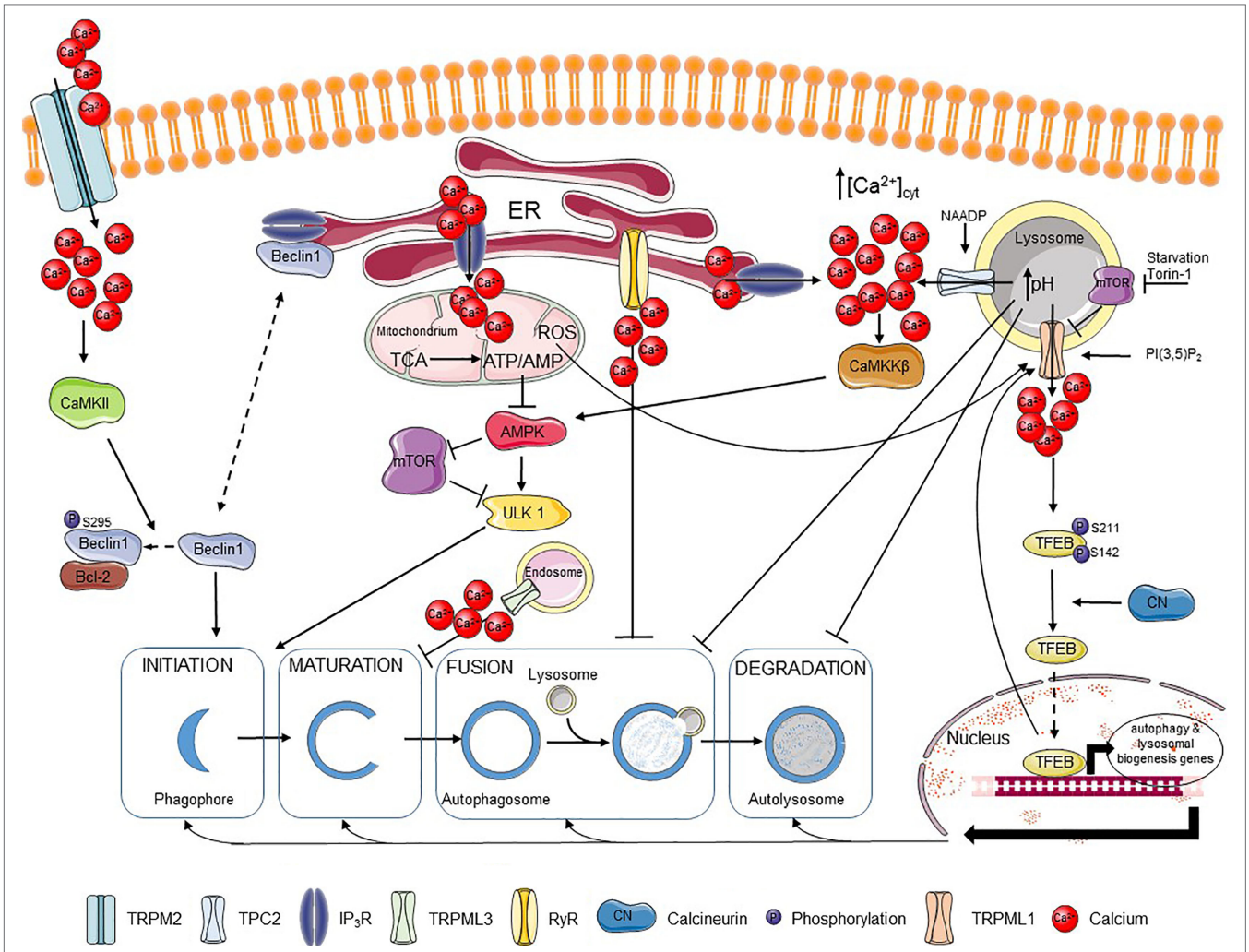

FIGURE 1 | Ca^{2+} regulates different steps of autophagy. Extracellular Ca^{2+} can enter the cell *via* the TRPM2 channel, where it activates CaMKII that phosphorylates Beclin 1, promoting its binding to Bcl-2 and blocking autophagy initiation. Beclin 1 can also be scaffolded to the IP_3R, which also limits autophagy promotion. IP_3Rs are responsible for fueling mitochondria with Ca^{2+}, which supports the tricarboxylic acid (TCA) cycle and the resulting ATP production. The presence of an adequate ATP/AMP ratio suppresses the AMP-activated kinase (AMPK)–mechanistic target of rapamycin (mTOR)–unc-51-like kinase 1 (ULK1) axis and thus autophagy induction. Autophagosome maturation can be inhibited by the TRPML3 channel mostly expressed in endosomes, while fusion of autophagosomes and lysosomes can be blocked by basal RyR activity. Ca^{2+} release through lysosomal nicotinic acid adenine dinucleotide phosphate (NAADP)-activated TPC2 channels can be further amplified by Ca^{2+} release from IP_3Rs resulting in stimulation of the CaMKKβ–AMPK pathway and autophagosome formation. Ca^{2+} release from the lysosomes can, however, affect lysosomal pH and so abrogate fusion of lysosomes with autophagosomes as well as the further lysosome-dependent degradation. TRPML1, another lysosomal channel, can be regulated by different factors, including lysosomal mTOR, $PI(3,5)P_2$ or reactive oxygen species (ROS). Ca^{2+} released from TRPML1 activates calcineurin, which binds and dephosphorylates TFEB and promotes its nuclear translocation, where TFEB induces transcription of various autophagy-related and lysosomal biogenesis genes. Plain black arrows indicate activatory and inhibitory pathways; dashed arrows indicate intracellular movement.

due to an increase in the activity of the AMP-activated kinase (AMPK), a positive regulator of autophagy (i) inhibiting mTOR and (ii) activating the unc-51-like kinase 1 (ULK1) complex (63). However, the increased basal autophagic flux triggered upon IP_3R inhibition appeared independent on mTOR (62) (**Figure 1**). Thus, IP_3Rs exert an inhibitory role on basal autophagy, and consequently IP_3R inhibition results in increased basal autophagy. An additional mechanism for this autophagy-inhibitory role of IP_3Rs has been attributed to its Beclin 1-scaffolding function (64, 65). Beclin 1 regulates autophagy by forming a complex with the class III phosphatidylinositide 3-kinase Vps34, thereby stimulating phagophore nucleation, an early step in the autophagy process (66–68). It has been shown that the IP_3R, independently of its Ca^{2+}-flux properties, could serve as a sink for Beclin 1 recruitment, reducing the availability of free Beclin 1 to drive autophagy (69) (**Figure 1**).

Besides IP_3Rs, RyRs have also been implicated in autophagy. In hippocampal neuronal stem cells, insulin withdrawal resulted in the upregulation of the RyR3 isoform, which triggered cell death through increased autophagy. Activation of RyRs using

caffeine increased autophagic cell death, while their inhibition using dantrolene suppressed this process (70). However, more recently, RyRs, both endogenously expressed in skeletal muscle cell lines and in dissociated rat hippocampal neurons or ectopically expressed in HEK cells, have been implicated in inhibition of autophagic flux, particularly at the level of autophagosome/lysosome fusion (**Figure 1**). RyR inhibition resulted in an increase of autophagic flux, independent of mTOR activity or of early autophagy regulators (71). This indicates that both IP_3Rs and RyRs could suppress basal autophagy, but by acting at a different level: IP_3Rs by suppressing autophagy at a proximal level by driving mitochondrial bioenergetics and thus decreasing AMPK activity, while RyRs block autophagy at a distal level by counteracting the fusion of autophagosomes and lysosomes.

In contrast to this, Ca^{2+} mobilization from the ER into the cytosol by itself can augment autophagic flux. Both physiological agonists as well as chemicals, such as Ca^{2+} ionophores and SERCA inhibitors, that provoke $[Ca^{2+}]$ rises in the cytosol originating from the ER result in the activation of Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ) that is an upstream activator of AMPK and autophagy (72). In fact, mechanisms that limited cytosolic $[Ca^{2+}]$ rise induced by these agents also limited autophagy induction. As such, Bcl-2, a known negative regulator of autophagy, was proposed not only to limit autophagy by scaffolding Beclin 1 but also by reducing the ER Ca^{2+}-store content, thus suppressing cytosolic $[Ca^{2+}]$ rises and the extent of CaMKKβ activation (**Figure 1**) (73).

In addition to this, cytosolic $[Ca^{2+}]$ rises can promote autophagosome formation by recruiting the phosphatidylinositol 3-phosphate [PI(3)P]-binding protein, encoded by autophagy-related gene (Atg) 18 (WIPI1/WIPI2), to autophagosomal membranes. Atg18, together with Atg16L and ULK1, are recruited to early autophagosomal structures and are critical for the formation of LC3-positive autophagosomes (74). Furthermore, the autophagosomal recruitment of Atg18 was blocked by cytosolic Ca^{2+} chelation. This mechanism allowed for an induction of autophagy by cytosolic $[Ca^{2+}]$ rise independently of AMPK activation or mTOR inhibition (75). However, apart from the autophagy-inducing properties of thapsigargin and Ca^{2+} ionophores, these agents have also been reported to suppress autophagosome biogenesis at steps in the autophagy pathway subsequently to WIPI1 punctae formation but preceding autophagosome closure (76). This may relate to the disturbance of critical Ca^{2+} fluxes from the ER during the distal steps in the autophagy process, including the closure of the autophagosomal vesicles.

Beyond these roles in modulating basal autophagy, the ER and more particular ER-derived Ca^{2+} signals mediated *via* IP_3Rs have been involved in driving starvation- and rapamycin-induced autophagy (58, 59). Starvation and rapamycin are two triggers that induce autophagy through inhibition of mTOR, a negative regulator of autophagy. Starvation- and rapamycin-induced autophagy resulted in enhanced Ca^{2+} signaling from the ER through a mechanism that involved complex formation between IP_3Rs and Beclin 1 and a subsequent direct IP_3R sensitization by Beclin 1 (58) (**Figure 1**). In turn, cytosolic Ca^{2+} and IP_3R activity were critical for cells to be able to increase their autophagic flux in response to nutrient starvation and rapamycin.

Resveratrol (RSV) is a polyphenol found in some food products and in red wine. Several health-promoting effects have been attributed to RSV, including longevity, anti-aging, and improved cardiovascular health (77, 78). These beneficial effects of RSV have been linked to its ability to induce autophagy. RSV induces autophagy in a pleiotropic manner *via* both mTOR-dependent and -independent mechanisms. RSV can activate the deacetylases sirtuins, a positive regulator of AMPK (79, 80). RSV can also directly inhibit mTOR by docking onto its ATP-binding pocket and thus competing with ATP. The inhibition of mTOR and the presence of ULK1 appeared to be critical for RSV-induced autophagy (81). However, RSV can also promote autophagy in a non-canonical manner, whereby RSV induces autophagosome formation independently of Beclin 1 or its binding partner Vps34 (82, 83). Also, Ca^{2+} signaling has been implicated in RSV-induced autophagy (27). RSV can deplete the ER Ca^{2+} stores independently of the presence or absence of IP_3Rs (27), which in part may be due to its inhibitory effect on mitochondrial ATP production, thereby suppressing SERCA-mediated ER Ca^{2+} uptake (84). Yet, although RSV triggered a Ca^{2+} leak from the ER independently of IP_3Rs, the ability of RSV to induce autophagy was critically dependent on the presence of IP_3Rs and on the availability of cytosolic Ca^{2+} (27). In this study, the inhibitory effect of RSV on mTOR activity was confirmed and did neither require cytosolic Ca^{2+} nor IP_3R expression (27).

Endolysosomal-Derived Ca^{2+} Signaling in Autophagy

A genetic analysis of mucolipidosis type IV (MLIV), a lysosomal storage disease associated with severe neurological deficiencies, implicated that mutations in TRPML1 play a role in autophagy deregulation (85). Fibroblasts derived from MLIV patients expressing mutant TRPML1 displayed an increased autophagosome formation accompanied with a delay in the fusion of autophagosomes with lysosomes. This was proposed to contribute to an accumulation of p62 and a defective removal of ubiquitinated proteins and/or defective mitochondria (85). Also, chaperone-mediated autophagy (CMA) was defective in MLIV fibroblasts, which could be attributed to TRPML1's ability to bind and recruit Hsc70 and Hsp40 proteins, two components critical for CMA, and a subsequent reduction in lysosomal LAMP2A-protein levels. As a consequence, oxidized proteins may accumulate in the cytosol due to their impaired degradation *via* CMA (86). However, in these studies, the role of TRPML1 channel activity and lysosomal Ca^{2+} release was not addressed.

One of the first data linking TRPML-mediated Ca^{2+} release and the autophagy pathway was provided from overexpression studies using TRPML3/MCOLN3 (87). This channel is mainly present in early endosomal compartments, where pH is not as low as in lysosomes. These endosomal compartments may host more functional TPRML3 channels than lysosomes, as low pH appears to inactivate TRPML3-mediated Ca^{2+} flux. Overexpression of TRPML3/MCOLN3 not only resulted in severe changes in the endosomal pathway, including increased endosomal pH, but also in defective autophagosome maturation (87) (**Figure 1**).

More recently, a direct role of lysosomal Ca^{2+} release through TRPML1 channels in upregulating autophagy upon mTOR inhibition has been elucidated (88). Nutrient starvation resulted in rapid peri-lysosomal [Ca^{2+}] rises, in the close proximity of TRPML1 channels. These [Ca^{2+}] rises were concentrated around lysosomes and could not be observed in the bulk cytosol. In turn, [Ca^{2+}] rises resulted in the activation of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, which can dephosphorylate the transcription factor TFEB. As a consequence, upon nutrient starvation, calcineurin dephosphorylated TFEB at two residues (Ser142 and Ser211) regulating TFEB nuclear translocation, resulting in nuclear accumulation of TFEB and activation of genes necessary for autophagy and lysosomal biogenesis (88) (**Figure 1**).

TRPML1 itself is also regulated during nutrient starvation. TRPML1 and the hereby associated Ca^{2+} flux from the lysosomes became upregulated upon nutrient deprivation, which was accompanied by transcription of autophagy-regulating genes (89). TRPML1 upregulation also occurred in response to complete inhibition of the mTOR complex 1 by Torin-1, while this was not observed upon treatment with rapamycin, a partial, allosteric inhibitor of mTORC1. A critical role for TFEB was found in the upregulation of TRPML1. Both starvation and Torin-1 treatment were able to induce TFEB dephosphorylation and its nuclear translocation, while rapamycin failed to do this. A more direct link between TFEB activation and TRPML1 upregulation was shown by overexpressing constitutively dephosphorylated and thus active TFEB in cells, which resulted in a functional upregulation of TRPML1 channels. The upregulation of TRPML1 activity by TFEB could be partially attributed to an increase in mRNA and protein expression of the channel, but likely also involved post-translational modifications or upregulation of TRPML1-interacting/modulating proteins. Moreover, a role of the lysosomal lipid $PI(3,5)P_2$, a TRPML1-activating lipid which levels decrease upon nutrient starvation, was proposed as part of a compensatory mechanism that causes upregulation of TRPML1. At the functional level, TRPML1 activity was critical for the increase in lysosomal proteolytic activity induced by nutrient starvation (89).

