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Targeting autophagy in breast cancer

Paola Maycotte, Andrew Thorburn

Paola Maycotte, Andrew Thorburn, Department of Pharmacology, University of Colorado School of Medicine, Aurora, CO 80045, United States

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Correspondence to: Andrew Thorburn, Professor and Chair, Department of Pharmacology, University of Colorado School of Medicine, Mail Stop 8303, 12801 East 17th Avenue, Aurora, CO 80045, United States. andrew.thorburn@ucdenver.edu

Telephone: +1-303-7243290 Fax: +1-303-7243664

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Core tip: Autophagy is thought to be a tumor suppressor pathway. However, once a tumor is formed, it may contribute to tumor cell survival in response to metabolic stress or to therapy. On the other hand, it has also been suggested that autophagy could be induced during breast cancer therapy to kill cells that avoid apoptosis. Here, we discuss some of the recent findings relating autophagy and cancer with a particular focus on breast cancer therapy. We conclude that there are important unresolved questions that should be addressed before autophagy can be successfully targeted for breast cancer treatment.

Abstract

Macroautophagy (referred to as autophagy here) is an intracellular degradation pathway enhanced in response to a variety of stresses and in response to nutrient deprivation. This process provides the cell with nutrients and energy by degrading aggregated and damaged proteins as well as compromised organelles. Since autophagy has been linked to diverse diseases including cancer, it has recently become a very interesting target in breast cancer treatment. Indeed, current clinical trials are trying to use chloroquine or hydroxychloroquine, alone or in combination with other drugs to inhibit autophagy during breast cancer therapy since chemotherapy and radiation, regimens that are used to treat breast cancer, are known to induce autophagy in cancer cells. Importantly, in breast cancer, autophagy has been involved in the development of resistance to chemotherapy and to anti-estrogens. Moreover, a close relationship has recently been described between autophagy and the HER2 receptor. Here, we discuss some of the recent findings relating autophagy and cancer with a particular focus on breast cancer therapy.

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INTRODUCTION

The term autophagy describes the lysosomal degradation, or eating (phagy) of part of the cell itself (auto). Several forms of autophagy have been described. Among them, macroautophagy (referred to here as autophagy) is a ubiquitous process in eukaryotic cells in which cytoplasmic components are engulfed in a double membrane structure called the autophagosome which delivers its contents for degradation to the lysosome. This process occurs at basal levels in all cells during nutrient rich conditions providing tissues with a housekeeping mechanism of cytoplasmic turnover and removal of damaged organelles as well as protein aggregates. Autophagy is also up-regulated in response to physiological conditions, such as starvation and in response to diverse pathological stresses, including hypoxia, formation of protein aggregates

or infection, and many others, thus allowing the cells to adapt to environmental and developmental changes^[1].

Alterations in autophagy are involved in several diseases including cancer^[2]. In cancer, autophagy is thought to have both tumor suppressive and tumor promoting functions. These paradoxical effects may be explained because the role of autophagy in cancer depends on the context and on the stage of tumorigenesis. The tumor suppressive functions of autophagy are most apparent during tumor initiation. Autophagy has been found to limit inflammation, tissue damage and genome instability which are known promoters of cancer initiation. Thus, it has been suggested that autophagy stimulation could be beneficial for cancer prevention. On the other hand, in later stages of cancer development, when tumor cells are exposed to stresses encountered during progression, metastasis and cancer therapy, autophagy is thought to be a tumor promoting mechanism by enabling survival of tumor cells^[3,4]. In this setting, it has been proposed that autophagy should be inhibited during cancer treatment in order to improve therapy, especially in those cancer cells that have high levels of autophagy and that are dependent on autophagy for survival under metabolic stress. Nevertheless, it is important to note that other studies suggest that excessive levels of autophagy could lead to cell death especially in those cells that are apoptosis deficient, which is the case of many cancer cells. It has also been suggested that autophagy modulation could have an effect on anti-tumor immune response^[5,6]. In this review, we discuss some of the recent evidence regarding the role of autophagy in cancer initiation, progression and therapy with a special emphasis on breast cancer.

AUTOPHAGIC PROCESS

The proteins mediating the formation of the autophagosome are known as Atg (autophagy-related) proteins. More than 30 Atg proteins have been identified in yeast and many have mammalian orthologs. The autophagic process (Figure 1 and Table 1) involves a series of steps which start with the initiation of the autophagosome, in which the phagophore (a cup-shaped structure that will later develop into the autophagosome) is formed. It is mediated by the Atg1/ULK kinase complex and is negatively regulated by mTORC1 (mammalian target of rapamycin complex 1), which integrates signals from growth factors, amino acids, glucose and energy status and allows autophagy to be induced in the absence of these stimuli. During nucleation, the Atg proteins are hierarchically recruited to the phagophore assembly site. This process is mediated by a complex integrated by beclin 1, hVps34/class III phosphatidylinositol 3-kinase (PI3K) and several other beclin 1 binding proteins (Figure 2). The elongation of the autophagosomal membrane is controlled by two ubiquitin-like protein conjugation systems: Atg12-Atg5 and Atg8/LC3. Autophagosomes may acquire membrane from multiple sources, including the endoplasmic reticulum (ER), the mitochondrial outer membrane, the plasma

membrane and the Golgi apparatus. Additionally, Atg8/LC3 also recruits adaptor proteins such as p62 and NBR1 to the autophagosomes mediating selective autophagy of different cellular structures, protein aggregates and microorganisms. Finally, autophagosomes move along microtubules towards the microtubule organizing center where lysosomes are enriched. Autophagosomes fuse with endosomes-lysosomes to form autolysosomes and are degraded together with their luminal content^[1,2,7].

At its basal rate, autophagy exercises quality control of the cytoplasm by removing damaged organelles and protein aggregates. Also, autophagy responds to a range of stimuli and in most cases protects the cell against stressful situations. In response to starvation, autophagy is important for lysosomal recycling of metabolites to the cytoplasm, where they are reused either as a source of energy or to provide building blocks for the synthesis of new macromolecules^[8]. Although the pro-survival functions of autophagy have been demonstrated at the cellular and organismal level in different contexts, it is also believed that autophagy can induce cell death. This has shown to be true particularly in lower eukaryotes, where autophagy seems to be directly involved in cell demise. Examples include *Dictyostelium discoideum*, a soil amoeba and midgut cell death occurring during development in *Drosophila melanogaster*^[9,10]. However, the existence of autophagic cell death in mammalian cells remains controversial. The bulk of the available data suggest that autophagic cell death occurs in specific *in vitro* models^[11-13] particularly in cells that are defective in apoptosis, or contributes to the induction of apoptosis or necrosis rather than being directly responsible for cell killing^[14,15]. Nevertheless, a recent report described a form of autophagic cell death, which the authors name autosis that is induced by an autophagy inducing peptide and is regulated by the Na⁺, K⁺-ATPase. Dead cells with morphological characteristics consistent with this type of cell death were found in a subpopulation of cells dying by starvation and in hippocampal neurons exposed to hypoxia-ischemia^[16], providing evidence of autophagic cell death in mammalian cells *in vivo*.