Interestingly, TRPML1 activity is not only controlled by $PI(3,5)P_2$ levels and *via* the TFEB transcription factor but also directly by mTOR (90). In nutrient-replete conditions, when mTOR was active and autophagy was suppressed, mTOR phosphorylated two serine residues in the C-terminal tail of TRPML1, resulting in TRPML1 channel inhibition. Upon mTOR inhibition by rapamycin, leading to autophagy induction, TRPML1 became dephosphorylated and active, resulting in lysosomal Ca^{2+} release. Moreover, rapamycin could induce Ca^{2+} release in cells expressing wild-type TRPML1 but not in cells expressing TRPML1 in which the two phosphorylable serine residues were mutated. Thus, loss of TRPML1 phosphorylation upon mTOR inhibition results in increased TRPML1 activity, driving autophagic flux (90). However, at this point, it is not clear which phosphatase is responsible for dephosphorylating the serine residues that are phosphorylated by the mTOR kinase.

Recently, it was shown that TRMPL1 can serve as a redox status sensor and can release Ca^{2+} upon stimulation by ROS or by mitochondrial uncouplers (91, 92). As a result of this Ca^{2+} release, also calcineurin-dependent TFEB activation and nuclear translocation occurred, which could be blocked by the ROS scavenger N-acetyl-cysteine (NAC), as well as by BAPTA-AM or synthetic TRPML1 inhibitors (ML-SIs). Since mitochondrial uncouplers failed to stimulate nuclear translocation of TFEB in TRPML1 knockout (KO) cells, this points out that TRPML1 is specifically required for ROS-induced activation of TFEB. By contrast, functional TRMPL1 was not required for TFEB nuclear translocation induced by mTOR inhibition through Torin-1 or nutrient starvation (91). In addition, ROS-induced autophagy and lysosome biogenesis could be impeded by NAC treatment as well as by ML-SIs or TRPML1 KO (91).

In contrast to TRPML1-mediated Ca^{2+} release from the lysosomes, thereby positively regulating autophagy, TPC2 has been implicated in the inhibition of autophagy (93). TPC2 channels are activated by NAADP. Overexpression of TPC2 resulted in the accumulation of autophagosomes, a phenomenon boosted by NAADP but antagonized by either the NAADP antagonist Ned19 or by knockdown (KD) of the essential autophagy gene Atg5. The effect of TPC2 on autophagosome accumulation could be attributed to an increase in lysosomal pH upon lysosomal Ca^{2+} release, which inhibits autophagy at the level of autophagosome–lysosome fusion (**Figure 1**). Therefore, lysosomal acidification could suppress TPC2-induced autophagosome accumulation (93). Similar findings of TPC2 have been observed in astrocytes, where its overexpression resulted in an increase in Beclin 1 and LC3-II levels (94). The latter may also relate to the accumulation of autophagosomes.

Ca^{2+} Influx from Extracellular Environment

Recently, oxidative stress has been implicated in autophagy inhibition through induction of melastatin-related transient receptor potential cation channel member 2 (TRPM2)-mediated Ca^{2+} influx (95). Ca^{2+} influx resulted in the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which phosphorylated Beclin 1 at Ser295 and abolished its autophagy-inducing properties. The mechanism involved a decrease in Vps34 complex formation with phospho-Beclin 1 and increased Bcl-2 binding of phospho-Beclin 1 (**Figure 1**). Consequently to autophagy inhibition, oxidative stress triggered cell death in cells expressing TRPM2, while TRPM2 KD resulted in upregulated autophagy as a survival pathway in these cells. In addition, TRPM2/CaMKII activation further increased ROS production and contributed to mitochondrial fragmentation and loss of mitochondrial potential (95, 96).

IP_3Rs AND AUTOPHAGY IN CANCER

IP_3Rs in Cancer

IP_3Rs control different hallmarks of cancer (97, 98). In particular, IP_3Rs impact cell death and survival by mediating Ca^{2+} release from the ER and subsequently affecting mitochondria-regulated processes, including bioenergetics and apoptosis (99). Moreover, several IP_3R isoforms can have distinct functions, dependent not only on their functional properties but also on their subcellular localization (100). For instance, IP_3R3 has been particularly

associated with pro-apoptotic Ca^{2+} flux from ER into mitochondria due to its localization at ER–mitochondrial contact sites (101, 102). As such, depending on the isoform and localization of the IP_3R that is modulated, opposite effects can occur. Enhanced basal IP_3R activity outside the MAMs may cause an increased passive Ca^{2+} leak from the ER. As a consequence, ER Ca^{2+} stores become less filled whereby less Ca^{2+} becomes available to be delivered to the mitochondria upon cellular exposure to a toxic, pro-apoptotic stimulus. By contrast, enhanced IP_3R activity at the MAMs can increase not only mitochondrial bioenergetics but also the likelihood for pro-apoptotic Ca^{2+} transfers. In addition, Ca^{2+} transfer into the mitochondria also participate in oncogene-induced and replicative senescence, a stable proliferation arrest accompanied with distinct features like increased apoptosis resistance and altered gene expression (103–105). Here, IP_3R2 and MCU were implicated in the enhanced mitochondrial Ca^{2+} transfer and accumulation that resulted in cellular senescence due to a decline in mitochondrial membrane potential and an increased ROS production and senescence. Therefore, loss of *ITPR2*, the gene encoding IP_3R2, or of *MCU* overcame the growth arrest and escape from senescence (105, 106).

IP_3Rs emerged as functional targets of an increasing number of oncogenes and tumor suppressors, which dynamically control IP_3R activity and thus Ca^{2+} flux from ER into mitochondria (97, 107). Several oncogenes can suppress pro-apoptotic Ca^{2+}-release events mediated by IP_3Rs and this can occur *via* different mechanisms. First, oncogenes can directly interact with IP_3Rs (97). For instance, anti-apoptotic Bcl-2 targets the central, modulatory domain of the IP_3Rs, thereby suppressing excessive IP_3R activity and protecting cells against pro-apoptotic Ca^{2+}-release events (108–111). Alternatively, oncogenes can exert post-translational modifications of IP_3Rs. Protein kinase B (PKB/Akt) phosphorylates IP_3R3 and dampens its Ca^{2+} flux, suppressing pro-apoptotic Ca^{2+} transfer (112). Oncogenes not only prevent excessive IP_3R-mediated Ca^{2+} release, but they can also promote basal Ca^{2+}-signaling events that are associated with increased mitochondrial bioenergetics and, thus, increased NADH and ATP output. As such, Bcl-XL, another anti-apoptotic Bcl-2-family member sensitizes all IP_3R isoforms, thereby promoting the occurrence of pro-survival Ca^{2+} oscillations and thus sustaining cell survival by boosting the mitochondrial metabolism (113–115).

Not only oncogenes but also tumor suppressors regulate IP_3Rs (97, 107). The product of the BRCA1 gene, frequently mutated in breast cancer, binds and promotes IP_3R activity. This underlies adequate apoptosis sensitivity of cells expressing wild-type BRCA1, while oncogenic mutations fail to engage IP_3Rs and, thus, promote apoptosis resistance (116). Tumor suppressors can also act *via* post-translational modification of IP_3Rs. As such, phosphatase and tensin homolog (PTEN) not only counteracts PKB/Akt activity by reducing phosphatidylinositol 3,4,5-trisphosphate (PIP_3) levels but also reverses the PKB/Akt-dependent phosphorylation of IP_3Rs, particularly at the MAMs where IP_3R3 becomes dephosphorylated and de-repressed (117).

In addition to these events, oncogenic mutations in cancer genes can also affect IP_3R expression levels. As such, the expression of mutant Ras in cells resulted in an IP_3R-isoform switch, thereby reducing the pro-apoptotic IP_3R3 isoform and increasing the pro-survival IP_3R1 isoform (118). IP_3R1 displays a higher IP_3 sensitivity than IP_3R3 and it is proposed to be less involved in pro-apoptotic Ca^{2+} transfers from ER into mitochondria than IP_3R3. The elevated IP_3R1-expression levels resulted in an increased Ca^{2+} leak from the ER and thus a slight decrease in ER Ca^{2+} content, which reduced Ca^{2+} availability for pro-apoptotic Ca^{2+} transfer at the ER–mitochondrial contact sites (118).

Finally, oncogenes and tumor suppressors can affect ER Ca^{2+} homeostasis by modulating other ER Ca^{2+}-transport systems. For instance, the tumor suppressor p53 accumulates at ER membranes, targeting and boosting SERCA in cells exposed to toxic, chemotherapeutic, and photodynamic agents (119, 120). As a consequence, ER Ca^{2+} stores became overfilled and the likelihood to flood mitochondria with excess Ca^{2+} increased, underlying cell death induction by these agents. Cancer cells lacking p53 or having mutated p53 failed to increase SERCA activity and, thus, did not display increased mitochondrial Ca^{2+} overload, which contributed to the resistance of these cells to these toxic agents (119, 120). Recently, in neuroblastoma cells, acute application of cisplatin, a DNA alkylating agent, and topotecan, a topoisomerase I inhibitor, resulted in a rapid Ca^{2+} release from the ER stores (121). In addition to this, long-term exposure of neuroblastoma to these drugs resulted in a remodeling of Ca^{2+}-transport systems, including an upregulation of IP_3R and RyR isoforms. Blocking Ca^{2+} release from the ER by inhibiting these channels or by chelating cytosolic Ca^{2+} using a cell-permeable Ca^{2+} buffer, suppressed cell death in neuroblastoma treated with cisplatin and topotecan (121). For a detailed discussion on the impact of oncogenes and tumor suppressors on ER Ca^{2+} signaling and IP_3R more specifically, we would like to refer to other recent reviews dedicated to this topic (107, 122).

In addition to this, altered IP_3R expression has been implicated in a variety of cancer-associated processes. For instance, a subset of tumor tissue samples derived from breast cancer patients express higher IP_3R2/IP_3R3 levels compared to the adjacent non-tumorigenic tissue, which has been related to subsequent alterations in metabolic products (123, 124). IP_3Rs were shown to be critical for the growth-stimulating effects of 17β-estradiol (E2) on tumorigenic MCF-7 breast cancer cells (125). E2 application exerted both acute and long-term effects on IP_3Rs and Ca^{2+} signaling in MCF-7 cells. Acute E2 application triggered IP_3R-mediated Ca^{2+} release, while prolonged E2 application resulted in an upregulation of IP_3R3 expression. Further work indicated that IP_3R3 and Ca^{2+}-dependent K^+ channels (BKCa) functioned in a concerted manner sustaining breast cancer cell proliferation by forming a macromolecular complex (126). Both IP_3R3 and BKCa were critical for the proliferation of MCF-7 cells. Excitingly, in non-tumorigenic MCF-10A cells, IP_3R3 and BKCa did not form such a complex and their expressions were dispensable for the proliferation of these cells (126). IP_3Rs have also been implicated in the migration of cancer cells, a process associated by an epithelial–mesenchymal transition and stimulated upon loss of cell–cell contact (127). In disconnected pancreatic ductal adenocarcinoma (PDAC) cells, IP_3Rs, together with Stim1-containing ER–PM junctions, redistributed to the leading edge of individual PDAC cells, supporting PDAC cell migration (127). Moreover, the selective inhibition of IP_3Rs and SOCE lead to reduced cell

migration underlying the importance of Ca^{2+} signaling in this process (127). Increased IP_3R3 expression and IP_3R-derived Ca^{2+} signals have also been shown to correlate with the invasive properties of glioblastoma cells (128, 129). Inhibition of IP_3Rs with caffeine inhibited the invasion and migration of glioblastoma cells and increased the survival of mice xenografted with glioblastoma cells (128). Interestingly, not only inhibition of IP_3Rs but also stimulation of IP_3Rs suppresses glioblastoma cell growth and invasion. Indeed, trifluoperazine (TFP), a FDA-approved antipsychotic drug, impeded proliferation, invasion, and motility of glioblastoma cells *in vitro* and *in vivo* by eliciting Ca^{2+} release from the ER through IP_3R1 and IP_3R2 channels, while IP_3R3 channels were dispensable for TFP-induced Ca^{2+} mobilization. TFP-induced Ca^{2+} rise also depended on the presence of the calmodulin subtype 2 (CaM2) protein, which correlates with previous work revealing TFP as a calmodulin-inhibitory molecule by inducing a conformational change in Ca^{2+}-calmodulin (130). Hence, it was proposed that TFP by targeting and antagonizing CaM2 alleviates CaM2's inhibitory action on IP_3Rs, resulting in a potent and irreversible Ca^{2+} release, responsible for the cell growth and invasion restraint of glioblastoma cells (129). More recently, IP_3R-mediated Ca^{2+} signaling has been shown to be critical for normal T-cell development through repression of Sox1, an antagonist of the transcription factor Tcf1, which is important for normal T-cell development. In the absence of IP_3R expression and activity, Notch signaling becomes active in T cells in post β-selection thymocytes, resulting in the development of aggressive T-cell acute lymphoblastic leukemia (131).

Autophagy in Cancer

Autophagy is a basic catabolic process, existing in all types of cells, where it functions mostly in controlling protein turnover and sustaining energetic balance (132). In cancer, however, the role of autophagy is more complex and can exert different effects depending on the stage of tumor progression, tissue origin, genetic background, etc. Therefore, autophagy in cancer can serve as both a tumor suppressive and a tumor-promoting mechanism (133–135).

The essential autophagy protein Beclin 1, encoded by the gene *BECN1*, has been shown to act as a haploinsufficient tumor suppressor protein (136). In fact, mice that are haplo-deficient for *BECN1* develop spontaneous tumors due to an impaired basal autophagy and in humans, mutations in *BECN1* occur in up to 75% of breast, ovarian, and prostate cancers (137). Also other autophagy-involved proteins, such as UV radiation resistance-associated gene (UVRAG), Atg5, and Atg7, were recently described as tumor suppressors (137). These findings strongly support an oncosuppressive role of autophagy especially at early stages of tumor development. Autophagy contributes to the maintenance of cellular homeostasis, largely by degradation of protein aggregates and dysfunctional mitochondria but also by supplying nucleotides for DNA repair processes (138, 139). This further protects cells against proteotoxicity, oxidative stress, and genomic instability—the conditions supporting tumor development. In some settings, autophagy was also shown to be necessary to execute cell death to prevent tumor transformation (140). In p53-mediated cell death, expression of the lysosomal protein damage-regulated autophagy modulator-1 (DRAM-1), responsible for autophagy induction, was critical for apoptosis to occur (141). DRAM-1 was also shown to be downregulated in a subset of epithelial cancers (141) possibly underlying a similar tumor-suppressive function as Beclin 1.

In contrast to this, cancer cells become addicted to autophagy at the later stage of tumorigenesis. Studies from Guo et al. (142, 143) revealed that in *Kras*-driven, genetically engineered mouse models of non-small-cell lung cancer (NSCLC), deletion of *Atg7* caused accumulation of mitochondria, suppression of tumor growth and the promotion of tumor cell death. These data underscore the role of Atg7, which was required for NSCLC growth, survival, and malignancy. Furthermore, systemic genetic ablation of Atg7 in mice with established NSCLC, promoted tumor regression before damage occurred to the normal tissues (144). These findings indicate that tumors can be selectively autophagy dependent and that there exists a "therapeutic window" for autophagy modulation (144). In particular, autophagy could compensate metabolic stress by providing bioenergetics substrates for the TCA cycle and nucleotides for biosynthetic pathways, thereby supporting cancer cell survival (139). In *Kras*-driven NSCLC, autophagy-mediated recycling was able to sustain the levels of amino acids and several metabolites during starvation. However, further autophagy ablation caused deficiencies in mitochondrial substrates. Supplementation of glutamine, glutamate, and nucleotides was, therefore, critical to overcome autophagy deficiency caused by *Atg7* deletion which indicates the role of autophagy in starvation survival (139). Autophagy can also serve as a tumor pro-survival mechanism in response to chemotherapy and other anticancer treatments. It is known that the use of anticancer agents, such as 5-fluorouracyl, bortezomib, or tamoxifen, results in elevated autophagy, which counteracts cell death induction and decreases therapy efficiency (145). Recently, it has been described that verapamil, an L-type calcium channel blocker, can induce a cytotoxic effect and autophagy in colon cancer cells. Cell death in these cells was further increased when verapamil treatment was accompanied by chloroquine, an autophagy inhibitor, or when autophagy was ablated by *Atg5* and *Atg7* deletion (146). Usage of chloroquine and hydroxychloroquine is a common strategy for autophagy inhibition and, therefore, the enhancement of anticancer therapy effectiveness (147). Recent studies, however, have shown that chloroquine can exert anticancer effects independently of autophagy (148). In fact, chloroquine acted by reducing intratumoral hypoxia and metastasis, specifically normalizing tumor vessels by a mechanism involving NOTCH-1. Nevertheless, the use of autophagy inhibitors, as well as genetic tools to abort autophagy, strongly support the hypothesis of tumor-promoting effects of autophagy at the later stages of tumor development.

As discussed above, autophagy is most likely playing a tumor-suppressive role at early steps of tumor development, while it tends to function as a tumor-promoting mechanism in established tumors. This division, however, is not always clear as the role of autophagy in cancer can also depend on other factors such as the genetic background of a particular tumor. Recent studies revealed that in a humanized genetically modified mouse model of PDAC, deletion of autophagy genes *Atg5* and *Atg7* can

play different roles according to the status of p53 (149). In mice possessing an oncogenic allele of *Kras*, ablation of *Atg5* and *Atg7* prevented further tumor development. However, in mice with mutated *Kras* and additionally lacking *p53*, blockage of autophagy significantly accelerated formation of tumor lesions (149, 150). This and other examples (151) indicate that the role of autophagy strongly depends on the tumor context and this should be considered while designing autophagy modulation-based therapies. For a further discussion on the role of autophagy in cancer, we refer to more detailed reviews (152–155).

IP_3Rs AND AUTOPHAGY CONTROL: A ROLE IN CANCER?

Autophagy Contribution to Modulation of Cancer Cell Death Induced by IP_3R Inhibition

Recently, it was shown that cancer cells may be addicted to basal IP_3R activity and its critical role in feeding mitochondria with Ca^{2+}, a regulator of the activity of several TCA cycle enzymes. Similarly to non-tumorigenic cells, in tumorigenic cells IP_3R inhibition or KD resulted in an increased autophagic flux (156). However, while this was sufficient to sustain cell survival in non-tumorigenic cells, the increase in autophagy was not sufficient for the survival of cancer cells. Thus, pharmacological inhibition using xestospongin B or genetic KD of IP_3Rs in cancer cells resulted in cancer cell death (156) (**Figure 2A**). Mitochondrial Ca^{2+} and its positive effect on the TCA cycle does not only serve to support ATP synthesis but also to support several anabolic pathways that use mitochondrial TCA cycle intermediates in their biosynthetic pathway (157). In non-tumorigenic cells, the lack of ER–mitochondrial Ca^{2+} transfers due to IP_3R inhibition dampened cell cycle progression, thereby arresting cells at the G1/S checkpoint. Indeed, adequate ATP production is an integral part of the G1/S checkpoint and a surge in ATP production is needed for cells to progress from the G1 phase to the S phase *via* a mechanism that involves cyclin E upregulation (158). This surge in ATP output is achieved by mitochondrial hyperfusion at that stage of the cell cycle (159). In cells experiencing blunted ER–mitochondrial Ca^{2+} transfer (like upon IP_3R inhibition), ATP output would be impaired and AMPK can be activated. One of

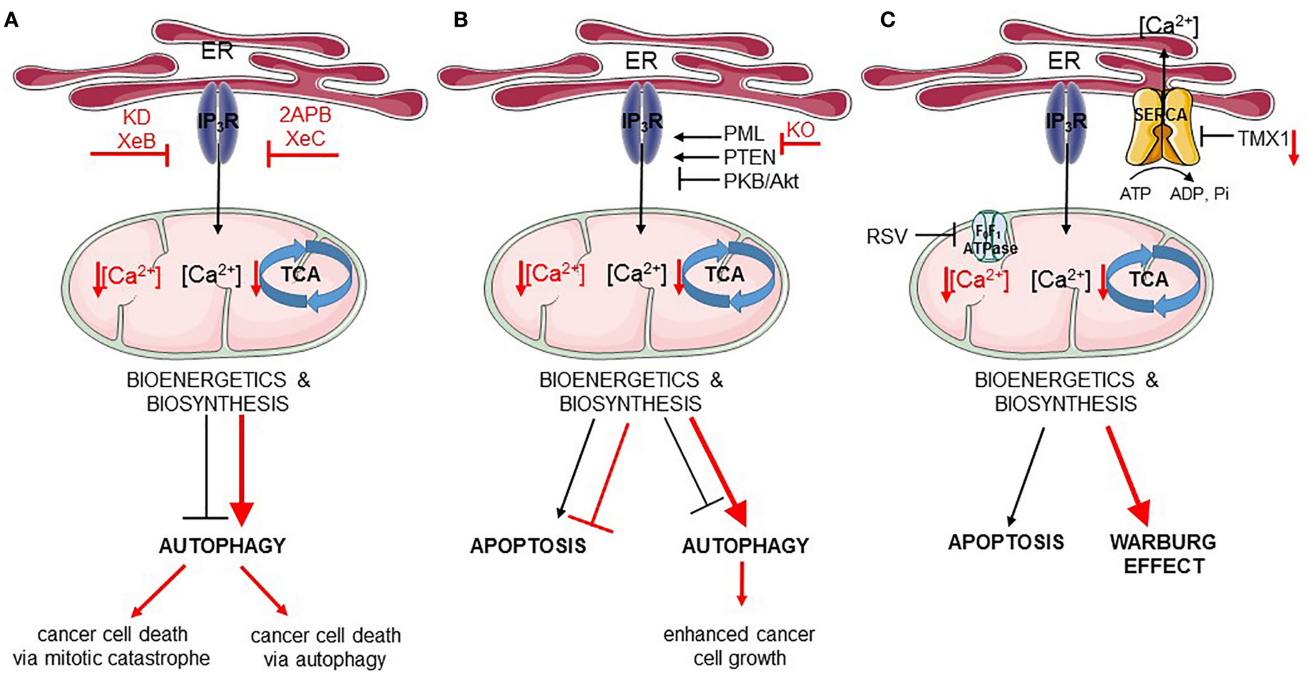

FIGURE 2 | Endoplasmic reticulum (ER)–mitochondria Ca^{2+} transfer regulates apoptosis and autophagy in cancer. In all panels, the black arrows mirror the basal cellular mechanisms while the red arrows indicate modulation of the pathways by the listed chemical compounds or genetic modifications. **(A)** IP_3Rs are engaged in autophagy and cell death regulation *via* the ER–mitochondrial Ca^{2+} flux. Ca^{2+} transferred to the mitochondria ensures proper tricarboxylic acid (TCA) function and, therefore, adequate bioenergetics and biosynthesis processes suppressing autophagy. Inhibition of IP_3Rs by Xestospongin B (XeB) or its genetic knockdown (KD) dampens ER–mitochondrial Ca^{2+} transfer, which inhibits the TCA cycle and ATP production. As a consequence, autophagy is increased, but this is not sufficient for the survival of cancer cells, which undergo mitotic catastrophe. In addition to this, inhibition of IP_3Rs by 2APB or Xestospongin C (XeC) also leads to impeded ER–mitochondrial Ca^{2+} fueling, and subsequently to further autophagy-dependent cancer cell death. **(B)** Fueling mitochondria with Ca^{2+} can be modulated by several oncogenes/tumor suppressors [promyelocytic leukemia protein (PML), PTEN, PKB/Akt]. In cancer cells lacking PML (KO, knockout), mitochondrial Ca^{2+} transfer is impeded resulting in downregulation of TCA cycle, stimulation of autophagy, and of cell growth. **(C)** Mitochondrial Ca^{2+} transfer can also be indirectly controlled by mitochondrial F_0F_1 ATPase. Its inhibition by resveratrol (RSV) impairs sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA) function, thereby increasing the net Ca^{2+} flux from the ER and promoting mitochondrial Ca^{2+} overload, eventually leading to apoptosis. In addition, SERCA is negatively regulated by thioreductase TMX1. Cancer cells having low levels of TMX1, exert high SERCA activity, which correlates with decreased ER–mitochondrial Ca^{2+} transfer. Consequently, the TCA cycle is limited and cells switch to aerobic glycolysis (Warburg effect).

the outcomes of AMPK activation besides autophagy induction is the activation of p53/p21, which downregulates cyclin E levels. As a consequence, cells cannot proceed from the G1 to S phase and are arrested (157). However, one feature of cancer cells is their uncontrolled proliferation. In fact, many cancer cells have mutations in p53 or display dysregulated cell cycle control (160). In that sense, these cancer cells become addicted to their mitochondrial metabolism to produce mitochondrial intermediates that are used for synthesis of lipids, nucleotides, and proteins, supporting the survival of the dividing cells. Cancer cells exposed to IP_3R inhibitors will, thus, not slow down their cell cycling. As a consequence, these cells will continue to divide irrespective of the surge in mitochondrial ATP output and the availability of mitochondrial intermediates needed for biosynthesis upon cell division. Without sufficient lipids and nucleotides, the daughter cells will not be able to survive. Hence, IP_3R inhibition caused a mitotic catastrophe in cancer cells. Consequently, cell death in these cells could be overcome by the addition of mitochondrial substrates, such as pyruvate, to the growth medium or by slowing down cell cycle progression (156). Yet, autophagy was not involved in the cell death process, as KD of essential autophagy genes did not modulate IP_3R inhibition-induced cell death. This indicates that cell death did not occur *via* autophagic cell death and also that autophagy activation could not support survival of the cells.

In contrast to these findings, another study revealed an important role for autophagic cell death in breast cancer cells exposed to IP_3R inhibition (123) (**Figure 2A**). Tumorigenic breast cancer cells were sensitive to treatment with the non-specific IP_3R inhibitors, 2-APB or xestospongin C, while non-tumorigenic breast cancer cells were resistant to this treatment. However, these compounds, as well as their derivatives, can indirectly cause depletion of the ER Ca^{2+} stores by inhibiting other Ca^{2+} transporters or by stimulating Ca^{2+} leakage out of the ER (161–164). Nevertheless, similar findings were obtained by genetically knocking down IP_3R2 or IP_3R3, which appear to be upregulated in tumorigenic versus non-tumorigenic breast cancer cell lines. Inhibition of IP_3Rs in the tumorigenic cells resulted in excessive autophagy activation, which could be attributed to a decrease in ATP production (and thus activation of AMPK), an upregulation of Atg5 and an increase in ROS production. In these cells, excessive autophagy was responsible for cell death, as inhibition of autophagy either at the level of the Vps34 complex formation using 3-methyladenine or at the level of lysosomal degradation by Bafilomycin A1 protected cells against cell death induced by IP_3R inhibition or IP_3R KD (123). These *in vitro* findings were also translated to *in vivo* xenografted breast tumor models. Interestingly, the increase in IP_3R2/IP_3R3 expression was not only found in breast cancer cell lines but also found in patient samples consisting of breast tumor tissue compared to adjacent non-tumorous tissue. Moreover, IP_3R2/IP_3R3 upregulation in breast tumors correlated with an increase in lipoproteins and several metabolites (such as lactate, alanine, and lysine) in the serum of these patients compared to breast cancer patients with low IP_3R2/IP_3R3 levels or healthy controls (124). Unfortunately, no autophagic markers were analyzed in these samples.

Downregulation of Autophagy and Increased Apoptosis Susceptibility in Response to IP_3R Modulation by Tumor Suppressors

As previously discussed, IP_3Rs suppress basal autophagy by promoting mitochondrial ATP production. In addition to this, IP_3Rs control the susceptibility of cells toward toxic, pro-apoptotic stimuli. By promoting Ca^{2+} transfer into the mitochondria, IP_3Rs participate in mitochondrial Ca^{2+} overload, a critical factor in the opening of the mitochondrial permeability transition pore and subsequent apoptosis. In fact, mitochondrial Ca^{2+} overload has been shown to be a critical component of several pro-apoptotic stimuli, including chemotherapeutic drugs (165). These stimuli can trigger Ca^{2+} release from the ER. In addition, these compounds can trigger ER Ca^{2+} overload by activating SERCA in a p53-dependent manner (120). As a consequence, such cells will display an increased likelihood for mitochondrial Ca^{2+} accumulation and thus cell death. In fact, cancer cells lacking p53 or expressing loss-of-function p53 mutants are resistant to chemotherapeutic drugs in part due to their lack of mitochondrial Ca^{2+} overload, as these cells can be re-sensitized to chemotherapeutics by overexpressing MCU (120, 166). In particular, the IP_3R3 isoform appears to participate in pro-apoptotic Ca^{2+} transfer into the mitochondria due to its presence in the MAMs (167). Moreover, IP_3R3 activity in the MAMs is subjected to functional modulation by survival/oncogenes and tumor suppressors (**Figure 2B**). IP_3R3 is phosphorylated by PKB/Akt, suppressing ER–mitochondrial Ca^{2+} flux and promoting apoptosis resistance (112). PKB/Akt-mediated phosphorylation of IP_3R3 is counteracted by PTEN, which dephosphorylates IP_3R3 particularly at the MAM fraction and, therefore, stimulates pro-apoptotic Ca^{2+} transfer from the ER into the mitochondria (117). Another regulator of PKB/Akt-dependent phosphorylation of IP_3R3 is the tumor suppressor promyelocytic leukemia protein (PML), which also resides at the MAMs, where it recruits PP2A, which suppresses PKB/Akt activity (168). In addition to this, downregulation of IP_3R3-protein levels has been implicated in cancer transformation and cell death resistance of isogenic cell pairs, in which an oncogenic mutant Ras allele was expressed (118).