Several signaling molecules and cascades modulate autophagy in response to numerous cellular and environmental cues. The best characterized modulator of autophagy is mTORC1. It negatively regulates autophagy by inhibiting the ULK1 complex through direct phosphorylation and is inhibited by rapamycin, which induces autophagy. The activity of mTORC1 is stimulated by a variety of anabolic inputs, including cellular energy status as well as the presence of amino acids and growth factors. Conversely, mTORC1 is inhibited when amino acids are scarce, when growth factor signaling is reduced (*e.g.*, decreased insulin receptor signaling as shown in Figure 1) and/or ATP concentration decreases, resulting in a de-repression of autophagy. In mammalian cells, ULK1 is also directly phosphorylated and activated by the AMP-activated protein kinase (AMPK) in response to energy restriction. The class III PI3K complex is another impor-

Table 1 Some autophagy-related proteins and other proteins implicated in autophagy mentioned in the text and their autophagy-independent functions

Protein	Role in autophagy	Autophagy-independent roles
ULK1/2	Protein kinase involved in autophagy induction and phagophore biogenesis ^[105]	
Atg2	Interacts with Atg18, possibly involved in phagophore biogenesis ^[105]	Regulation of lipid droplet morphology and dispersion ^[106]
Atg3	E2-like enzyme for Atg8/LC3 ubiquitin-like conjugation system. Involved in phagophore expansion ^[105]	Atg12-Atg3 complex formation does not affect starvation-induced autophagy, increases mitochondrial mass and inhibits cell death mediated by mitochondrial pathways ^[107]
Atg4	Cysteine protease involved in Atg8/LC3 processing (removal of amino acid residues to expose the C-terminal glycine for lipidation) and in lipid removal from Atg8/LC3-PE (lipidated LC3 or LC3 II). Involved in phagophore expansion ^[105]	
Atg5	Atg12-Atg5 conjugation system, involved in phagophore expansion ^[105]	Its expression increases during DNA damage induced by chemotherapy. It induces cell cycle arrest and mitotic catastrophe ^[108]
Atg6/beclin 1	Component of the class III PI3K complex, involved in the induction of autophagy and phagophore biogenesis ^[105]	Interacts with Bcl-2 family proteins. Regulates the stability of USP10 and USP13 thus controlling p53 levels ^[92]
Atg7	E1 (ubiquitin-activating)-like enzyme for the two ubiquitin-like conjugation systems (Atg12-Atg5 and Atg8/LC3) ^[105]	Binds p53 and regulates cell cycle arrest upon metabolic stress ^[109]
Atg8/LC3	Ubiquitin-like protein that is conjugated to PE. It is involved in cargo recruitment into, biogenesis of autophagosomes and phagophore expansion ^[105]	
Atg9	Transmembrane protein that may act as lipid carrier for phagophore expansion ^[105]	
Atg10	E2 (ubiquitin-conjugating)-like enzyme for the Atg12-Atg5 ubiquitin-like conjugation system ^[105]	
Atg12	Ubiquitin-like protein that gets covalently linked to Atg5 in the Atg12-Atg5 conjugation system. Involved in phagophore expansion ^[105]	Atg12-Atg3 complex formation does not affect starvation-induced autophagy, increases mitochondrial mass and inhibits cell death mediated by mitochondrial pathways ^[107]
Atg13	Binding partner and regulator of ULK1/2, involved in induction of autophagy and phagophore biogenesis ^[105]	
Atg14/ATG14L	Component of the class III PI3K complex, involved in induction of autophagy and phagophore biogenesis ^[105]	
Atg16	Associates with Atg12-Atg5 and acts as an E3 ligase to direct LC3 lipidation, involved in phagophore expansion ^[105]	
Atg17/FIP200	Binding partner and regulator of ULK1/2 involved in induction of autophagy and phagophore biogenesis ^[105]	
Atg18/WIPs	PI3P-binding proteins possibly involved in phagophore biogenesis ^[105]	Retrograde transport from the vacuole to the Golgi complex. Regulation of PI(3,5)P ₂ synthesis ^[105]
mTORC1	Serine/threonine kinase rapamycin-sensitive complex, main down-regulator of autophagy that responds to growth factor and nutrient availability ^[105]	Regulates cell growth (accumulation of cell mass) through coordination of protein anabolism, nucleotide biosynthesis, lipogenesis, glycolysis and autophagy ^[110]
Vps34	Class III PI3K, produces PI3P and allows recruitment of PI3P-binding proteins WIP1/2 and of the two ubiquitin-like conjugation systems ^[105]	Regulation of vesicular trafficking in the endosomal/lysosomal system. Regulates signaling by recruiting proteins that bind PI3P ^[111]
Vps15	Regulatory kinase subunit of the class III PI3K ^[105]	
p62	Selective substrate of autophagy that functions as an adaptor protein that links ubiquitinated proteins to LC3 ^[105]	Serves as a scaffold to promote NFκB signaling in TNFR, IL-1βR or NGFR signaling by binding RIP1 or TRAF6. Can bind caspase-8 and stimulate apoptosis. Binds mTORC1 and Rag GTPases on the lysosomal surface to signal amino acid availability ^[112] . Activates transcription factor Nrf2, which drives expression of antioxidant and detoxifying enzymes by competitively binding to Keap1, its ubiquitin ligase ^[113]
NBR1	(Neighbor of BRCA1 gene 1), selective substrate of autophagy with structural similarity to p62 ^[105]	Negatively regulates receptor tyrosine kinase endocytic traffic ^[114]
AMPK	(AMP-activated protein kinase), a sensor of energy that is activated by an increase in the AMP/ATP ratio ^[105,115]	AMPK regulates metabolism, decreases energy expenditure, mediates cell cycle checkpoints, inhibits pro-survival growth pathways and modulates mitotic progression ^[115]
Bcl-2/Bcl-XL	Members of the Bcl-2 family of proteins that inhibit macroautophagy (by binding beclin 1) and pro-apoptotic BH3-only proteins ^[105]	Antiapoptotic proteins that inhibit pro-apoptotic BH3-only proteins (BNIP3, Bad, Bik, Noxa, Puma and BimEL) ^[105]
Survivin	Interacts with beclin-1 ^[103]	Member of the Inhibitor of Apoptosis (IAP) family of proteins that inhibits caspases. It is also involved in chromosome segregation during cell division ^[116]
PINK1	(PTEN-induced kinase 1/PARK6), mitochondrial protein that spans the outer mitochondrial membrane upon mitochondrial depolarization, recruiting Parkin to facilitate mitophagy ^[105]	Mitochondrial protein (mutated in some forms of Parkinson disease) that is processed in a membrane potential-dependent manner to maintain mitochondrial function ^[105]

VMP1	(vacuole membrane protein 1), localizes to the plasma membrane of the ER. Interacts with beclin 1 and is required for autophagy ^[105]	Required for protein secretion and Golgi organization, regulates cell proliferation, anchorage-independent growth and secretory membrane transport. It is a component of initial cell-cell contacts and tight junctions ^[117]
DAPK	Death associated protein kinase, it phosphorylates beclin 1 to activate autophagy by causing dissociation from Bcl-2 ^[105]	Regulates cell death, inhibits cell motility and adhesion, promotes membrane blebbing and stress fiber formation ^[118]
Bif-1	Bax-interacting factor 1)/endophilin B1, protein that interacts with beclin 1 <i>via</i> UVRAG and is required for macroautophagy ^[105] . It has membrane curvature-inducing activity, indicating that it may play a role in biogenesis of isolation membranes ^[119] .	Involved in mitochondrial fission and coat protein complex I (COPI)-vesicle formation ^[119] . Regulates receptor degradation and cytokinesis when present in a class III PI3K subcomplex containing Vps15, Vps34, beclin 1 and UVRAG ^[120]
UVRAG	UV irradiation resistance-associated gene, component of the class III PI3K complex that activates autophagy. It disrupts beclin 1 dimers and forms a heterodimer that activates autophagy. It binds Bif-1 to activate class III PI3K and competes with Atg14L for binding to beclin 1, directing class III PI3K to function in the maturation step of autophagy ^[105]	Regulates receptor degradation and cytokinesis when present in a class III PI3K subcomplex containing Vps15, Vps34, beclin 1 and Bif-1 ^[120] . Regulates coat protein complex I (COPI)-vesicle tethering in the ER ^[121]
Ambra1	Activating molecule in beclin 1-regulated autophagy, binds beclin 1 and positively regulates autophagy ^[105] . Regulates ULK1 stability and kinase activity and is phosphorylated and inactivated by mTOR, inhibiting its action on ULK1 ^[122] . Also, changes in its subcellular localization are important during autophagosome formation ^[123]	
HMGB1	High mobility group box 1, a chromatin-associated nuclear protein that translocates to the cytoplasm in response to stress. Binds to beclin 1, displacing Bcl-2 and promoting autophagy. Autophagy also promotes the release of HMGB1 from the nucleus and the cell which further induces autophagy ^[105]	In the nucleus it is a DNA chaperone, sustains nucleosome dynamics and chromosome stability, modulates gene transcription, recombination and participates in DNA repair and telomere maintenance. It regulates mitochondrial function and when secreted regulates inflammation, immunity, migration, proliferation, metabolism and apoptosis ^[124]
NAF-1	Nutrient-deprivation autophagy factor-1, integral membrane component of the IP3 receptor complex. It binds Bcl-2 at the ER and is required for Bcl-2 to bind beclin 1, resulting in the inhibition of autophagy ^[105]	Required for Bcl-2 dependent regulation of the IP3 channel at the ER and regulates Ca ²⁺ homeostasis ^[125]