More recently, further insights into the contribution of IP_3R modulation in the tumor suppressive function of PML have been revealed (168, 169). PML was recruited at the MAMs in a p53-dependent manner supporting efficient ER–mitochondrial Ca^{2+} transfer (**Figure 2B**). As a consequence, cells expressing the tumor suppressor PML were susceptible to engage apoptosis upon cell stress or damage and to maintain an adequate production of ATP, preventing the growth of damaged or malignant cells. These conditions dampened AMPK activity and, thus, result in a regular basal autophagic flux. The combination of adequate apoptosis sensitivity and regular autophagy flux allows for a normal and balanced cell growth. However, in cells lacking PML, ER–mitochondrial Ca^{2+} transfer was suppressed, resulting in excessive apoptosis resistance and an upregulation of basal autophagy due to suppressed ATP production followed by AMPK activation and thus increased ULK1 activity. The increase in autophagy upon PML deletion in cells could be

attributed to the decreased mitochondrial Ca^{2+} signaling, since overexpression of MCU in these cells could suppress basal autophagic flux (168). Moreover, PML-deficient cells displayed a growth advantage compared to PML-proficient cells, particularly in stress conditions such as nutrient starvation that engage the autophagy pathway. Interestingly, PML-deficient cells could be sensitized to chemotherapeutic drugs such as 5-fluorouracil by co-administration of chloroquine, an autophagy inhibitor that acts at the level of the lysosomal proteolysis. Moreover, in some promyelocytic leukemia cells, PML became fused to retinoic acid receptor α (RARα), abrogating wild-type endogenous PML function and causing neoplastic transformation. This oncogenic PML fusion was degraded by stimulating the proteasome using arsenic trioxide. Treatment of cells expressing oncogenic PML fusion protein with this drug not only resulted in PML-RARα degradation but also rescued wild-type PML levels, which was then present in the MAMs and able to promote ER–mitochondrial Ca^{2+} transfer (168).

Another tumor suppressor actively involved in autophagy regulation is Beclin 1. Interestingly, a target of Beclin 1 is the IP_3R, which becomes sensitized upon Beclin 1 binding, a process enhanced during nutrient starvation (58). Thus, cancer cells, commonly deficient in Beclin 1, will not only be able to form less complexes with the lipid kinase Vps34 but also with the IP_3Rs, what may lead to decreased autophagy. However, at this point, it is not clear how decreased IP_3R/Beclin 1-complex formation contributes to basal autophagy and how this impacts tumorigenesis.

Indirect Impact of IP_3R on Autophagy in Cancer Cells: Effects of ER Ca^{2+} Modulation

Resveratrol, a natural polyphenol, is well known to induce cancer cell death by engaging autophagy induction *via* different mechanisms (82, 170–172), including the modulation of Ca^{2+} signaling (173). More recently, further insights into RSV-induced cancer cell death *via* Ca^{2+} signaling have been obtained (84). In particular, cancer cells can display increased ER–mitochondrial contact sites, potentially to facilitate the transfer of basal Ca^{2+} signals to accommodate their increased need for mitochondrial TCA cycle activity, which also provides substrates for several biosynthetic pathways. Exposing cancer cells to RSV resulted in a rapid depletion of the ER Ca^{2+} stores. The underlying mechanisms appeared to involve the direct inhibition of the mitochondrial F_0F_1-type ATP synthase, resulting in a rapid drop in ATP levels, in particular at the ER–mitochondrial contact sites (**Figure 2C**). SERCA activity is thereby impaired, leading to a net increase of Ca^{2+} delivery to the mitochondria, as for a given IP_3-induced Ca^{2+} release less Ca^{2+} will be pumped back in the ER. Thus, mitochondrial Ca^{2+} levels will increase, thereby promoting cell death. The concept of SERCA modulating ER–mitochondrial Ca^{2+} transfer in cell death and tumor biology has been nicely illustrated in a recent study of Raturi et al. (174). It has been shown that SERCA activity was dynamically regulated at the ER–mitochondrial contact sites by different factors, including palmitoylated calnexin, which positively regulated SERCA, and the thioreductase TMX1, which negatively regulated SERCA. In normal cells, TMX1 levels are high, thereby suppressing SERCA activity and thus promoting ER–mitochondrial Ca^{2+} transfer. This supports mitochondrial metabolism on the one hand and adequate apoptotic susceptibility on the other hand. Interestingly, many tumors display low TMX1 levels, which results in increased SERCA activity, particularly at the MAMs, leading to dampened ER–mitochondrial Ca^{2+} transfer (**Figure 2C**). This was proposed to contribute to the Warburg effect and increased glycolysis, as the activity of TCA cycle enzymes became suppressed, while the need for glucose metabolism remained high to sustain cell growth and proliferation (174, 175). Nevertheless, it is possible that these findings relate to concepts identified for PML at the ER–mitochondrial interface. Indeed, tumors with low TMX1 levels may display increased autophagy and decreased apoptosis susceptibility due to suppressed ER–mitochondrial Ca^{2+} fluxes. However, further work is needed to reconcile these concepts.

IP_3R-Regulated Autophagy As a Protection against Natural Killer (NK)-Induced Cancer Cell Death

Recently, *ITPR1*, the gene encoding IP_3R1, has been implicated as a major resistance mechanism of renal carcinoma cells against the lytic action of NK cells by activating autophagy (176–178). Many renal carcinoma cells are characterized by a dysfunctional von Hippel–Lindau gene (*pVHL*), which encodes a protein that has many functions, including targeting the family of hypoxia-inducible factor transcription factors for degradation by the proteasome. Thus, in cells lacking pVHL, HIF1α/HIF2α become stabilized. Currently, an emerging concept in anticancer therapies is the use of NK cells. Tumor cells contain several resistance factors against NK-induced cancer cell killing, including stabilized HIF2α. Strikingly, *ITPR1* appeared to be one of the most important target genes of HIF2α in a renal carcinoma cell line with dysfunctional *pVHL* gene and it conferred resistance against NK-induced lysis. In particular, renal carcinoma cells upregulated pro-survival autophagy in a HIF2α/IP_3R1-dependent manner in response to NK treatment, while cells in which IP_3R1 was knocked down failed to stimulate autophagy and became susceptible to NK-induced lysis. The IP_3R1-dependent induction of autophagy protected the carcinoma cells against the deleterious action of NK cells by degrading the lytic granzyme B. In renal carcinoma cells with functional pVHL, HIF2α levels are very low due to its targeting to the proteasome, thus failing to upregulate *ITPR1* expression and abrogating autophagy induction as resistance mechanism against NK cells and the lytic action of granzyme B. Thus, antagonizing IP_3R function in renal carcinoma cells lacking functional pVHL may provide a manner to sensitize these cells to lysis by NK cells by counteracting the induction of autophagy as a resistance mechanism. Furthermore, also NK cells by themselves require functional autophagy for maturation and survival (179). Upon deletion of *Atg5*, NK cells accumulated damaged mitochondria, which lead to their death due to excessive ROS production. Furthermore, silencing *Atg7* and the resulting disruption of the interaction between Atg7 and phosphorylated forkhead box O1 (FOXO1) prevented autophagy and contributed to incomplete maturation of NK cells (179).

CONCLUSION

IP_3Rs play a critical role in autophagy due to their localization at the ER and the ER–mitochondrial contact sites and the resulting Ca^{2+}-signaling regulation. On the one hand, they suppress autophagy by continuously sustaining the mitochondria with Ca^{2+}, needed for mitochondrial metabolism and energy production. On the other hand, they participate in the increased autophagic flux induced upon cellular stress, including nutrient starvation, chemical mTOR inhibition, or RSV treatment, by providing cytosolic Ca^{2+} that is needed to drive autophagic flux. Given autophagy's critical role in tumor development and progression, it is not surprising that IP_3R function can affect these processes through autophagy modulation. Cancer cells appear to be addicted to IP_3R-mediated Ca^{2+} release to sustain their mitochondrial metabolism and related anabolic pathways. Cancer cells exposed to IP_3R inhibition can undergo cell death, which in some cases could be due to excessive autophagy induction, while in other cases cell death could be the result of an uncontrolled cell proliferation and thus be due to mitotic catastrophe. Also, IP_3Rs are modulated by tumor suppressors, such as PML, as part of a homeostatic program to support normal cell growth by balancing adequate apoptosis susceptibility and regulated autophagy flux. However, loss of these tumor suppressors results in dampened IP_3R function and thus defective ER–mitochondrial Ca^{2+} transfer. As a consequence, cells become resistant to cell death inducers, including genotoxic stress and cell damage, by a combination of increased apoptosis resistance and an increased autophagy flux serving as a pro-survival function. This phenomenon will contribute to neoplastic transformation and tumorigenesis. The role of IP_3Rs in autophagy seem also to be exploited by renal carcinoma cells with dysfunctional pVHL, which induce autophagy in a HIF2α/IP_3R-dependent manner as a resistance mechanism that protects these cancers against NK-induced killing. Finally, it should be noted that IP_3R function and the net IP_3R-mediated Ca^{2+} delivery into the mitochondria is dependent on the activity of SERCA, which is also present in the MAMs. Thus, SERCA modulation at the ER–mitochondrial contact sites will affect the net Ca^{2+} transfer into the mitochondria and, thus, ultimately affect the Ca^{2+}-dependent mitochondrial functions, such as bioenergetics, autophagy, and apoptosis. SERCA inhibition in the MAMs will increase mitochondrial Ca^{2+} accumulation, which will drive the mitochondrial metabolism and bioenergetic output and thus suppress autophagy. In cancer cells, de-inhibition of SERCA at the ER–mitochondrial contact sites can be part of the Warburg effect but also of the increase in basal autophagy that could promote neoplastic behavior by promoting cell survival and excessively protecting cancer cells against cell stress. Overall, IP_3Rs impact several cancer hallmarks through autophagy modulation.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception of the work. GB and EK drafted the manuscript. EK produced the figures. GR, TV, and JP critically revised manuscript and figures and provided important intellectual content. All authors concur with the final version of the manuscript and agree to be held accountable for all aspects of the work.

FUNDING

GR and TV are recipients of a Ph.D. fellowship and a postdoctoral fellowship of the Research Foundation-Flanders (FWO), respectively. Work performed in the laboratory of the authors was supported by FWO grants G.07310.9N, G.0634.13 and G.0927.15, by the Research Council of the KU Leuven *via* concerted action 09/012 and grant OT/14/101 and by the Interuniversity Attraction Poles Program (Belgian Science Policy; IAP-P7/13). JP and GB are part of the Scientific Research Community "Calcium Signaling in health, disease & therapy" (CaSign) supported by the FWO (W0.019.17N).

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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.

frontiers in Oncology

REVIEW
published: 03 March 2017
doi: 10.3389/fonc.2017.00028

Molecular Pathways Controlling Autophagy in Pancreatic Cancer

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Pancreatic ductal adenocarcinoma (PDAC) is one of the few cancer types where the 5-year survival rate shows no improvement.

Despite conflicting evidence, the majority of data points to an essential role for autophagy in PDAC growth and survival, in particular constitutively activated autophagy, can provide crucial fuel to PDAC tumor cells in their nutrient-deprived environment.

Autophagy, which is required for cell homeostasis, can both suppress and promote tumorigenesis and tumor survival in a context-dependent manner. Protein by protein, the mystery of how PDAC abuses the cell's homeostasis system for its malignant growth has recently begun to be unraveled. In this review, we focus on how autophagy is responsible for growth and development of PDAC tumors and where autophagy and the mechanisms controlling it fit into PDAC metabolism. Understanding the range of pathways controlling autophagy and their interplay in PDAC could open the way for new therapeutic avenues.

Keywords: autophagy, pancreatic cancer, PDAC, metabolism, autophagy inhibition

OPEN ACCESS

Edited by:
Patrizia Agostinis, State University of Leuven, Belgium

Reviewed by:
Eric Chevet, INSERM, France
Manuela Côrte-Real, University of Minho, Portugal
Kasper Rouschop, Maastricht University, Netherlands

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *17 November 2016*
Accepted: *15 February 2017*
Published: *03 March 2017*

Citation:
New M, Van Acker T, Long JS, Sakamaki J-i, Ryan KM and Tooze SA (2017) Molecular Pathways Controlling Autophagy in Pancreatic Cancer. Front. Oncol. 7:28. doi: 10.3389/fonc.2017.00028

INTRODUCTION

Pancreatic cancer is a disease in which malignant cells originate in pancreatic tissue, leading to over 200,000 deaths per year worldwide—making pancreatic cancer the ninth leading cause of death from cancer (1). Eighty-five percent of pancreatic cancer cases are pancreatic ductal adenocarcinomas (PDACs), and there is currently no effective screening tool to detect early malignant or premalignant tumors. This makes PDAC one of the most deadly common cancers, as diagnosis is most likely to be at an advanced stage, with metastatic or locally advanced disease (2). Median patient survival is only 6–9 months (3) and only about 4% of patients live 5 years after diagnosis (4).

Defining features of PDAC include a high rate of *KRAS* activating mutations (>90%), a reprogramming of cellular metabolism, a hypervascular and hypoxic microenvironment, and susceptibility to both local invasion and metastasis (2). Therapeutic resistance of PDAC to radiotherapy, targeted agents, and chemotherapy means that new therapeutic avenues are urgently needed. One avenue would be to target the autophagic pathway as a number of studies have linked autophagy to PDAC survival and progression.

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved membrane-mediated process that delivers cytoplasmic constituents to lysosomes for degradation and component recycling. This complex process is mediated by at least 18 autophagy genes (Atg genes) in mammals (5). Upon autophagy induction triggered by cell stress, double membrane autophagosomes form and engulf cytosolic proteins and damaged organelles, either through a non-selective process or a selective receptor mediated autophagy, such as mitophagy (6). Autophagy initiation is controlled by the ULK kinase complex and the VPS34 phosphoinositol-3-phosphate (PtdIns3P)-kinase complex

containing Beclin-1, which integrate stress signals from the mTOR complex 1 (mTORC1). When mTORC1 activity is inhibited, the ULK and the Beclin-1 complex translocate to the initiation site marked by ATG9 (7). The production of PtdIns3P by the Beclin-1 complex allows binding of WIPI2, recruitment of ATG12-5-16, and lipidation of the LC3/GABARAP family (8). Lipidated LC3 (LC3-II) is required for autophagosome formation, and detection of LC3-II by immunoblotting or immunofluorescence is the most established method of monitoring autophagy.

In normal conditions, autophagy is a homeostatic mechanism that serves to degrade damaged proteins and organelles that may diminish cellular fitness and integrity. The levels of autophagy can also be changed in response to a variety of intracellular and extracellular stresses, such as starvation, ER stress, hypoxia, oxidative stress, and pathogen invasion. The role of autophagy in cancer is complex with both tumor-survival and tumor-suppressive roles, which are dependent on tumor type, stage, and genetic lesions. Autophagy is thought to inhibit malignant transformation under normal conditions and is required for anticancer immunosurveillance (9). However, autophagy in cells which are already malignant frequently supports tumor progression and anticancer therapy resistance, by providing a means for cells to survive intracellular and extracellular stress (9).

Autophagy is tightly regulated starting from transcriptional activation to posttranslational protein modification (10), and the regulation of autophagy in PDAC is gradually becoming elucidated. Transcriptional control of autophagosome–lysosome function has been shown to drive PDAC metabolism (11), whereas starvation-induced vacuolar protein 1 (VMP1) expression in pancreatic acinar cells drives early autophagy through VMP1 association with the early autophagic structures on the ER membrane (12, 13). Autophagy inhibition or loss has been shown to lead to tumor regression in PDAC xenograft models and death in PDAC cell lines (14). Autophagy supports PDAC cell survival by a range of mechanisms, including autophagic secretion of alanine by pancreatic stellate cells (PSCs) for tumor metabolism (15) and prevention of ER stress (16). The well documented role of autophagy for survival of PDAC and the potential for therapy through autophagy modulation has been explored in PDAC cell lines, where autophagy blockage has been shown to reduce chemoresistance (14). In one study involving a small number of human patients, inhibition of autophagy did not show any significant therapeutic effect (17).

The focus of this review will be the role of autophagy in PDAC, a cancer type in which extensive evidence currently points to a dependence on autophagy for tumor growth, development, and metabolism (14, 18), although there are also studies highlighting autophagy-independent PDAC cell line and tumors (19, 20).

DUAL ROLE OF AUTOPHAGY IN CANCER

It is now accepted that autophagy can suppress or promote tumorigenesis and tumor survival depending on cellular context and stage in tumor development, this characteristic is referred to as a "double-edged sword" (21) (**Figure 1**).

Defects in the autophagic machinery in mouse cancer models have been connected to malignant transformation in a number of studies, and indeed, the tumor-suppressive properties of Beclin-1 provided the first evidence of this (22). More recently, mice heterozygous for activating molecule in Beclin-1-regulated autophagy (AMBRA1) were also shown to have an increased rate of tumorigenesis (23). The scaffold protein AMBRA1 was shown to promote the binding of protein phosphatase 2A to the c-Myc transcription factor and, when mTOR is inhibited, causes c-Myc to be dephosphorylated consequently followed by a reduction in cell division (23). Other mechanisms through which autophagy functions in an oncosuppressive role include protection of the cell from mutagenic reactive oxygen species (ROS) accumulation, DNA damage, genomic instability, and oncogenic proteins, hyperactivation of which activates autophagy (9). While this suppressive function typically allows the cells to survive, prolonged autophagy activation may result in caspase-independent autophagic-programmed cell death (24). Autophagic cell death is poorly defined but is associated with autophagosome formation and depends on autophagy proteins, although it is controversial whether cells truly die *via* autophagy, particularly as there are no distinct markers of the process (25). Autophagy may also contribute to oncogene-induced senescence, demonstrated by depletion of ATG5 by shRNA which inhibits oncogene-induced senescence in human fibroblasts (26). There is a growing body of evidence showing that defects in autophagic machinery prevent malignant cell proliferation, for example, metastatic carcinoma cell lines where Beclin-1 or ATG5 is downregulated are unable to survive (27), and siRNA depletion of the essential autophagy gene ATG7 enhances apoptosis in colon cancer cells (28).

In contrast, autophagy may allow established tumors to survive and progress by reducing their sensitivity to stress and cell death signals. Enhanced autophagic response in advanced human tumors correlated with an invasive phenotype and poor prognosis (29). Autophagy also supports tumor cell survival by increasing ATP levels during hypoxia, nutrient deprivation, and detachment from the extracellular matrix, all of which may occur in tumors and would usually result in cell death (9). A number of anticancer therapies have been shown to induce autophagy in human cancer cell lines (30), which may cause cells to become resistant to the therapy, and autophagy inhibition can re-sensitize previously resistant cells to therapy (31).

In summary, the role of autophagy in cancer appears to change during tumor progression. Autophagy protects healthy cells from malignant transformation by maintaining cellular homeostasis and normal metabolism, but after malignant transformation, when presumably autophagy has been suppressed, restoration of autophagy promotes tumor progression, invasion, and metastasis (9). The pro-survival role of autophagy in tumors has been explored as a potential therapeutic target in a number of cell-based studies and clinical trials.

MOLECULAR CONTROL OF AUTOPHAGY IN PANCREATIC CANCER AND ITS DEVELOPMENT

Autophagy is crucial for maintaining cellular homeostasis and has a dual role in cancer as discussed above. It is therefore

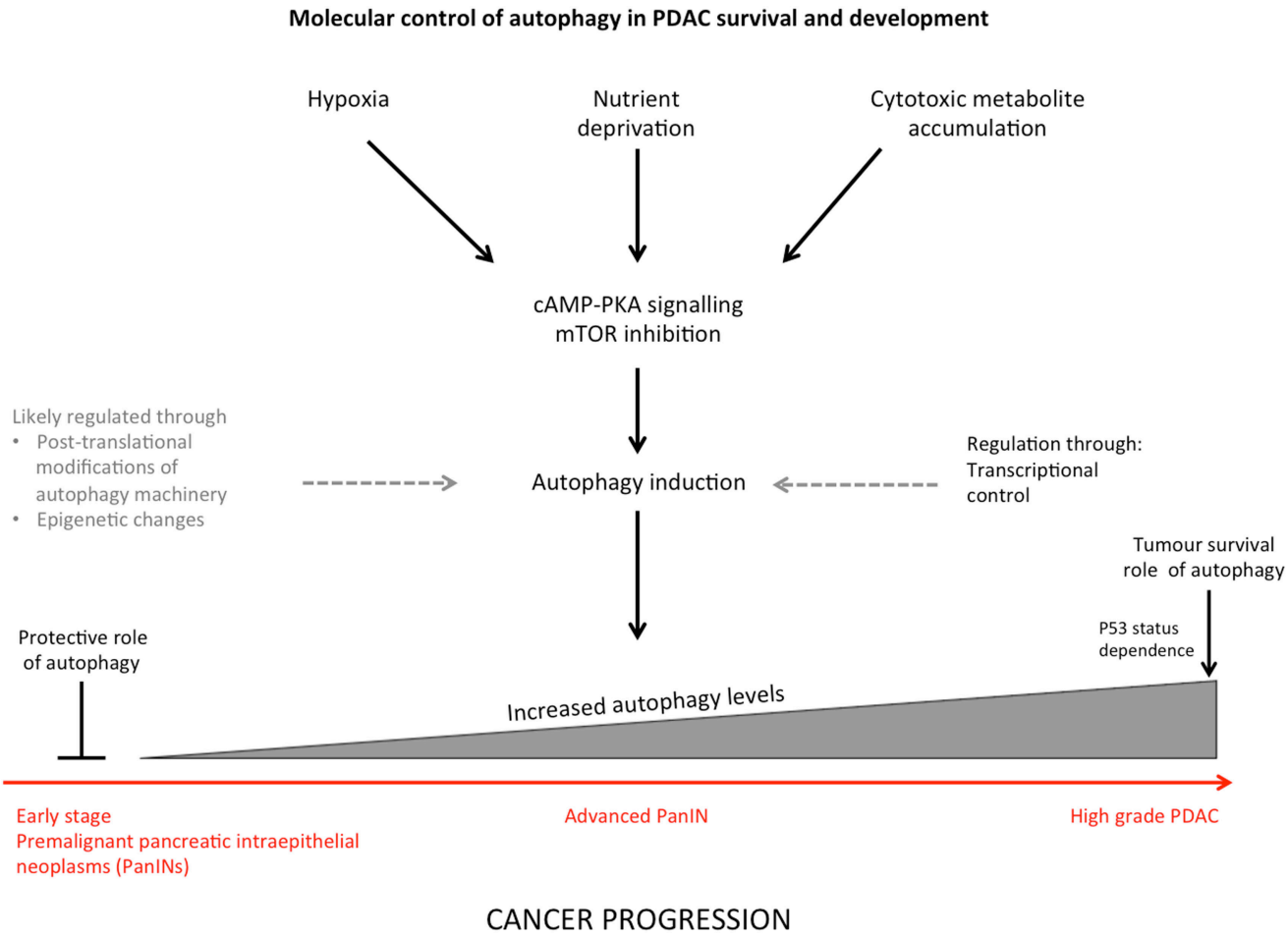

FIGURE 1 | Summary diagram indicating the stimuli that induce autophagy in pancreatic ductal adenocarcinoma (PDAC) and regulation of autophagy through different stages of cancer progression.

important to understand autophagy regulation as a degradation and stress-control pathway. The TOR and RAS–cAMP–PKA signaling cascades negatively regulate autophagy and sense nutrient deprivation (which activates autophagy), although details on how multiple signaling mechanisms coordinate in order to control autophagy are not fully understood (32) (**Figure 1**). Molecular control of autophagy has been widely studied, and the process is tightly regulated at various levels (10).

One of the levels of autophagy regulation is transcriptional control. Transcription of LC3 is upregulated during starvation in mammalian cells (10), a process dependent on the FoxO3 transcription factor (33). Epigenetic changes have also been shown to regulate autophagy, such as the hyperacetylation of histones through histone deacetylase inhibitor treatment, which activates autophagy (34). Posttranslational modification of the autophagy machinery includes phosphorylation of Beclin-1 in response to autophagic stimuli, which is required for maximal autophagy (35).

Despite the accumulation of information on molecular control of autophagy, evidence is just emerging showing these mechanisms (transcriptional, epigenetic, or posttranslational) controlling autophagy are active in PDAC. The evidence on autophagy control in PDAC will be summarized for the purpose of this review.

Molecular Control of Autophagy in PDAC Survival

A major route in the development of PDAC is through acinar cell damage and dysfunction. Pancreatic acinar cells produce and secrete digestive enzymes and proteases, which require a very high protein biosynthetic rate and an extensive rough endoplasmic reticulum network. Consequently, acinar cells are prone to accumulation of misfolded proteins and ER stress (36, 37). The latter can be involved in the pathogenesis of pancreatitis, which in turn causes inflammation of the exocrine pancreas that may lead to development of PDAC (38). This is particularly likely in the case of chronic pancreatitis (39).

Autophagy is required for the maintenance of acinar cell physiology, as demonstrated by *in vivo* loss of ATG7 in pancreatic epithelial cells leading to pronounced acinar cell damage and

loss followed by chronic pancreatitis (16). Primary acinar cells depleted of ATG7 displayed an impaired autophagic canonical flux as LC3-I and p62 protein levels were elevated. Impaired autophagy can lead to an increase in misfolded proteins that undergo ubiquitination and are bound by p62, leading to ER stress and mitochondrial damage (40). Conditional ATG7 knockout mice, in which ATG7 was lacking in all pancreatic epithelial cells, displayed an increase in damaged mitochondria and ER stress, resulting in accumulation of ROS in the pancreata. To counteract these disruptive processes, ATG7-depleted primary acinar cells and pancreata upregulate the transcription nuclear factor erythroid 2-related factor 2 (NRF2), which can stimulate an antioxidative response (16, 41).

A way in which autophagy in acinar cells may be controlled is through VMP1, which triggers the formation of LC3 positive vacuoles when stably expressed in the pancreatic acinar cells of transgenic mice (12). Cell starvation and mTORC1 inhibition induce VMP1 expression (12), and VMP1 is thought to function through interaction with Beclin-1 and recruitment of the PtdIns3P-kinase complex at the phagophore (42). VMP1 transiently localizes with early autophagic structures on the ER membrane (13) and co-localizes with ULK1 at early autophagic structures (13). Furthermore, RNAi experiments in PANC1 cells show that oncogenic KRAS requires VMP1 to induce autophagy. In PDAC cells, VMP1 is upregulated *via* a KRAS–PI3K–AKT1–GLI3-p300 pathway (43). This is of particular interest given that VMP1 was originally identified in rats as a pancreatitis-induced protein restricted to acinar cells (44), so expression of this protein is likely to be an autophagy regulator in PDAC.

As discussed above, accumulation of misfolded proteins and ER stress can be involved in pathogenesis of pancreatitis. ROS generation, which has been shown to regulate autophagy, might contribute to this process (45). In PDAC cell lines, ROS inhibition with an antioxidant significantly reduced basal autophagy levels and, conversely, autophagy inhibition resulted in an increase in ROS levels, confirming a cross-regulation of ROS and autophagy in PDAC (14). The role of ROS and autophagy in PDAC may be biphasic—during early stages of cancer low autophagy levels allow ROS to promote pro-tumorigenic genomic instability required for transformation, whereas in more progressed PDAC cells, autophagy protects the cells from cytotoxic ROS accumulation (14). This has been demonstrated by an increase in markers of double-strand breaks such as 53BP1 foci in PDAC cells where autophagy is inhibited, and this DNA damage in PDAC is thought to allow increasing tumor growth (14).

Molecular Control of Autophagy in PDAC Development

There is a range of evidence showing that PDAC tumors have constitutively activated autophagy and are dependent on autophagy for survival and development. Measurement of LC3 puncta and LC3-II levels in PDAC cell lines shows elevated basal autophagy levels compared to non-cancerous pancreatic cells and other cancer cell lines (14). Immunohistochemistry analysis of samples from a range of human pancreatic tumors has shown an increase in autophagy levels during the progression from premalignant pancreatic intraepithelial neoplasms (PanINs) to more advanced PDAC (14). The role of autophagy in PDAC progression was probed further by the use of the chloroquine (CQ), which raises the lysosomal pH and thereby inhibits autophagy, to treat mice with advanced PanIN or PDAC, which suppressed tumor growth *in vivo* (14).

However, in a separate mouse study, it has been shown that autophagy deficiency increases PanIN development and tumor initiation, although it makes PanIN progression to PDAC less likely (46). This is supported by evidence showing that autophagy-deficient *ATG7*$^{-/-}$ mice show enhanced RAS-driven PanIN formation but do not develop PDAC (20).

The functions of the RAS oncogene and TP53 tumor suppressor in tumorigenesis have been described in detail elsewhere, and aberrations of both of these proteins appear to be cooperative in their contribution to malignancy (47). As well as the high rate of *KRAS* activating mutations, sequence analysis has shown that PDACs demonstrate a mixture of tumor suppressor gene mutations, with TP53 being mutated or inactivated in 75% of PDAC and mutant TP53 being shown to drive pancreatic cancer (48). Autophagy inhibition by CQ treatment or RNAi has been shown to inhibit growth of PDAC cell lines harboring *TP53* mutations. Furthermore, patient-derived xenografts with *TP53* mutations grow slower after autophagy inhibition (46).

In contrast, TP53 status has been shown to determine the role of autophagy in tumor development in mice KRAS mutant pancreatic tumors, where PDAC formation is accelerated by autophagy inhibition in cases where TP53 is absent (20). This may be because TP53-deficient tumors and cell lines have lower numbers of autophagosomes, so their viability is not dependent on the process (20). This indicates that autophagy is not always critical to PDAC tumor development.

Another study indicates that autophagy is dispensable for growth of KRAS mutant tumors and cell lines (19). Forty-seven human cancer cell lines were treated with the CQ derivative Lys01 or shRNA to remove autophagic machinery components such as ATG7, revealing that KRAS-mutated cells are no more dependent on autophagy than their wild-type counterparts (19). This was supported by *in vivo* experiments where autophagy inhibition did not reduce growth of a KRAS mutant tumor derived from the PDAC cell line Panc10.05 (19). These findings raise questions regarding the assumption that inhibition of autophagy reduces cell growth and viability of KRAS mutant PDAC cells and could mean that the function of autophagy is to support tumor growth through host tissues, such as cancer-associated fibroblasts (49).

Hypoxia-Induced Autophagy in PDAC

Preexisting vasculature of normal tissue has been shown to be insufficient to support the requirements of tumors for nutrients and oxygen, and in particular, the pancreatic tumor microenvironment has been found to be hypoxic (50). Higher tumor levels of hypoxia as measured by hypoxia-inducible factor 1α (HIF-1α) expression have been shown to correlate with poor prognosis in patients with PDAC (51, 52).