tant point of regulation for autophagy induction. Beclin 1 is one of the subunits of the complex and its incorporation, which is essential for its kinase activity, is regulated by its association with other proteins, such as Bcl-2, Survivin, PINK1 or VMP1 (Figure 2). The phosphorylation of beclin 1 by death-associated protein kinase (DAPK) or phosphorylation of Bcl-2 by c-Jun N-terminal kinase (JNK) triggers the dissociation of beclin 1 from Bcl-2 in response to various stimuli, thereby inducing autophagy. AMPK can also stimulate autophagy in response to glucose starvation by phosphorylating beclin 1 on a residue that promotes its incorporation into the PI3K complex^[1,17]. Recent evidence also suggests that the Atg proteins are substrates for transcriptional regulation as well as post-translational modifications such as phosphorylation, ubiquitination and acetylation^[18]. The activity of the class III PI3K complex can be manipulated by pharmacological activators (such as BH3 mimetics) and inhibitors (such as spautin). Inhibitors of lysosomal enzymes (such as cystatin B) and lysosomotropic agents that increase the lysosomal pH (such as chloroquine, hydroxychloroquine and Bafilomycin A) are also used to block the degradative activity of autolysosomes and block autophagy at the degradation step (Figure 1)^[2,17].

AUTOPHAGY AND CANCER

Alterations in the autophagic pathway have been observed in several disorders, including metabolic diseases, myopathy, neurodegenerative disorders, infectious and

inflammatory diseases, autoimmune disorders, aging and cancer^[2,19]. Cancer was one of the first diseases genetically linked to an impairment of autophagy with the proposal that *beclin 1* functioned as a haploinsufficient tumor suppressor. *Beclin 1* was found to be monoallelically deleted in a high percentage of ovarian, breast and prostate cancers and its heterozygous disruption in mice results in increased spontaneous malignancies including lung cancers, liver cancers, lymphomas and mammary precancerous lesions^[20-22]. More recently, studies have established a relationship between beclin 1 expression and cancer prognosis since low levels of beclin 1 are associated with a worse prognosis in gastric, colorectal, pancreatic, oesophageal and breast cancers as well as in chondrosarcoma, whereas high levels of its expression are associated with improved survival in high-grade gliomas, hepatocellular carcinomas and B cell lymphomas^[2]. Nevertheless, deletions of *beclin 1* have recently been found mostly associated with *BRCA1* in breast and ovarian human tumors (suggesting that *BRCA1* loss is the driver mutation and that *beclin 1* is lost because of its proximity to it). Furthermore, in a recent comprehensive study no evidence was found for *beclin 1* mutations or loss in other cancers, casting doubt on whether it is a real tumor suppressor in human cancers^[23].

Other ATG proteins have been shown to be involved in the suppression of tumorigenesis. For instance, *Atg 4C* knockout mice do not develop more spontaneous tumors than their wild-type littermates but they are more prone to develop chemically-induced fibrosarcomas^[24] and *LC3* is localized to 16q24.1, a locus frequently deleted in liver,

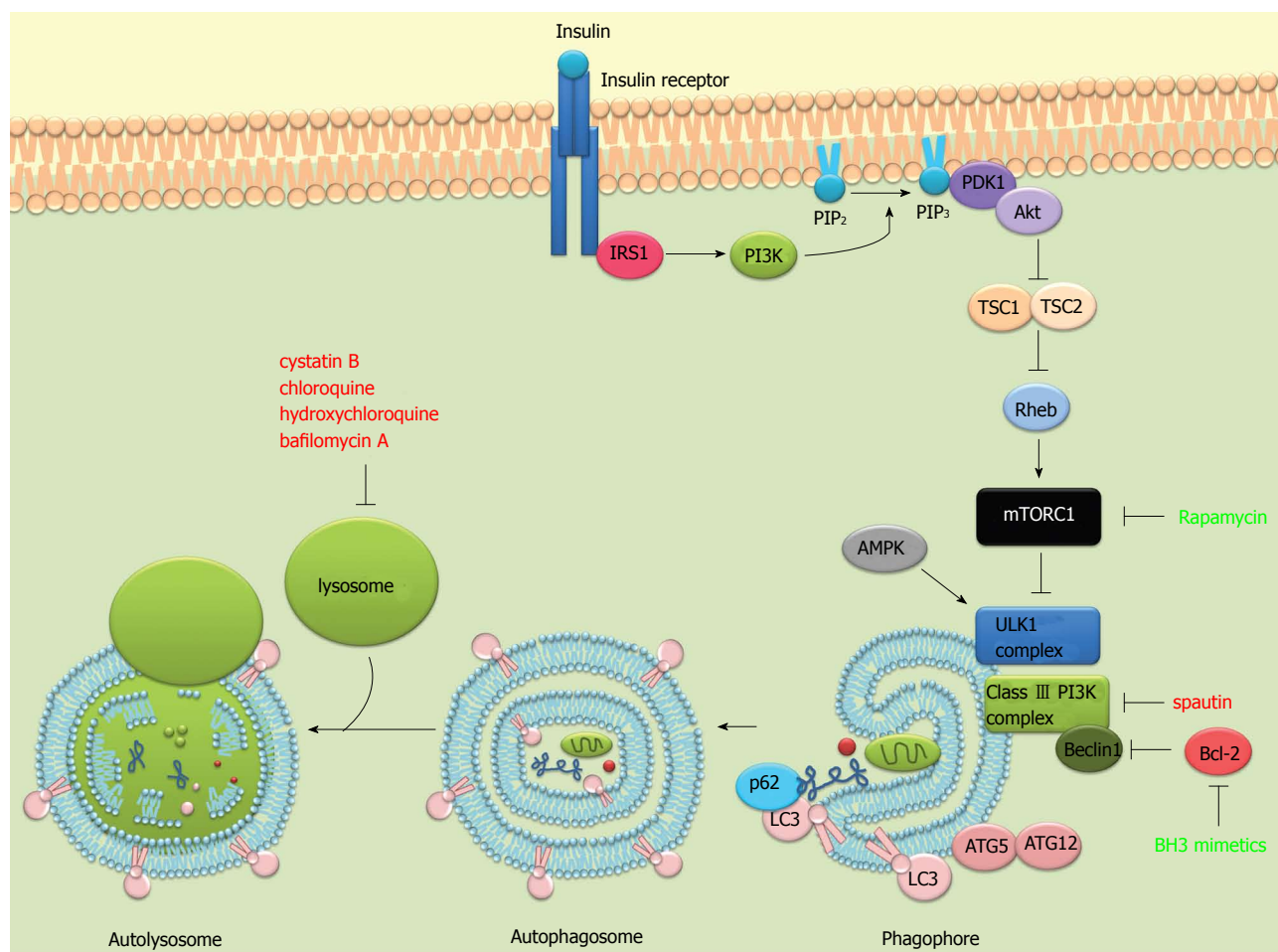


Figure 1 The autophagic process and some of its major regulators. The phosphatidylinositol 3-kinase (PI3K) pathway is triggered by the binding of insulin or growth factors to the insulin receptor, activating PI3K. Activated PI3K converts PIP₂ to PIP₃ which then recruits PDK1 and Akt to the plasma membrane. Activated Akt then phosphorylates and inactivates TSC 1/2, leading to the activation of Rheb and mammalian target of rapamycin complex 1 (mTORC1). Under nutrient-rich conditions, mTORC1 suppresses autophagy by phosphorylating and inhibiting the ULK1 complex. Under starvation conditions or rapamycin treatment, mTOR dissociates from the ULK1 complex and autophagy gets activated. ULK1 is also directly phosphorylated and activated by the AMP-activated protein kinase (AMPK) in response to energy restriction. The autophagic process involves the degradation of cytosolic proteins and organelles in the lysosomes via autophagosomal delivery. Two complexes regulate the formation of the phagophore, the ULK1 complex and the beclin 1-VPS34 (class III PI3K) complex. The elongation of the autophagosomal membrane is mediated by two ubiquitin-like protein conjugation systems: ATG12-ATG5 and ATG8/LC3. LC3 can additionally recruit adaptor proteins such as p62 to autophagosomes mediating selective autophagy of cellular structures, protein aggregates and microorganisms. LC3II (LC3 bound to phosphatidylethanolamine) is recruited to both the inner and outer autophagosomal membrane. Autophagosomes fuse with lysosomes to form autolysosomes where they are degraded together with their luminal content. Pharmacological modulators of autophagy are labeled according to their effect on autophagy. Red: Inhibits autophagy; Green: Induces autophagy.

breast, prostate and ovarian cancers^[25]. Also, p62 has been found to be upregulated in human tumors^[26], *Bif-1* (a protein part of the beclin 1/class III PI3K complex that enhances its activity) knockout mice have a higher incidence of tumors than wild-type mice, especially lymphomas^[27], and *UVRAG* (a beclin 1 binding protein also part of the class III PI3K complex) is localized to the 11q13 human chromosomal region which is frequently implicated in the development of malignancies, including breast and colon cancers^[28].