The cellular response to hypoxia may contribute to elevated basal autophagy levels in PDAC, as autophagy can be induced by hypoxia in several ways. First, HIF-1α has been shown to upregulate Bcl-2/adenovirus E1B 19-kDa protein-interacting

protein 3 (BNIP3) and BNIP3 like protein (BNIP3L). BNIP3 and BNIP3L subsequently disrupt the Bcl-2–Beclin-1 complex in an mTOR-independent way, which induces autophagy (53). This mechanism has been demonstrated in various cancer cell lines, including prostate cancer and salivary adenoid cystic carcinoma, and we speculate that this process may occur in PDAC (54, 55). In contrast, another study suggests hypoxia-induced autophagy in tumor cells is dependent on AMP-activated protein kinase and mTOR, thus excluding a role for HIF-1α, BNIP3, and BNIP3L (56). In a hypoxic tumor microenvironment, the unfolded protein response can facilitate autophagy. This mechanism involves the PKR-like endoplasmic reticulum kinase-activating transcription factor 4 (ATF4) pathway. ATF4 is able to bind a cyclic AMP response element binding site in the LC3B promoter inducing LC3B transcription (57). This ATF4-mediated transcriptional LC3B induction results in replenishment of LC3B levels during extended periods of hypoxia characterized by high autophagic flux (58).

Evidence for the connection between autophagy and hypoxia in PDAC tumors is high levels of LC3, which has been shown to be associated with the hypoxic marker carbonic anhydrase IX at the peripheral area of the pancreatic cancer tissue (59). Under intermittent hypoxia, pancreatic cancer cells demonstrated enhanced invasive ability and increased levels of the cancer stem cells (CSC) marker CD133. In these cells, enhanced autophagy was correlated with elevated HIF-1α levels. The metastatic ability and epithelial-to-mesenchymal transition of pancreatic CSC was also associated with HIF-1α and autophagy (60). These findings are consistent with a previous report showing autophagy to increase survival and migration of pancreatic tumor-initiating CSCs under hypoxic conditions (61). Recent research in the pathways underlying hypoxia in PDAC revealed that hypoxia induces ROS production which subsequently inhibits the pAKT/mTORC1 pathway, inducing autophagy. This process results in a decrease in MUC4 protein levels (an oncogenic transmembrane protein expressed during the early preneoplastic stage). MUC4 degradation decreases growth and survival, potentially providing other stressed cells with required metabolites (62).

In conclusion, although there is significant evidence linking hypoxia and autophagy in PDAC and the translational relevance of this connection, the precise mechanism for hypoxia-induced autophagy in PDAC is not fully elucidated.

MOLECULAR PATHWAYS INVOLVED IN AUTOPHAGY AND ITS IMPACT ON PANCREATIC CANCER METABOLISM

Autophagy plays a major role in PDAC metabolism, although not all pathways involved in activating and reprogramming autophagy in this context are fully elucidated. Autophagy in PDAC can be seen as part of a broader transcriptional program that coordinates lysosome function and nutrient sensing by the MiT/TFE subclass of basic helix–loop–helix transcription factors including TFE3, MITF, and TFEB, ensuring sufficient levels of intracellular amino acids (11). PDAC cells display an increased lysosomal biogenesis accompanying their expanded autophagosome compartment. In normal cells under nutrient stress, biogenesis of autophagy–lysosome proteins is under control of the MiT/TFE transcription factors (63). RNAseq data across 10 tumor types revealed a high relative expression of these transcription factors in PDAC (11). MiT/TFE proteins act selectively in PDAC cells to regulate a broad autophagy–lysosome program under basal conditions. Despite displaying intact mTORC1 signaling, which phosphorylates MiT/TFE proteins in fed conditions in non-PDAC cells and ensures their cytoplasmic retention (64), PDAC cells show constitutive nuclear localization of each MiT/TFE protein. The cytoplasmic retention mechanism of MiT/TFE in PDAC cells is overwritten by importin-8 (IPO8), a member of the importin-β family of nucleocytoplasmic transporters (65). In PDAC cells, in contrast to non-PDAC cells, IPO8 binds TFE3 resulting in its nuclear translocation and upregulation of its transcriptional program regardless of the nutritional condition (11). Endogenous binding of IPO8 to MITF or TFEB was not shown; however, a combinational depletion of IPO8 and its homolog IPO7 in PDAC cells decreased MITF and TFEB protein levels.

Depletion of MiT/TFE proteins across several PDAC cell lines revealed a regulatory role for MiT/TFE proteins in autophagic flux and lysosomal catabolism. This enables efficient processing of cargo from autophagy and macropinocytosis. Thus, the MiT/TFE protein system provides PDAC cells with both intracellular and extracellular nutrient supplies (11). In nutrient-depleted conditions, PDAC cells rely on the autophagy–lysosome system to maintain intracellular amino acid pools. *In vitro*, silencing of MiT/TFE proteins impaired growth of PDAC cells. TFE3 and MITF were also required for *in vivo* xenograft growth of several PDAC cell lines (11).

In summary, overriding MiT/TFE inactivation by mTORC1 *via* IPO8-driven nuclear import enables PDAC cells to maintain their intracellular amino acid pool by activation of both autophagy and lysosomal catabolism (**Figure 2**) (11). This system resembles the constitutive nuclear import of the pro-oncogenic protein eIF4E (a downstream target of mTORC1) in acute myeloid leukemia patients by IPO8 (66) and might be a general mechanism used by several cancer types.

In addition to MiT/TFE-regulated autophagic-lysosomal catabolism, PDACs are also dependent on mitochondrial oxidative phosphorylation for their energy supply. Using an inducible mouse model of mutated KRAS in a *TP53* heterozygous background, Viale et al. showed that repression of mutant KRAS resulted in regressed growth of implanted cells isolated from primary tumors, followed by a relapse after 4–5 months (67). This suggests that a fraction of dormant tumor cells survive oncogene ablation [surviving cells (SCs)]. SCs may possess CSC characteristics as only $CD133^{+}$ $CD44^{high}$ cells were able to avoid apoptosis (67–69). Transcriptomics analysis showed genes involved in the mitochondrial electron transport chain (ETC), lysosome activity, and autophagy are upregulated in SCs. A hyperactive ETC and increased ROS production are hallmarks of SCs. SCs operate close to their maximum respiratory chain capacity and fail to increase glycolysis upon oxidative phosphorylation inhibition in a manner sufficient to maintain ATP production. Furthermore, SCs seem to rely more on pyruvate and palmitate than glucose and glutamine to generate TCA intermediates (67). This is

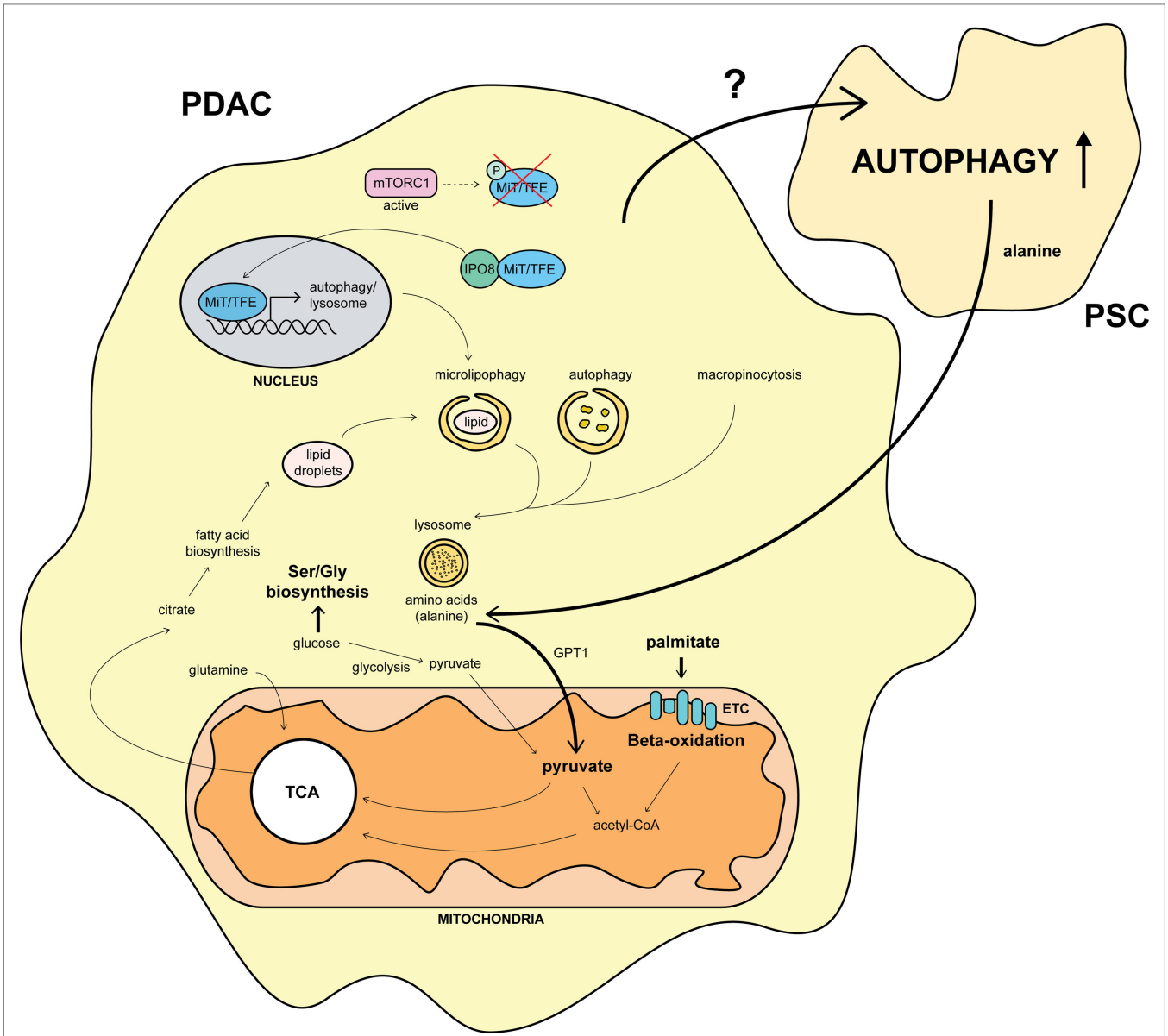

FIGURE 2 | **Overview of the molecular pathways involved in autophagy and its impact on pancreatic cancer metabolism**. Constitutive nuclear import of MiT/TFE proteins by importin-8 (IPO8) upregulates the autophagic-lysosomal machinery in pancreatic ductal adenocarcinoma (PDAC) (11). Autophagy fuels the TCA cycle by amino acid production, shifting the usage of glucose from glycolysis to Ser/Gly biosynthesis (67). This mechanism is further strengthened by pancreatic stellate cells (PSCs) secreting alanine upon autophagy stimulation by unknown PDAC factors (15). Furthermore, the necessity for palmitate feeding oxidative phosphorylation is upregulated in PDAC. Citrate, produced during the TCA cycle, is used for fatty acid biosynthesis, leading to lipid droplet formation and microlipophagy (67). This process, together with the macropinocytotic uptake of extracellular proteins, amplifies the autophagic system in PDAC (11, 67). Arrows and names in bold mark an upregulated pathway in PDAC.

consistent with previous data reporting activation of anabolic glucose and glutamine metabolism in PDAC by oncogenic KRAS (70, 71).

The dependence on oxidative phosphorylation by SCs for their survival was demonstrated by oligomycin treatment of a tumor regression mouse model. Tumors were grown in a mutant *KRAS/TP53* heterozygous-inducible mouse model and regressed upon doxycycline withdrawal. When reintroducing mutant KRAS, 25% of oligomycin-treated mice survived longer than 60 days while vehicle-treated mice survived on average 15 days (67). Mitochondrial respiration would thus make an attractive druggable target to eradicate SCs in PDAC. The ETC dependence of SCs is consistent with previous reports showing both normal and leukemic stem cells rely on mitochondrial respiration (**Figure 2**) (72).

The role of autophagy in pancreatic cancer metabolism is not restricted to just PDAC cells, but cells surrounding tumors also use autophagy for their energy supply. The surrounding environment heavily influences PDAC metabolism, for example, the stroma enveloping PDAC cells impairs vascularization of

tumors leading to a hypoxic, nutrient-poor environment (73). Recently, a new role has been described for stroma-associated PSCs in governing PDAC metabolism. When treating PDAC cells with conditioned medium from a human PSC cell line, the oxygen consumption ratio in PDAC cells increased independent of the presence of serum (15). This effect was attributed to alanine secreted by PSCs. The increase in intracellular alanine concentrations in PDAC cells by PSC-derived alanine could even be further induced by silencing of GPT1, the alanine transaminase responsible for transamination of alanine to form pyruvate and glutamate. Alanine-derived pyruvate did not contribute to glycolytic intermediates but was used in mitochondria as a major source for the TCA cycle as citrate was the main recipient of carbon originated from alanine (15). This alanine-derived carbon would then further fuel fatty acid biosynthesis and could supplant glucose-derived carbon in TCA cycle metabolism, enabling glucose to be used for additional biosynthetic functions (for example, serine/glycine biosynthesis) (15, 71).

Surprisingly, treatment of PSCs with PDAC-conditioned medium significantly increased autophagic flux in PSCs and depletion of ATG5 and ATG7 in PSCs abolished alanine secretion. These findings reveal a two-way intra-tumor metabolic crosstalk in which PDAC signals to PSCs resulting in autophagy induction in the latter, followed by PSC-derived alanine secretion which can fuel the TCA cycle in PDAC. This process is of significant importance under low-nutrient conditions, which mimic the nutrient-deprived PDAC environment. *In vivo*, co-injection of PDAC cells with autophagy-impaired PSCs decreased tumor growth and kinetics, also in orthotopic assays.

An unresolved question in the PDAC–PSC crosstalk is how PDAC stimulates autophagic flux in PSCs (**Figure 2**). Just as autophagy inhibitors in PSCs may be effective, this could be a new avenue for therapeutic intervention, restraining PDAC growth and sensitizing tumors to chemotherapy. One possible mechanism may be regulated by TGF-β1 secretion from PDAC. Activation of PSCs by TGF-β1 transforms them to an activated myofibroblast-like phenotype where synthesis of excessive amount of extracellular matrix proteins causes fibrous tissue formation (74). Furthermore, TGF-β1 was shown to induce autophagy in hepatic stellate cells (75) so a similar mechanism may be at play in PSCs although other cytokines secreted by PDAC cells could also be involved in this process.

Future therapies targeting the metabolism of PDAC are likely to be directed toward fighting a multifront battle. The autophagic component is one important aspect of this battle but will need to be part of a combined approach to drain or disrupt PDAC energy supplies.

POTENTIAL FOR PDAC THERAPY THROUGH AUTOPHAGY MODULATION

Autophagy inhibition is a promising avenue for therapeutic treatment of PDAC. One of the first clinical trials aimed at inhibiting autophagy in PDAC used hydroxychloroquine (HCQ), which did not demonstrate a significant therapeutic effect as a monotherapy (17). However, the HCQ doses tested in this study may have been inadequate to consistently inhibit autophagy, and patients tested were suffering from previously treated metastatic tumors. It should be noted at this point that the antiproliferative effects of CQ were shown to be autophagy independent as both ATG7-deficient and -proficient cells were equally sensitive to CQ (19). This implies that data from clinical trials involving CQ as an autophagy inhibitor should be interpreted with caution. More promising were results from Yang et al. who showed CSCs *in vivo* were more susceptible to gemcitabine treatment upon autophagy inhibition, and combined treatment was more effective than either agent alone in preventing pancreatic tumor formation (18). In addition, as Viale et al. proved in SCs that ETC, lipophagy, and autophagy are all critical for the survival of SCs (67), inhibition of autophagy alone still leaves alternative pathways for PDAC energy production. Thus, the potential efficacy of a monotherapy inhibiting autophagy in PDAC is low, and combinational therapies are preferential. An overview of autophagy-related PDAC therapies currently being tested in clinical trials listed on the US website http://Clinicaltrials.gov can be found in **Table 1**.

Investigation is ongoing into a number of possibilities for combinational PDAC treatment involving autophagy inhibition. MAPK and NF-κB inhibition could be a promising strategy. PANC1 and MIA-PaCa-2 PDAC cell lines were treated with U0126 (a MAPK inhibitor) or caffeic acid phenethyl ester (CAPE, an NF-κB inhibitor), producing a strong inhibition of tumor cell growth without inducing apoptosis. Autophagy inhibition by (3-MA, an inhibitor of PI3K, which blocks autophagosome formation) followed by PDAC treatment with U0126 or CAPE

TABLE 1 | Overview of autophagy-related pancreatic ductal adenocarcinoma (PDAC) therapies currently being tested in clinical trials listed on the US website http://Clinicaltrials.gov.

Aim	Agent	Trial design	NCT number
Autophagy inhibition in PDAC and assessment of JNK1 as PDAC biomarker	Hydroxychloroquine (HCQ) Gemcitabine	Phase I/II	NCT01506973
Determine the ability of HCQ to improve a pre-operative regime of gemcitabine/nab-paclitaxel in patients with potential resectable PDAC	HCQ Gemcitabine Abraxane	Randomized phase II	NCT01978184
Test efficacy of HCQ/gemcitabine combined treatment in PDAC patients before surgery	HCQ Gemcitabine	Phase I/II	NCT01128296
Determine whether a combinational therapy of HCQ/radio therapy/capecitabine can control tumor growth	HCQ Capecitabine Proton/photon radio therapy	Phase II	NCT01494155

caused a significant apoptotic response (76). A combinational treatment including MAPK/NF-κB/autophagy inhibitors might thus be an interesting avenue.

More evidence supporting a combinational therapy involving NF-κB inhibitors was provided by Yang et al. (18). This study also emphasizes the need for reliable prognostic markers for PDAC. The high metastatic potential and resistance to chemotherapy and radiation therapy in several cancers have been linked to CSCs (77–79). Presence of CSCs is associated with poor outcome for patients diagnosed with pancreatic cancer (80). A putative marker for CSCs, other than the aforementioned CD133 and CD44, is aldehyde hydrogenase 1 (ALDH1) (81). Another marker associated with poor prognostic outcome in several cancers is osteopontin (OPN), a secreted glycoprotein able to interact with CD44 and activate several downstream signaling pathways such as growth factor receptor signaling *via* PI3K/AKT, NF-κB, and MEK/ERK (82–85). High expression of LC3 combined with high levels of ALDH1 is associated with shorter overall survival and disease-free survival in pancreatic cancers patients, making coexpression of LC3/ALDH1 a valuable prognostic PDAC marker (18). Autophagy inhibition by silencing of ATG5, ATG7, or Beclin-1 *in vivo* rendered tumors markedly more susceptible to gemcitabine treatment. A combined treatment of CQ and gemcitabine was more effective than either agent alone in preventing pancreatic tumor formation *in vivo* (18). Autophagy blockade boosted the susceptibility of pancreatic CSCs to gemcitabine and thus enhanced the efficacy of gemcitabine against pancreatic cancer. OPN was found to upregulate CSC activity by activating autophagy. OPN can exert its functions by triggering the NF-κB, MEK/ERK, and p38 MAPK in PDAC cells. Pretreatment with BAY 1170–82, an NF-κB inhibitor, could effectively block the OPN-mediated LC3-II increase in PANC1 cells (18). A role for OPN is also found in breast cancer, where its expression associates with cancer aggressiveness. Depletion of OPN in breast cancer cells inhibited the class I PI3K/AKT/mTOR pathway, promoted expression of LC3 and Beclin-1, and increased apoptosis (86). Pharmacological autophagy and NF-κB inhibition have not been tested in this context.

KRAS was considered another interesting prognostic marker for PDAC. As oncogenic KRAS has been described as a contributing factor in PDAC addiction to autophagy, it was suggested that the mutation status of RAS could identify patients who would be more susceptible for HCQ treatment (87). This biomarker avenue turned out not to be beneficial for patient selection as oncogenic KRAS did not always promote autophagy (88). As mentioned above, oncogenic KRAS can have both stimulating and repressive effects on autophagy, and these differing effects are tumor cell-specific and context-dependent. Considering CQ sensitivity, KRAS activation rendered some cell lines more susceptible to CQ while others became more resistant (88). This is in line with the findings of Rosenfeldt et al. who suggest that in the absence of TP53, autophagy is no longer required for KRAS-mediated tumor development in PDAC, although this study does not indicate that p53 status predicts the response to anti-autophagic therapy for a developed tumor (20). Thus, the quest for suitable biomarkers identifying PDAC patients susceptible to autophagy inhibition is currently still ongoing.

CONCLUSION AND FUTURE DIRECTIONS

Autophagy has roles both in protection from malignant transformation and in promotion of tumor progression and survival. In the case of PDAC, a significant body of evidence points to a pro-tumorigenic autophagy role, where the constitutive activation of this process allows cell survival and promotes metabolism.

The mechanisms for this are diverse and require consideration of both the tumor itself and the surrounding tissue, such as stroma-associated PSCs, which provide metabolic support for the tumor by secreting alanine through cancer cell-stimulated autophagy, hence fueling the TCA cycle, Ser/Gly biosynthesis, and fatty acid synthesis in PDAC cells (15). Within the PDAC cells, it is thought that autophagy is constitutively active and is regulated through transcriptional control (11) and ROS-related signaling (45). Surrounded by a stressful environment, therefore, one way PDAC cells can upregulate their energy production components to fuel their expansion and migration is through autophagy. Autophagic genes and flux are upregulated in PDAC, as are the lysosomal and oxidative phosphorylation systems (67). MiT/TFE proteins play a crucial role in the basal transcriptional upregulation of autophagy in PDAC (11). Furthermore, upregulated autophagy is important for survival of these cells, as demonstrated by studies where autophagy is either pharmacologically or genetically impaired, resulting in loss of viability in PDAC cell lines and pancreatic cancer xenograft regression (14). PDAC progression has also been shown to rely on autophagy, although this appears to be dependent on TP53 status. In cases where TP53 is absent, tumors and cell lines are actually accelerated by autophagy inhibition (20), highlighting the need for biomarkers to report autophagy inhibition in PDAC.

Pancreatic cancer is a cancer of unmet need (89). The requirement of many pancreatic cancers for constitutively activated autophagy makes targeting this pathway an attractive new therapeutic avenue. However, due to the various feedback loops, crosstalk and parallel energy supply systems in PDAC, it might be challenging to impair PDACs' energy metabolism by autophagy inhibition on its own. Early clinical trials have shown that autophagy inhibition as a monotherapy may not be sufficient (17), but clinical trials involving combination treatment of an autophagy inhibitor and chemotherapy treatments are ongoing. In this light, the development of new, more effective upstream autophagy inhibitors of autophagy also has great potential.

AUTHOR CONTRIBUTIONS

MN and TVA wrote most of the manuscript and made the figures. JL, JS, and KR thoroughly revised and amended the manuscript. ST conceived, thoroughly revised, and amended the manuscript.

FUNDING

The work done in the lab of ST was supported by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001187), the UK Medical Research Council (FC001187), the Wellcome Trust (FC001187), and Astellas Pharmaceuticals (both ST and KR).

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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.

frontiers in Oncology

MINI REVIEW
published: 09 November 2016
doi: 10.3389/fonc.2016.00236

Prognostic Impact of Autophagy Biomarkers for Cutaneous Melanoma

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Prognosis and survival for malignant melanoma is highly dependent on early diagnosis and treatment. While the American Joint Committee on Cancer (AJCC) criterion provides a means of staging melanomas and guiding treatment approaches, it is unable to identify the risk of disease progression of early stage tumors or provide reliable stratification for novel adjuvant therapies. The demand for credible prognostic/companion biomarkers able to identify high-risk melanoma subgroups as well as guide more effective personalized/precision-based therapy is therefore of paramount importance. Autophagy, the principle lysosomal-mediated process for the degradation/recycling of cellular debris, is a hot topic in cancer medicine, and observations of its deregulation in melanoma have brought its potential as a prognostic biomarker to the forefront of current research. Key regulatory proteins, including Atg8/microtubule-associated light chain 3 (LC3) and BECN1 (Beclin 1), have been proposed as potential prognostic biomarkers. However, given the dynamic nature of autophagy, their expression *in vitro* does not translate to their use as a prognostic biomarker for melanoma *in vivo*. We have recently identified the expression levels of Sequestosome1/SQSTM1 (p62) and activating molecule in Beclin 1-regulated autophagy protein 1 (AMBRA1) as novel independent prognostic biomarkers for early stage melanomas. While increasing followed by subsequent decreasing levels of p62 expression reflects the paradoxical role of autophagy in melanoma, expression levels additionally define a novel prognostic biomarker for AJCC stage II tumors. Conversely, loss of AMBRA1 in the epidermis overlying primary melanomas defines a novel prognostic biomarker for AJCC stage I tumors. Collectively, the definition of AMBRA1 and p62 as prognostic biomarkers for early stage melanomas provides novel and accurate means through which to identify tumors at risk of disease progression, facilitating earlier patient therapeutic intervention and stratification tools for novel personalized therapeutic approaches to improve clinical outcome.

Keywords: autophagy biomarkers, malignant melanoma, Ambra1, p62, prognostic biomarkers

OPEN ACCESS

Edited by:
Patrizia Agostinis, State University of Leuven, Belgium

Reviewed by:
Boris Zhivotovsky, Karolinska Institutet, Sweden
Esther Hoste, Ghent University, Belgium

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *28 September 2016*
Accepted: *24 October 2016*
Published: *09 November 2016*

Citation:
Tang DYL, Ellis RA and Lovat PE (2016) Prognostic Impact of Autophagy Biomarkers for Cutaneous Melanoma. Front. Oncol. 6:236. doi: 10.3389/fonc.2016.00236

Malignant melanoma, the most aggressive form of skin cancer arising from the malignant transformation of melanocytes, is an increasing public health concern worldwide with incidence rates doubling every 10–20 years (1), which now renders this malignancy accountable for 75% of all skin cancer deaths and the most common cause of cancer-related mortality in young individuals between 20 and 35 years of age (2).

As with many cancers, prognosis and survival for melanoma is highly dependent on early detection, diagnosis, and treatment. In line with this need and coupled with the emergence of novel

targeted and immunotherapies, current interest is focused on the discovery of predictive and prognostic biomarkers. A biomarker refers to any measurable diagnostic indicator that is used to assess the risk or presence of disease (3). While predictive biomarkers are able to indicate which patient subgroups are likely to benefit from certain treatments (4), prognostic markers enable stratification of patients at initial diagnosis according to eventual outcome, which can be used clinically to guide patient management including the earlier initiation of adjuvant therapies in patients at high risk of disease progression, potentially preventing the development of untreatable metastatic disease (5).

Some of the best established prognostic biomarkers for melanoma are incorporated into the current American Joint Committee on Cancer (AJCC) 2009 staging criterion, the most comprehensive staging system for melanoma to date, which remains the international standard for disease staging and as a guide for treatment approaches. AJCC staging combines several prognostic factors for melanoma, including the depth of invasion (Breslow depth), rate of mitoses, presence of ulceration (loss of the epidermis overlying the tumor), evidence of metastatic spread, and changes in serum lactate dehydrogenase (LDH) to allow risk stratification of morbidity and mortality at the initial diagnosis (6). In general, this divides malignant melanoma into four stages: stages I–II comprising primary tumors of distinct thickness (defined as early stage disease), stage III where locoregional spread of disease (mainly to local lymph node basins) is present, and stage IV where there is presence of distant metastasis.

While early stage melanoma is largely curative by surgical excision, metastatic disease represents the cause of death from melanoma in the vast majority of cases due to a lack of consistently beneficial treatment regimens for late stage disease. Furthermore, despite its comprehensiveness, AJCC staging as a prognostic biomarker is limited by the inability of its criteria to accurately identify high-risk melanoma subgroups that will go on to progress; a particular problem in seemingly "low risk" AJCC stage I melanomas where up to 10% of tumors subsequently metastasize. This emphasizes the urgent need for novel credible biomarkers to identify high-risk tumors as well as the stratification of such patients for more efficacious and earlier therapeutic approaches (6).

Observations of deregulated autophagy in many cancers, including melanoma, have brought this key signaling mechanism to the forefront of much research (7, 8), including its potential capacity as a prognostic biomarker. Autophagy, the principle catabolic process for lysosomal-mediated degradation of intracellular components to sustain cellular energy and survival, is regulated by a complex signaling cascade involving ubiquitin-like conjugation systems, autophagy regulatory proteins [BECN1/Beclin 1 (Beclin-1), activating molecule in Beclin 1-regulated autophagy protein 1 (AMBRA1), Atg8/microtubule-associated light chain 3 LC3 (LC3), and Sequestosome1/SQSTM1 (p62)], and the inactivation of mammalian target of rapamycin (mTOR) to induce activation. Cellular debris within the cytoplasm is sequestered into double-membrane autophagosomes, which are then delivered to lysosomes for degradation and recycling (9). In cancer, however, autophagy plays a paradoxical role; on the one hand, preventing build-up of toxic cellular components that result in genomic stress and instability, thereby promoting tumorigenesis, while, on the other hand, promoting tumor survival of advanced stage solid tumors such as melanoma in a nutrient-deprived hypoxic environment (10). Consistent with the paradoxical role of autophagy and in contrast to observations in benign nevi, electron microscopy studies have shown an increased presence and vacuolization (suggesting degradation) of double-membraned autophagosomes in the cytoplasm of metastatic melanoma cells, thus supporting the notion of increased autophagic activity in advanced stage disease (11, 12).

BRAF is a member of the RAF group of serine/threonine protein kinases, and as such functions to regulate the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) cellular growth pathway (13). Activating mutations in BRAF, present and in up to 70% of all melanomas along with NRas mutations (present in approximately 15–20% of melanomas), result in constitutive activation of MAPK signaling, promoting growth, survival, and chemoresistance (14–17). Interestingly, BRAF mutational status in melanoma has also been shown to variably influence autophagy. Following treatment of BRAF wild-type melanoma cells with endoplasmic reticulum (ER) stress-inducing agents *in vitro*, autophagy is activated in line with its pro-survival role; however, when autophagy is inhibited exogenously, this leads to increased cell death (12, 18). Conversely, oncogenic BRAF induces a chronic ER stress status, resulting in enhanced basal autophagy [as evidenced by increased Atg8-PE/LC3-II (LC3-II) expression], resistance of melanoma cells to apoptosis, and insensitivity to further autophagy induction. This suggests that although melanomas rely on increased innate autophagic activity, BRAF-mutated tumors are resistant to further mTOR-dependant stimulation of autophagy and that while combined inhibition of autophagy with chemotherapy might be a viable therapeutic avenue for BRAF wild-type melanomas, targeted therapies that attenuate ER stress may prove a more effective treatment strategy for BRAF mutant melanomas (12, 18, 19).