The previous observations suggest a tumor suppressive role of autophagy. However, mosaic deletion of *Atg5* in mice resulted in benign tumor development only in the liver^[29]. When taken together, these studies do suggest that autophagy has tumor suppressor functions particularly in the liver and that its inhibition predisposes to the development of cancer induced by additional

agents or mutations in other tissues. However, it is important to note that since all the autophagy regulators also affect other things too, it is difficult to be sure that any effects seen when a particular autophagy regulator is inactivated are due to autophagy as opposed to other functions. Consistent with this, the more pronounced effects observed with heterozygous disruption of *beclin 1* when compared with other *ATG* genes might be caused by effects on autophagy together with other autophagy-independent mechanisms. The different phenotypes observed in the *Atg5* or *Atg7* knockout mice (which are viable but die after birth presumably due to nutrient and energy depletion) when compared to *beclin 1* knockout mice (which are embryonic lethal) indicates that beclin 1 has important functions independent of its role on the autophagic pathway^[30]. Indeed, beclin 1 can bind anti-apoptotic proteins of the Bcl-2 family (such as Bcl-2 and

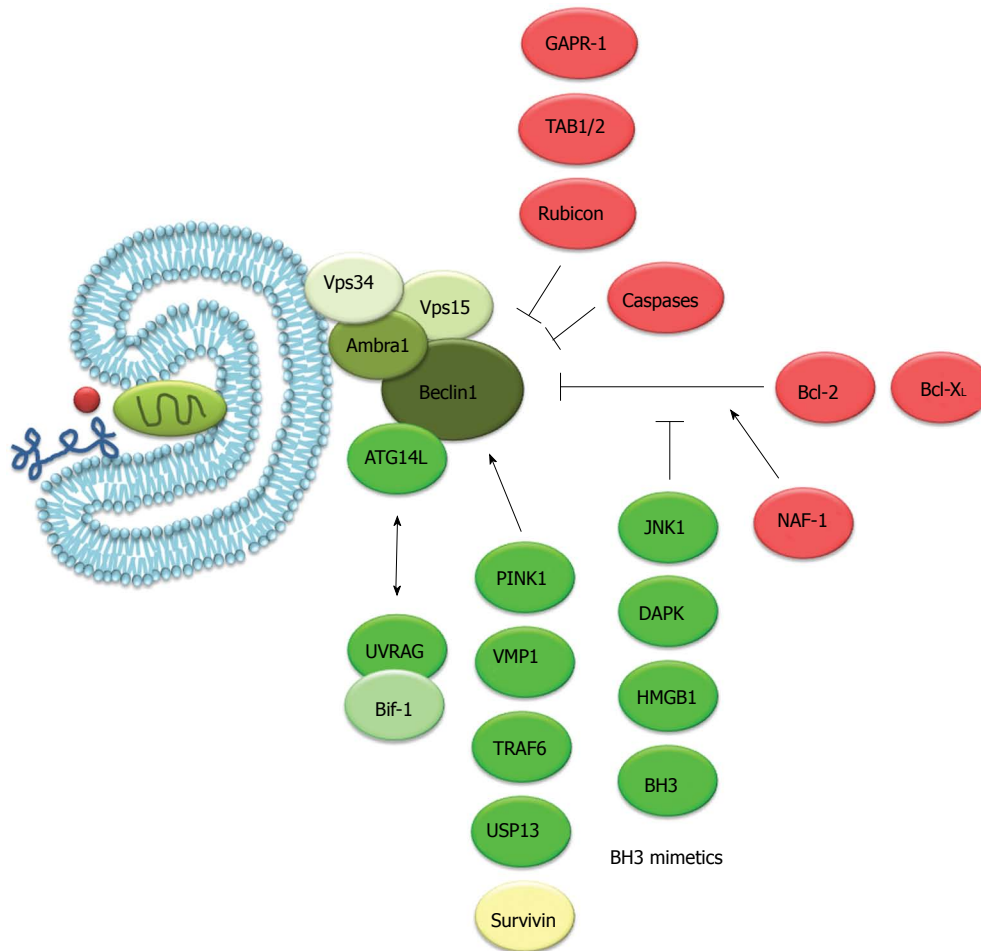


Figure 2 Beclin 1, its protein interactions and its role in autophagy. Beclin 1 has been found to form part of different class III phosphatidylinositol 3-kinase (PI3K) complexes. Each complex consists of beclin 1, Vps34, Vps15 and Ambra1. ATG14L activates the complex and induces the formation of autophagosomes. UVRAG and ATG14L are present in mutually exclusive class III PI3K complexes and UVRAG and Bif-1 have been shown to activate the complex. UVRAG has also been shown to function in autophagosome maturation and endocytic trafficking possibly independent from its interaction with beclin 1. Rubicon has also been shown to bind beclin 1 and can inhibit autophagosome formation and maturation. TAB1/2, two upstream activators of the TAK1-IKK signaling pathway interact with beclin 1 and their dissociation seems to be necessary for autophagy induction. GABR-1 can also bind beclin 1 and inhibit autophagy probably through beclin 1 tethering in the Golgi apparatus. Bcl-2/Bcl-XL can bind beclin 1 and inhibit autophagy. JNK1 phosphorylates Bcl-2 while DAPK phosphorylates beclin 1 and disrupt their interaction. Additionally, BH3-only proteins (tBid, Bad, BNIP3) or BH3 mimetics (ABT737) bind Bcl-2 and release beclin 1. Beclin 1 has also been found to be a substrate of caspases-3, 7 and 8 during apoptosis, in which cleavage of beclin 1 suppresses autophagy. Other beclin 1 interacting proteins that induce autophagy are PINK1 and VMP1. TRAF6 and USP13 have been shown to regulate beclin 1 ubiquitination. Survivin, an anti-apoptotic protein can also bind beclin 1 and regulate TRAIL induced apoptosis. NAF-1, a component of the IP3 receptor complex contributes to the interaction of Bcl-2 with beclin 1 at the ER^[32,99-104]. Proteins and drugs shown are color-coded according to their effect on autophagy. Red: Inhibits autophagy; Green: Induces autophagy; Yellow: Unknown.

Bcl-XL^[31]) and is also known to bind USP13 and regulate p53 levels (Figure 2)^[32]; these other functions could contribute to tumor suppression effects.

The mechanisms by which autophagy itself can decrease tumor formation have been suggested to involve degradation of damaged mitochondria that could otherwise induce oxidative stress, DNA damage and genomic instability (Figure 3). This situation of chronic tissue damage can also provoke an inflammatory response that can further promote tumor growth through cytokine production, and autophagy inhibition has been shown to increase cytokine production in some cases^[6,33,34]. Also, autophagy suppression leads to up-regulation of p62 which activates a NRF2 mediated antioxidant survival response that could lead to tumor promotion^[4].

Once a tumor is formed, tumor cells are exposed to

many and varied stresses including hypoxia, starvation and lack of growth factors, all known to be autophagy inducers. Increasing evidence in the literature suggests that cancer cells robustly activate autophagy to survive such stresses as they are encountered during tumor progression and metastasis. For example, autophagy is known to be most prominent in hypoxic tumor regions, where it sustains survival of tumor cells under metabolic stress^[3,4] and autophagy is also needed for survival during detachment from the extracellular matrix so that tumor cells can avoid anoikis^[35].

Many cancer cells have high levels of basal autophagy even in fed conditions. This is in contrast to normal cells in which autophagy normally occurs at low levels and is only up-regulated in response to stresses like starvation^[4]. In this regard, it has been suggested that transformation

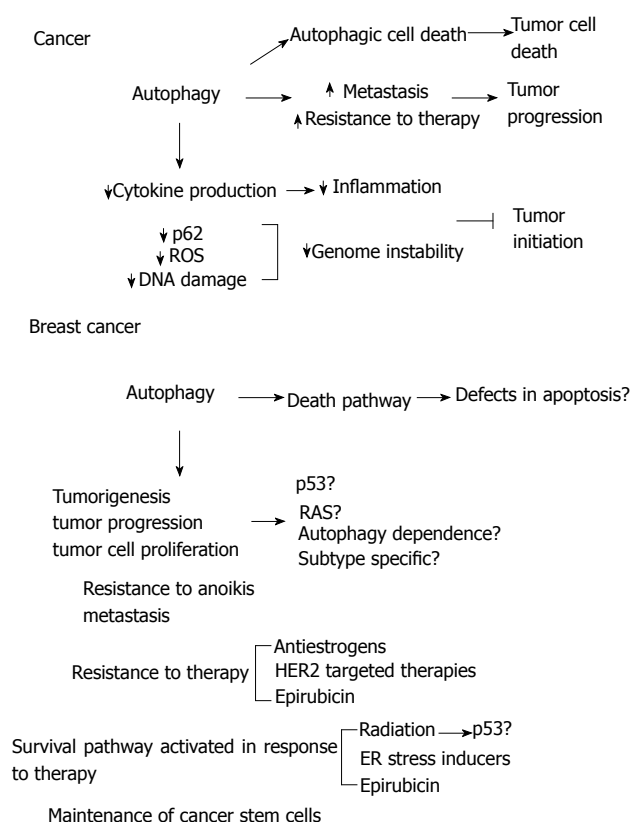


Figure 3 Autophagy in cancer and breast cancer. Different roles have been described for autophagy in cancer. Autophagy can limit tumor initiation by decreasing inflammation and genome instability. Once a tumor is established, autophagy can induce tumor progression by facilitating metastasis and resistance to therapy. On the other hand, it has also been proposed that autophagy could function as a cell death process that could be induced during therapy. In breast cancer, although some evidence suggests that autophagic cell death occurs in apoptosis defective cells, most of the evidence in the literature suggests a tumor promoting role for autophagy. Autophagy has been shown to promote tumorigenesis, tumor proliferation and progression. This could depend on the p53 or RAS mutation status of the cells. Also, if breast cancer is an autophagy-dependent disease remains to be determined as well as if this dependence is subtype specific. Autophagy has also been involved in resistance to anoikis, facilitation of metastasis, resistance to therapy and maintenance of breast cancer stem cells. Moreover, when cancer cells are made resistant to the therapies shown in the figure, they show increased autophagy and inhibition of autophagy reverts this resistance. Therapies that are known to induce autophagy in breast cancer are also shown. With these treatments, autophagy inhibition has been shown to increase cell death. Regarding radiation, results are controversial and the role of autophagy could depend on the p53 status of the cell.

mediated by certain oncogenes, like RAS, induces a metabolic switch in cancer cells that makes them addicted to autophagy under basal, but especially under starvation conditions and that autophagy inhibition would be particularly beneficial for the treatment of these tumors^[36,37]. This has been shown to be true in chloroquine-treated mouse xenografts models of pancreatic ductal adenocarcinoma (PDAC), in which activating Kras mutations are frequent^[38] and in a mouse model of Kras^{G12D} driven non-small-cell lung cancer (NSCLC). In the latter, autophagy inhibition diverted tumor progression from adenomas to oncocytomas, a more benign type of tumor, particularly in p53 deficient tumors^[33]. On the other

hand, in another model of NSCLC driven by Braf^{V600E}, the downstream effector of Kras, autophagy inhibition increased tumor burden. In this work, “autophagy dependence” became apparent only at later time points, when the tumors had been established. This study also shows a change in tumor type to more benign oncocytomas and a greater survival advantage than Kras^{G12D} driven tumors by autophagy inhibition^[39]. Similar to the previous study, autophagy inhibition by mosaic deletion of *Atg5* or *Atg7* in the pancreas induced increased formation of pre-cancerous lesions in a Kras^{G12D} mouse model of pancreatic ductal adenocarcinoma, but these lesions did not progress to carcinoma. Interestingly, this only occurred in a p53 wt background^[40] suggesting that a single oncogenic event such as Ras mutation may only cause dependence on autophagy in some circumstances and that the full spectrum of tumor mutations may ultimately determine just how autophagy-dependent a tumor will be. Further support for this idea comes from other studies where, in contrast to Ras-driven autophagy-dependence, it has been shown that autophagy restrains the proliferative potential of Ras transformed cells so that autophagy inhibition dramatically increased clonogenic growth^[41]. Moreover, autophagy has also been shown to restrict proliferation of cells carrying activated PI3K^[42], suggesting that autophagy might have different, and even opposing effects, depending on the cellular context and the transformation event. In conclusion, the role of autophagy in cancer is complex and is both context and probably tissue dependent (Figure 3). Thus, although numerous studies have addressed this issue, it remains inconclusive in which tumors autophagy should be targeted. This has important practical implications because current clinical trials are using chloroquine or hydroxychloroquine with the purpose of inhibiting autophagy in a variety of cancers without selecting patients who would more likely benefit from this treatment. We believe that further studies are needed to identify the tumors where autophagy inhibition will be effective before autophagy can be successfully targeted in the context of cancer treatment.

BREAST CANCER

Estimates of the worldwide incidence and mortality of cancer ranked breast cancer as the most frequent cancer among women with an estimated 1.67 million new cases diagnosed in 2012 (25% of all cancers) with slightly more cases in less developed than in more developed regions. Breast cancer mortality rates have declined in part due to therapeutic advances and it ranked as the fifth cause of death from cancer overall in 2012. However, it was still the most frequent cause of cancer death in women in less developed regions and the second in more developed regions after lung cancer^[43], underscoring the need for better strategies in both prevention and therapy.

Breast cancer is a heterogeneous disease. Clinically, it is classified as hormone receptor positive, HER2 (ERBB2) positive and triple negative breast cancer. Hor-

hormone receptor positive breast cancers are the most numerous and diverse accounting for 60%-70% of breast cancers. They express the estrogen receptor (ER) and usually respond to endocrine therapy. HER2 positive breast cancers overexpress the HER2 receptor tyrosine kinase and respond to targeted therapies against this receptor such as trastuzumab (monoclonal antibody targeting HER2) or lapatinib (a dual-kinase inhibitor targeting both the epidermal growth factor receptor and the HER2 receptor)^[44,45]. This type of cancer accounts for 10%-15% of breast cancer patients. Finally, triple negative breast cancers (TNBC) do not express hormone receptors (ER or progesterone receptor, PR) or HER2. Although the metastatic potential in these cancers is similar to that of other breast cancer subtypes, they are associated with a shorter median time to relapse and death. They have an increased incidence in younger women, in patients with germline *BRCAl* mutations or of African ancestry^[46]. Cytotoxic chemotherapy remains the mainstay of treatment of triple-negative disease since there is not a clear agent that targets a defining vulnerability in this disease^[47].

More recently, gene expression analysis has led to the definition of five molecular “intrinsic” subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched and basal-like) and a Normal Breast-like group which have differences in incidence, survival and response to treatment^[48]. Luminal cancers include the ER positive group and are subdivided in luminal A and luminal B subtypes. Luminal B tumors have a lower expression of ER or estrogen-regulated genes, low or no PR expression, higher tumor grade, higher expression of proliferation-related genes, and activation of growth factor receptor signaling, such as IGF-1R and PI3K/AKT/mTOR pathways when compared to Luminal A tumors. They are also considered to have a lower sensitivity to endocrine therapy and higher sensitivity to chemotherapy than luminal A tumors^[49]. The increased aggressiveness of luminal B tumors has been attributed to inactivation of the p53 pathway and hyper activation of *MYC* and *FOXM1*^[46]. Prospective trials are now evaluating the validity of prognostic gene signatures to distinguish between these two subtypes and identify those women (with luminal A cancers) who can be spared adjuvant chemotherapy^[49].