To date, several markers of autophagy, including LC3 and Beclin 1, have been identified as potential prognostic biomarkers for melanoma. Under normal homeostatic conditions, exogenous LC3 is cytoplasmic but upon autophagy induction becomes conjugated to phosphatidylethanolamine (PE) to form the membrane bound form LC3-II, thus acting as a marker of autophagy induction (20). Increased immunohistochemical expression of LC3 has been shown in malignant melanomas compared to benign nevi (21) and is associated with the development of metastatic disease and poorer outcomes (22). In addition, studies of Beclin 1 expression suggest its' downregulation parallels melanoma disease stage progression, further supporting a role for autophagy in tumor invasion and metastasis (23, 24). However, although the expression of LC3 provides an indication of autophagy status in melanoma, it is important to note that conversion of LC3-I to LC3-II is a dynamic process, thus limiting the capacity of endogenous LC3 expression as an accurate biomarker of autophagy status and importantly, the reflection of autophagic flux (15). Moreover, although reported to be downregulated in melanoma, there is also conflicting evidence of Beclin 1 overexpression in

advanced melanoma, thus questioning its expression as a reliable prognostic biomarker (25).

Sequestosome1/SQSTM1 is a scaffold protein that shuttles ubiquitinated proteins into the autophagosome, later degraded along with other autophagosomal contents upon fusion with a lysosome. Impairment of autophagy is therefore reflected by an associated accumulation of p62, a process reported to be a key to the onset of tumorigenesis (17). Conversely, decreased levels of p62 reflect active autophagy, as observed in advanced stage melanomas where autophagy is commonly reactivated to enhance tumor survival (**Figure 1A**). Data from our lab have further defined p62 expression as a prognostic biomarker for melanoma where a stepwise increase in expression is observed in early AJCC stage melanomas (increased above basal levels in benign nevi and reflecting deregulated autophagy) but which is subsequently decreased in advanced metastatic tumors, consistent with the reactivation of autophagy and its paradoxical role in cancer [**Figures 1B,C**; (26)]. Furthermore, univariate analysis showed a significantly increased risk of metastasis in AJCC stage II tumors with low p62 expression (<20% median p62 expression) compared to those with high expression (>20% p62 expression) [**Figure 1D**; (26)]. Moreover, since there was no association with

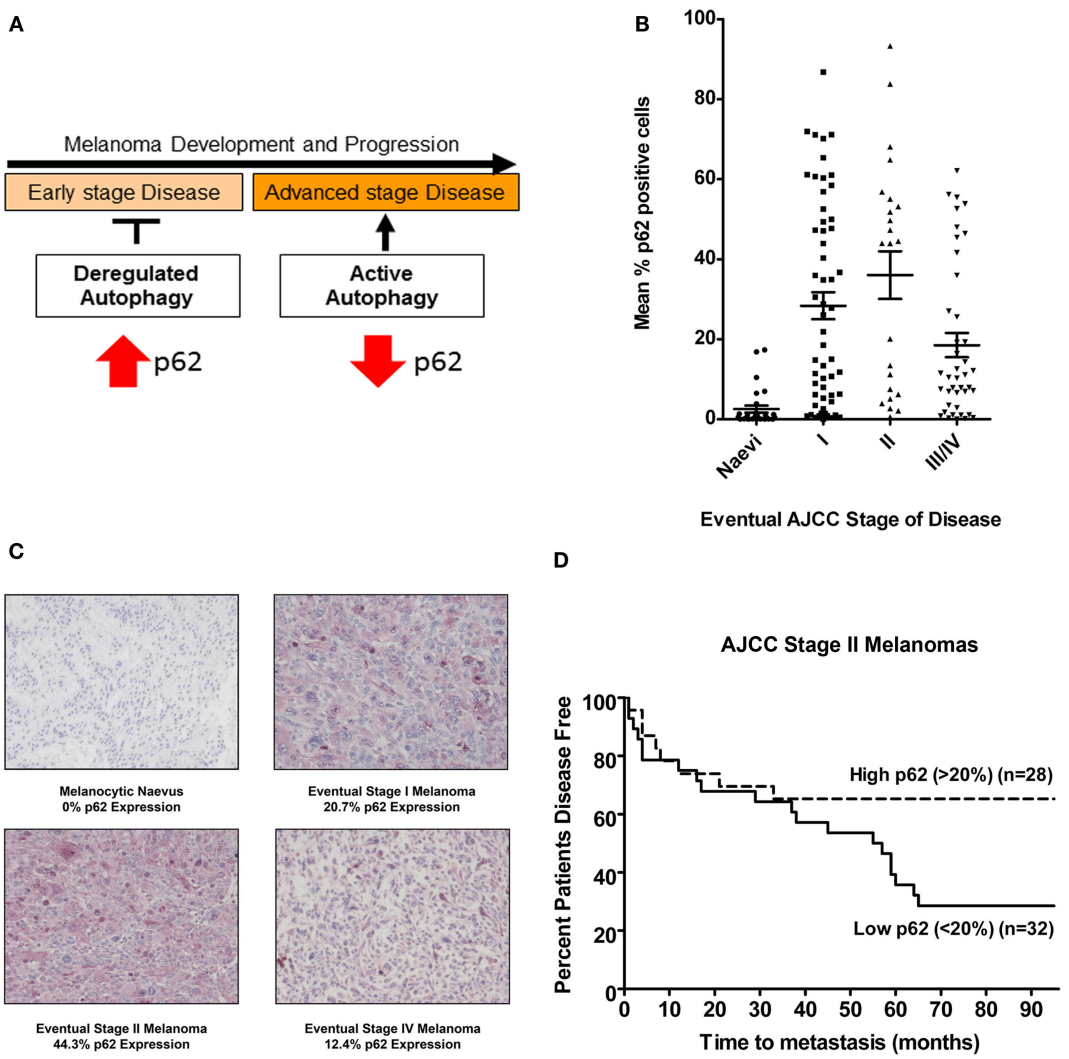

FIGURE 1 | p62 expression is a prognostic biomarker for AJCC stage II melanomas. **(A)** Schematic of the paradoxical role of autophagy in melanoma in the context of p62 expression; impairment of autophagy drives tumorigenesis of early stage melanomas reflected by p62 accumulation, whereas decreased levels of p62 seen in advanced disease reflect autophagy reactivation. **(B)** Mean % p62 expression in a cohort of eventual AJCC stage I, II, III, and IV melanomas or benign nevi after a minimum 5-year follow-up. Each point represents the mean % of p62 positive cells. Horizontal lines representing median p62 expression levels indicate an increase in median p62 expression levels between benign nevi and AJCC stage I or II melanomas and a relative decrease in expression in advanced AJCC stages III and IV tumors (Kruskal–Wallis $P < 0.0001$) (26). **(C)** Photomicrographs of immunohistochemical p62 expression and mean % in a melanocytic nevus or an eventual AJCC stage I, II, or IV melanoma. Scale bar = 100 μm. **(D)** Univariate analysis of mean p62 expression in AJCC stage II primary tumors demonstrating an increased risk of metastasis in tumors expressing >20% p62 [Log-Rank (Mantel–Cox) $P = 0.031$, HR 2.29 (95% CI 1.08–4.86)], and highlighting the potential of p62 as a prognostic biomarker (26).

Breslow depth or tumor ulceration, p62 expression defines an independent stratifying variable from AJCC staging prognosticators. Collectively, these data highlight p62 as a novel independent prognostic biomarker for AJCC stage II melanomas, providing a powerful tool for refining the risk of disease progression and enabling earlier patient therapeutic intervention. In addition, p62 expression may represent a companion biomarker of response to autophagy modulation *in vivo*, an important concept in view of emerging autophagy modulator therapies (27) and their potential to improve overall clinical outcome for patients with metastatic melanoma.

Activating molecule in Beclin 1-regulated protein 1 (AMBRA1) is a component of the Beclin 1/VPS34 complex and involved in the formation of PI3K rich membranes during the nucleation phase of autophagy [**Figure 2A**; (28)]. As a key autophagy initiating regulatory protein, AMBRA1 represents a potential marker of autophagy induction as well as a possible therapeutic target for autophagy inhibition. However, in addition to its functional role in autophagy, a growing body of evidence supports a role for AMBRA1 in cellular differentiation (29, 30) including in the early differentiation of neuronal stem cells in which autophagy is activated to fulfill the high energy demands of this process (28, 31). In line with these findings, we have recently demonstrated the role of AMBRA1 in epidermal differentiation with the expression *in vivo* increasing in line with keratinocyte differentiation from the basal layer of the epidermis to the uppermost layer, the *stratum corneum* (32). Unlike p62, however, the expression of AMBRA1 in primary melanomas is variable and as such its value as a tumoral biomarker remains undefined. Strikingly, however, our recent data demonstrate the decreased or even complete loss of AMBRA1 expression in the epidermis overlying many AJCC stage I melanomas (**Figure 2B**), which did not correlate with the degree of epidermal invasion and was not observed in benign nevi (32). These data suggest that the expression of AMBRA1 in the melanoma microenvironment may have prognostic potential. Univariate analysis of an initial cohort of 129 all AJCC stage melanomas further revealed decreased or loss of epidermal AMBRA1 expression was significantly associated with decreased disease-free survival, with stratification for AJCC stage I disease, additionally revealing epidermal AMBRA1 expression as a putative biomarker of disease progression [**Figure 2C**; (32)]. Again, there was no correlation with Breslow depth suggesting, such as

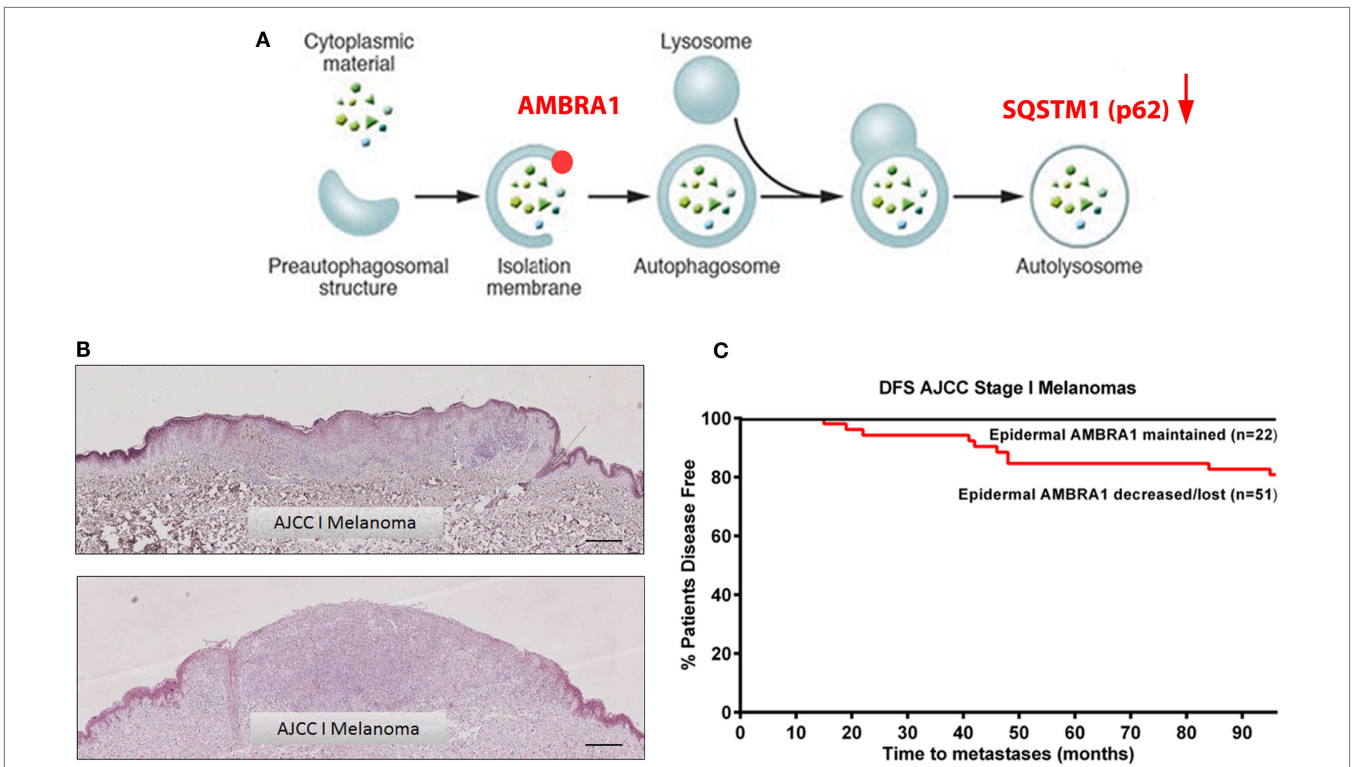

FIGURE 2 | Loss of epidermal AMBRA1 identifies a high-risk AJCC stage I melanoma subgroup. **(A)** Schematic representation of the autophagy pathway highlighting the role of AMBRA1 in the nucleation phase of autophagy and indicating interplay of p62. **(B)** Representative immunohistochemistry images of epidermal AMBRA1 expression depicting maintained (top image) or loss of AMBRA1 expression (bottom image) in the epidermis overlying AJCC stage I melanomas. Loss of epidermal AMBRA1 expression overlying the tumor tissue creates a "watershed" area, where the epidermis distant to the tumor reveals a normal pattern of AMBRA1 expression. Scale bar = 100 μm. **(C)** Kaplan–Meier curve showing decreased 7-year disease-free survival in 51 AJCC stage I tumors where epidermal AMBRA1 was decreased or lost as compared with 22 tumors where AMBRA1 expression was maintained [Log-Rank (Mantel–Cox) test $P < 0.03$, HR 4.3 (95% CI 1.14–16.51)]. Epidermal AMBRA1 expression of each tumor was recorded as being either maintained, decreased, or lost based on the perceived degree of loss of epidermal AMBRA 1 expression overlying the tumor bulk compared to normal epidermis within the same section.

p62, that epidermal AMBRA1 expression is also a biologically distinct marker for AJCC stage I melanomas. This is a striking finding considering that these tumors are normally regarded as low risk, with currently no alternative means of identifying specific individuals whose tumors are likely to progress.

Since the current and universally adopted AJCC staging system is unable to identify the risk of disease progression in seemingly "low risk" early stage melanomas, such tumors are only identified after the onset of metastatic disease progression, at which point treatment options are limited and frequently ineffective. Critically, identifying, refining, and validating prognostic biomarkers for early stage melanomas such as the proposed biomarkers of autophagy will thus enable the identification of high-risk tumor subgroups. Both p62 and AMBRA1 expression exemplify how autophagy can be harnessed as prognostic biomarkers for melanoma, each providing clinically relevant information over and above AJCC staging, which in particular will be useful for refining the risk of melanoma progression in patients with AJCC stage I or II melanomas. Ultimately, further validation of these biomarkers will allow application in a clinical context, facilitating both earlier therapeutic intervention and the refinement of personalized therapies for malignant melanoma to improve clinical outcome and the prevention of premature loss of life.

AUTHOR CONTRIBUTIONS

DT: main author of the paper and corresponding author involved in all aspects of authorship. RE: substantial contributions to the acquisition, analysis, and interpretation of data for the work including production of figures for the paper. PL: senior author, contribution to the analysis of data and the writing of the manuscript; accountable for all aspects of the work. All the authors have undertaken final approval of the final manuscript version to be published.

ACKNOWLEDGMENTS

DT is sponsored by a fellowship from The British Skin Foundation, RE and PL are supported by research grants from Melanoma Focus, North Eastern Skin Research Fund, and The Newcastle Healthcare Charity.

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