The majority of HER2+ tumors fall into the HER2 enriched subtype. About 30%-40% of these tumors are ER+, most are ER- and some of these tumors are clinically triple negative but still express the gene signature of HER2 activation. It remains unknown whether those patients with the HER2 enriched subtype but having triple negative cancers would also benefit from HER2 targeted therapies^[50].

Most basal-like tumors are triple negative and further molecular profiling has shown extensive heterogeneity of both TNBC and basal tumors. Molecularly, most, if not all of these cancers show a high frequency of *TP53* mutations or loss of the p53 pathway activity, loss of *RB1* and *BRCAl*. They show increased signaling of the PI(3)K/AKT, *MYC* and HIF1/ARNT pathways and

hyper activation of *FOXM1*^[46]. The high prevalence of these mutations together makes them highly proliferative, aneuploid tumors with a very high apoptotic rate and geographic or central tumor necrosis^[48,50,51] with an inferior prognosis when compared to the luminal subtypes^[52,53]. The heterogeneity of TNBC is widely acknowledged. Another subtype belonging to this group, the claudin-low subtype, is characterized by the low expression of genes involved in cell-cell adhesion like claudins and E-cadherin, increased expression of immune response genes, high mesenchymal features, low epithelial differentiation and a stem cell-like phenotype^[48]. More recently, Lehmann *et al*^[53] defined six TNBC subtypes: basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor, providing a broader classification for those TNBC tumors that do not fit in the basal-like group. In this work, the molecular signature of each subtype was used to predict which therapy should be used in cell lines belonging to each subtype with promising results, opening up the opportunity to use better molecular classification of tumors to improve the choice of treatments in the clinic^[53].

Some novel therapies in current clinical trials in TNBC include the use of fibroblast growth factor receptor (FGFR), vascular endothelial growth factor (VEGF), PI3K-AKT-mTOR pathway and PARP [poly (ADP-ribose) polymerase] inhibitors as well as the use of androgen receptor (AR) antagonists in AR positive breast cancers^[54]. Also, signaling pathways that are thought to be important for resistance to conventional breast cancer therapy are currently under investigation in clinical trials including: EGFR, HER2 and HER3 inhibitors, AMPK-activating compounds, restoration of the p53 signaling pathway by MDM2 inhibitors and inhibitors of the following proteins and signaling pathways: PI3K-AKT-mTOR, Notch signaling, CDK4/6, HDAC, HSP-90, BCR-Abl, VEGFR/PDGFR/Raf, VEGFR/PDGFR/c-kit, c-MET/VEGFR2, FGFR, Hedgehog signaling and MEK^[55].

AUTOPHAGY AND BREAST CANCER

The suggestion that beclin 1 is a tumor suppressor was particularly important for breast cancer since it was found to be deleted in 40%-75% of sporadic human breast and ovarian cancers. Beclin 1 expression is frequently low in human breast epithelial carcinoma cell lines and tissues and expressed at high levels in normal breast epithelia. However, as discussed previously, the *beclin 1* gene maps to 17q21, a region that is deleted in approximately 50% of sporadic breast carcinomas^[56], and *beclin 1* is next to *BRCAl*, a tumor suppressor gene whose loss is a driver of some breast and ovarian cancers^[23]. In this regard, recent evidence suggests that although *beclin 1* is lost in breast and ovarian cancers, it is co-deleted with *BRCAl*, consistent with *BRCAl* loss being the primary driver mutation in these cancers and suggesting that *beclin 1* itself may not be a tumor suppressor in this context^[23]. Howev-

er, it would be interesting to explore if breast and ovarian cancers with loss of *BRCAl* and *beclin 1* have a different outcome than those that only lack *BRCAl*.

Even though *beclin 1* might not be a tumor suppressor gene in human tumors, overexpression of *beclin 1* in the MCF7 breast cancer cell line decreases proliferation, *in vitro* clonogenicity and tumorigenesis^[20], and the *beclin 1* loss that occurs in breast cancer could have important effects independent of autophagy through its interaction with Bcl-2. This relationship would be particularly important since Bcl-2 is itself overexpressed in 50%-70% of cancers, including breast cancer^[57]. An inverse correlation of *beclin 1* and Bcl-2 expression has been described in breast cancer tissue and Bcl-2 expression was correlated with histological grade, tubule formation, nuclear pleomorphism, mitotic count, estrogen receptor and distant metastasis^[58]. These findings suggest that the interaction between these two proteins might be particularly important for breast cancer tumorigenesis since loss, or low levels of *beclin 1* would increase free Bcl-2 and an antiapoptotic response. Moreover, it has also been shown that the growth-promoting activity of Bcl-2 correlates with its ability to bind *beclin 1* and inhibit autophagy rather than with its anti-apoptotic function, thus contributing to tumorigenesis^[59].

In contrast to the tumor suppressive role of autophagy discussed previously, autophagy has been shown to have a tumor promoting role in breast cancer. Two recent papers studied the role of autophagy in mammary tumorigenesis. In the first one, conditional knockout of the essential autophagy protein FIP200 in mammary epithelial cells in the MMTV-PyMT mouse model of breast cancer (which is known to induce mammary adenocarcinomas following PyMT-mediated activation of Ras, Src, and PI3K) reduced tumorigenesis, metastasis and increased survival. Gene expression profiling of mammary tumors in this study revealed increased expression of immune responsive genes in the autophagy deficient tumors, suggesting that FIP200 deletion might trigger enhanced anti-tumor immune response and contribute to the suppression of mammary tumorigenesis and progression^[6]. In the second study, the authors used mice deficient in *Palb2* in the mammary gland, which produced tumors with diverse histology but generally of high grade, invasive, with increased DNA double strand breaks, DNA damage and mutations in p53. In this work, autophagy inhibition by allelic loss of *beclin1* delayed tumor formation. The authors proposed that autophagy was being activated in response to DNA damage and oxidative stress and mediated survival of tumor cells in collaboration with p53, since allelic loss of *beclin1* did not have an effect in tumor formation when p53 was also deleted from the mammary gland^[60]. Both studies found a tumor promoting role of autophagy in oncogene-driven breast cancer models and thus suggest that autophagy addiction might be a potential therapeutic target in breast cancer (Figure 3).

As mentioned above, autophagy addiction has been

particularly linked to strong oncogenic insults like RAS transformation or alterations in the RAS pathway, which induce alterations in metabolic pathways to meet biosynthetic demands. Although RAS transformation is not a common event in breast cancer, other oncogene pathways activated in breast tumors, including HER2, Myc and activated PI3K, produce metabolic alterations similar to RAS transformation. Moreover, components of the PI3K and RAS-RAF-MEK pathway are amplified in basal-like cancers and autophagy addiction can be induced by RAS transformation in breast cancer cells^[3,37,46]. For instance, HRas^{V12} expressing MEFs were found to need autophagy for anchorage-independent growth and the MDAMB231 cell line (KRAS mutated) was found to need autophagy for proper proliferation in both anchorage-independent and in attached, nutrient rich conditions. In both cases, autophagy promoted survival through facilitation of glycolysis^[37]. In agreement with these observations, autophagy is necessary for lumen cell survival in breast cancer MCF10A cells expressing the oncogenic PI3KCA mutant in 3D acini. Interestingly, in this work autophagy inhibition promoted adhesion-independent proliferation in soft agar transformation assays in 3D culture but not in 2D conditions and was suggested to be mediated by an increase in p62 after autophagy inhibition^[42].

Autophagy is induced in non-transformed and oncogene-transformed breast cell lines following matrix detachment and protects them from anoikis or detachment-induced cell death^[35]. Also, chloroquine treatment, a pharmacological agent that blocks autophagy by accumulating in the lysosome and blocking the degradation of autophagosomes, decreased tumor growth, increased survival and decreased metastasis in the 4T1 model of breast cancer^[61]. Additionally, most breast cancer tumors will very likely have alterations in the autophagic pathway since mutations commonly present in breast cancers are known to be important regulators of the autophagic pathway. This is particularly the case of PI3K mutations, alterations in the PI3K-mTOR pathway, p53, EGFR and Bcl-2^[46,58].

AUTOPHAGY AND BREAST CANCER TREATMENT

Endocrine therapy is the mainstay in ER positive disease. While adjuvant endocrine therapy reduces breast cancer mortality, many ER+ tumors develop resistance and eventually recur. Although the mechanisms of endocrine therapy resistance are not well understood, autophagy has been suggested to be involved in them^[62]. In this regard, autophagy is induced in response to anti-estrogen therapy in the MCF7 breast cancer cell line and its inhibition sensitized to tamoxifen treatment in a tamoxifen-resistant cell line^[63]. It has also been shown that blocking autophagy sensitizes to restoration of antiestrogen sensitivity by Bcl-2 and BCL2L2 co-inhibition in a MCF7-derived anti-estrogen resistant cell line. Importantly, in

the former work, autophagy inhibition with 3-MA or beclin 1 knockdown, decreased necrotic cell death and induced apoptosis in the resistant cells after treatment with the antiestrogen ICI 182780 in response to Bcl-2/BCL2L2 co-inhibition^[64]. These observations suggest that autophagy is not only important for the development of anti-estrogen resistance but also for defining the type of cell death, which could also be important for a successful therapy.

Several important roles for autophagy have been described for HER2 positive breast cancers. Therapies targeting the HER2 receptor like trastuzumab (an antibody targeting HER2) or lapatinib (a small molecule tyrosine kinase inhibitor that targets both EGFR and HER2) are known to induce autophagy in both sensitive and resistant cells^[65,66]. Autophagy has also been implicated in the development of resistance to treatment since cells that have been made resistant to trastuzumab exhibit high levels of autophagy and its pharmacological or genetic inhibition sensitized to trastuzumab in cells that have acquired or inherent resistance^[66,67]. Also, a significant association has been found between loss of beclin 1 and HER2 amplification in breast cancers and this work found that loss of beclin 1 predicted response to trastuzumab alone or in combination with other drugs^[56] suggesting an important role for autophagy in this type of cancer. Additionally, a recent study found a direct interaction between both proteins. In this regard, beclin 1 overexpression was found to enhance HER2 phosphorylation and to decrease response to lapatinib whereas beclin 1 knockdown enhanced lapatinib-induced apoptosis^[65]. Also, ATG12 has been reported to be upregulated in trastuzumab resistant cell lines and its knockdown sensitized JIMT1 resistant cells to trastuzumab treatment both *in vitro* and *in vivo*^[68].

Although the previous evidence suggests that autophagy inhibition would be a promising therapeutic target in combination with HER2 targeted therapies, a recent study found conflicting results in NSCLC where a direct association between beclin 1 and EGFR was also found^[69]. Although these findings are in line with the previous observations on beclin 1 and HER2 receptor, in this work, active EGFR was found to bind and phosphorylate beclin 1 decreasing its association with VPS34 and autophagy in NSCLC cell lines. Tyrosine kinase inhibitor (TKI) treatment disrupted beclin 1 association with EGFR and restored autophagy in TKI sensitive cells. These results associated autophagy inhibition with an enhanced cancer cell survival in response to TKI inhibitors and resistance *in vitro*. Also, cells expressing a tyrosine phosphorylation mutant of beclin 1 (EGFR-phosphorylated sites), which decreased binding to VPS34 and autophagy, showed enhanced tumorigenesis and reduced response to erlotinib *in vivo*. The conclusions of this study were that autophagy should not be inhibited in combination with TKI therapy since they found that the tumors with the least autophagy and the greatest amount of cell death also were the most aggressive. It is unclear if these major differences are due to effects that are specific to lung cancer *vs* breast cancer

or if the interaction of beclin 1 with HER2 is different to the one with EGFR.

Chemotherapy in breast cancer often includes the use of anthracyclines, taxanes (docetaxel, paclitaxel), and DNA damaging agents like cyclophosphamide, fluorouracil or platinum-based compounds^[70,71]. Adjuvant systemic chemotherapy is used particularly in the management of TNBC, which lacks a targeted treatment, and is also given with or preceding trastuzumab for patients with HER2-positive, invasive breast cancer^[72]. Most of these agents are known to be good autophagy inducers in different types of cancer^[15]. In breast cancer cell lines, specifically in the MCF7 cell line, epirubicin was found to induce autophagy and a non-apoptotic form of cell death. In this work, autophagy inhibition increased drug toxicity by inducing apoptosis. Moreover, sensitivity to epirubicin was partially restored by autophagy inhibition in an MCF7 cell line that was made epirubicin-resistant^[73]. On the other hand, pharmacological inhibition of autophagy was found to enhance doxorubicin induced cardiotoxicity. Importantly, autophagy induction with rapamycin increased survival of doxorubicin-treated mice in the same work^[74]. Although the mechanisms by which rapamycin increased mice survival could have involved autophagy-independent mechanisms, this work brings up an important point about trying to manipulate autophagy systemically during cancer treatment, since it could have undesirable side-effects in other tissues even if it has the desired effects in tumor tissue and the overall effect could still be disadvantageous for treatment of a patient.

Microtubule stabilizing agents (taxanes) are used in cancer therapy because of their ability to inhibit mitosis. However, they are also known to have profound effects on autophagy. Microtubules support the assembly of pre-autophagosomal structures, direct their movement possibly to mediate formation of mature phagophores and also mediate trafficking of autophagosomes towards lysosomes. Microtubules also regulate two major complexes involved in the initiation of the autophagic response: mTORC1 and class III PI3K complex^[75]. Indeed, part of the cytotoxic effect of taxol has been shown to be dependent on its ability to block autophagosome transport and maturation, since treatment with 3MA or knockdown of ATG7 or VPS34 decreased cell death induced by paclitaxel in MCF7 and SKBR3 breast cancer cell lines. Importantly, this work also found increased expression of *ATG* genes in docetaxel sensitive primary breast tumor biopsy samples (when compared to resistant samples), probably indicating higher levels of autophagy and suggesting that levels of ATG proteins could be investigated as possible prognostic markers for clinical taxane effectiveness^[76]. Notably, other microtubule targeting drugs like vinblastine, a microtubule depolarizing drug, was found to stimulate formation of autophagosomes, decrease fusion with endosomes and disrupt autophagosome motility^[77]. So, despite having different effects on the autophagic pathway, drugs that disrupt microtubules certainly decrease autophagic flux. This is important

since it is often thought that autophagy is always induced in response to stress induced during chemotherapy, underscoring the need to understand the molecular mechanisms by which specific therapies affect the autophagic pathway.

Genotoxic stress as a result to ionizing radiation or chemotherapeutic drugs is known to increase p53 levels and induce cell cycle arrest, apoptosis, senescence or autophagy^[78]. In this regard, p53 has recently been shown to be an important regulator of autophagy with dual roles depending on its subcellular location. In the nucleus, p53 is pro-autophagic in a transcription dependent and independent manner and in the cytoplasm it can act as a repressor of autophagy^[8]. It is thus not surprising that DNA damaging agents and radiotherapy used for breast cancer treatment can induce autophagy in breast cancer cell lines^[79-81]. However, genetic inhibition of autophagy was not found to sensitize to cisplatin treatment and chloroquine treatment only mildly sensitized one mouse breast cancer cell line (67NR) but not another one (4T1)^[79]. Moreover, autophagy inhibition sensitized the MCF7^[81] but not the 4T1 cell line^[80] to radiation therapy. These different effects could be due to the p53 status (MCF-7 cells have wild type p53 and 4T1 cells are p53-null), possibly suggesting that autophagy inhibition together with DNA damaging agents should be used especially in those cancers with wild type p53 or with p53 mutants that maintain certain functions. So, despite numerous reports in the literature reporting a cytoprotective role of autophagy during DNA damaging agents in other types of cancer^[82], whether this is true for breast cancer and if this is dependent on the p53 status remains to be determined. This last statement is of particular importance since *p53* is frequently mutated in basal-like breast cancer, where the combination of *p53* mutations together with inferred pathway activity suggests that loss of p53 function occurs within most, if not all basal-like cancers^[46] and in which chemotherapy is most frequently used.

As mentioned above, triple negative breast cancers lack a molecular-directed therapy. However, recent studies have described molecular characteristics of the disease that are now targets of experimental agents, including poly (ADP-ribose) polymerase (PARP, for BRCA-associated tumors) inhibitors, angiogenesis blockers, EGFR inhibitors^[52,83] and endoplasmic reticulum stress inducers^[84]. With regards to endoplasmic reticulum stress, TNBC are known to be highly proliferative, aneuploid tumors^[48,50], conditions that are known to lead to proteotoxic stress in cancer cells and that could lead to the induction of autophagy. In this regard, autophagy inhibition seems to be a promising target since combination of autophagy inhibitors plus endoplasmic reticulum aggravators nelfinavir and celecoxib was synergistic in enhancing tumor cell killing particularly in TNBC cells^[84] and autophagy inhibition sensitized MCF7 cells to bortezomib treatment^[85]. Also, treatment with HDAC6 inhibitor panobinostat induced endoplasmic reticulum stress and autophagy in TNBC cells. Moreover,

chloroquine treatment synergized with panobinostat and induced cell death both *in vitro* and *in vivo*^[86].

Although most of the evidence described above suggests that autophagy should be inhibited in order to improve breast cancer therapy, some studies also suggest that autophagy could be involved in the cell's demise implying the opposite—that one should sometimes increase autophagy during breast cancer treatment. For example, Bcl-2 knockdown in MCF7 cells has been shown to induce autophagy and non-apoptotic cell death, which was decreased by knocking down ATG5, suggesting that autophagy could be involved in cell death in this model^[87]. These studies were performed in the MCF7 cell line, a widely used model for breast cancer and which is known to lack the executioner caspase-3 and thus have reduced susceptibility to apoptosis^[82]. Although this raises the caveat that pro-death effects of autophagy in this experimental model are due to its unusual deficiency in apoptosis, it is a relevant model for breast cancer since Bcl-2 is known to be overexpressed in breast cancers, resistance to apoptosis is thought to be an intrinsic property of many tumor cells and anti-apoptotic members of the Bcl-2 family have been found to be amplified in human cancers, including breast cancer^[88].

AUTOPHAGY AND BREAST CANCER STEM CELLS

According to the cancer stem cell hypothesis, tumorigenic cancer stem cells or tumor initiating cells (TICs) are those cells within a tumor that have self-renewal and tumorigenic capacities. They can “differentiate” into cancer cells with limited proliferative potential, creating a hierarchical organization within a tumor^[89]. Breast cancers are known to follow the cancer stem cell model, where tumors can be separated into tumorigenic (CD44⁺/CD24^{-low} or ALDH1⁺) and non-tumorigenic components, suggesting that only a minority of cells in a tumor can proliferate extensively and that some therapies that shrink tumors might not be curative because they fail to eliminate TICs. Breast cancer claudin-low tumors and cell lines have features of tumor-initiating cells since they express the same gene signature as the CD44⁺/CD24⁻ tumor fraction^[50]. Moreover, recent evidence suggests that TICs are also enriched in basal tumors and cell lines and their abundance has been associated with a worse overall survival^[90,91].

It is predicted that autophagy should be especially crucial for quality control mechanisms and maintenance of cellular homeostasis in stem cells due to their unique ability to self-renew and differentiate and relatively long life^[92]. Recent studies suggest that autophagy does in fact play a crucial role in the origin, maintenance and systemic distribution of TICs. The first paper linking autophagy to TICs described higher levels of LC3B and ATG5 in DCIS (ductal carcinoma *in situ*) derived tumorigenic spheroids when compared to epithelial cells in the same culture. Moreover, treatment of DCIS culture cells

with chloroquine suppressed xenografts tumor formation^[93]. The authors suggest that autophagy is activated in DCIS cells to persist and proliferate in the metabolically stressed intraductal space^[94]. Another recent paper reported that autophagy inhibition by ATG12, ATG8 knockdown or chloroquine treatment could decrease the number of CD44⁺/CD24⁻ cells in the MDAMB231 and JIMT1 breast cancer cell lines by increasing CD24 transcription^[95]. Finally, a recent study found increased autophagic flux in ALDH⁺ cells from mammospheres of the MCF7 cell line when compared to the bulk population of cells, increased beclin 1 expression in mammospheres when compared to adherent cells and decreased mammosphere size and formation by knockdown of beclin 1 as well as decreased tumor-forming ability in beclin 1 knockdown cells from mammosphere cultures of MCF7, SKBR3 and SK-3rd breast cancer cell lines^[96]. All these studies suggest that autophagy inhibition could preferentially target the TIC population in a tumor suggesting that combination of autophagy inhibition with other therapies would eliminate stem cells that survive the original treatment.

CONCLUSION

Research on autophagy and breast cancer treatment has dramatically increased in the past years. Despite being an area with many unanswered questions, recent discoveries in breast cancer biology and the autophagic pathway have increased our understanding on the implications and importance of trying to manipulate autophagy during breast cancer treatment. For example, it has been suggested that autophagy should be preferentially inhibited together with treatments that increase the tumor cell's dependency on it, like proteasomal inhibitors. Such combination treatment should probably be especially used to treat highly proliferative, aneuploid tumors with high proteotoxic stress, *e.g.*, basal-like tumors. On the other hand, autophagy inhibition could perhaps be avoided in combination with those therapies that themselves are known to negatively target the autophagic pathway, like microtubule-targeting agents. Therefore, autophagy manipulation will probably not be a generally applicable mechanism of sensitization to therapy in breast cancer and it will most likely depend on the type of breast cancer and on the treatment used. Moreover, evidence in the literature suggests that breast cancers (at least those with oncogenic events similar to the ones in the MMTV-PyMT or *Palb2*^{-/-} mouse models) display a certain degree of autophagy dependence and we recently found that many TNBC cell lines are particularly sensitive to autophagy inhibition when compared to luminal cells^[97], indicating that autophagy dependence in breast cancer may be subtype-dependent.

There are, however, some unresolved questions on autophagy and cancer that should be carefully addressed. For example, the fact that autophagy modulation can regulate the type of cell death in response to therapy is

an important thing to consider. In this regard, autophagy inhibition was shown to decrease necrosis and induce apoptosis in an MCF7 anti-estrogen resistant cell line in response to ICI 182780 treatment and Bcl-2/BCL2L2 co-inhibition^[64]. However, it has also been shown that although autophagy inhibition decreased survival in apoptosis deficient epithelial cells exposed to metabolic stress, it also changed the mode of cell death from apoptosis to necrosis, stimulating cytokine and chemokine production, macrophage infiltration and tumor growth *in vivo*^[34]. It has also been shown that autophagy induces ATP secretion during chemotherapy and that this is necessary for the establishment of a therapeutic immune response^[5]. Moreover, a recent study found that while autophagy inhibition sensitizes to radiotherapy both *in vitro* and *in vivo* in immune deficient mice, it increases tumor volume in an immune competent model *in vivo*. This work suggests that autophagy inhibition can decrease irradiation-induced release of ATP from tumor cells and impair an anti-tumoral immune response^[98]. This raises important questions about the immunogenicity of cell death and the possible final outcome in therapy when autophagy is inhibited-in a person, it may be better to directly kill slightly fewer tumor cells while allowing a robust anti-tumoral immune response than to obtain more direct tumor cell killing but losing the immune response. In breast cancer, autophagy has been shown to inhibit rather than stimulate the immune response (since autophagy inhibition was found to induce cytokine release) and autophagy deficient tumors had increased immune cell infiltration and decreased tumor growth and metastasis^[6]. The different effects might be related to the specific interplay of the immune system with cancer cells and to the type of therapy employed. Thus, if these effects are particular to the type of cancer (or the subtype of breast cancer) and the treatment used remains an important open question.

Finally, although most of the evidence in the literature suggests that autophagy should be inhibited in combination with breast cancer therapies, the fact that autophagy seems to be involved in cell death at least with some treatments should also be considered. If this effect is particular to a subtype of breast cancer, to specific mutations found in the tumor or to a certain treatment is something that needs to be studied further. Also, the fact that autophagy is involved in tumor suppression and that protective effects of autophagy have been described for diseases other than cancer (such as neurodegenerative, infectious diseases and ageing) raises concerns with regard to whether autophagy inhibition during cancer treatment could induce tumor formation in other tissues or promote other diseases in patients^[2]. Thus, a careful analysis of the full spectrum of effects of autophagy on cancer will be needed in order to successfully modulate it for therapeutic purposes. This may be a particular challenge for breast cancer therapy due to the high heterogeneity of this type of cancer but holds the opportunity for personalizing treatment protocols to maximize the benefit to breast cancer patients.

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