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The Multifaceted Roles of Autophagy In Tumors--Implications For Breast Cancer

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Abstract

Autophagy is an evolutionarily conserved lysosomal degradation process that is crucial for adaptation to stress as well as in cellular homeostasis. In cancer, our current understanding has uncovered multifaceted roles for autophagy in tumor initiation and progression. Although genetic evidence corroborates a critical role for autophagy as a tumor suppressor mechanism, autophagy can also promote the survival and fitness of advanced tumors subject to stress, which has important implications during breast cancer progression and metastasis. Here, I discuss the mechanisms and the evidence underlying these diverse roles for autophagy in cancer and speculate on specific circumstances in which autophagy can be most effectively targeted for breast cancer treatment.

Introduction

Cancer cells face a wide array of environmental and cellular stresses. As biologists begin to appreciate the importance of effective stress adaptation during cancer initiation and progression, this has led to the intriguing hypothesis that tumor cells under duress are uniquely dependent on specific pathways that promote cellular fitness. Interestingly, these same pathways are comparatively dispensable in normal cells, which previously led to their premature dismissal as housekeeping functions. This intriguing idea, recently termed “non-oncogene addiction,” forms the biological rationale behind the growing number of unbiased screens to identify molecules engaged in “synthetic lethal” interactions with established oncogenes and tumor suppressors (Luo et al., 2009).

As part of this research arena, increasing scrutiny has been directed toward modulating fundamental cellular stress response pathways to prevent the survival and expansion of tumor cells. One such process is macroautophagy, a tightly regulated lysosomal degradation process conserved in all eukaryotic cells. The degradation and recycling of proteins, organelles, and other cytoplasmic components is vital for the maintenance of cellular homeostasis and is commonly observed in cells under various forms of duress (Levine and Kroemer, 2008). Two principal mechanisms of protein degradation have been described—the ubiquitin-proteasome system, for the degradation of short-lived proteins; and autophagy, which mediates the delivery of long-lived cytoplasmic proteins and organelles to the lysosome for destruction (Levine and Kroemer, 2008). Importantly, one should recognize that multiple routes of autophagic degradation exist within cells, including: 1) macroautophagy, in which cytoplasmic contents are sequestered in double membrane autophagosomes, and subsequently delivered to the lysosome; 2) microautophagy, where cytoplasm is directly engulfed by lysosomal membrane; and 3) chaperone-mediated

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autophagy, where proteins with a specific signal sequence are transported to the lysosomal lumen by a receptor-mediated process (Mizushima et al., 2008). Of these routes, macroautophagy has been most extensively studied for its potential functions in cancer; as a result, this process will be the exclusive focus of this review and henceforth be referred to as autophagy (Roy and Debnath, 2010).

Autophagy is tightly regulated by a limited number of highly conserved genes called *ATG*s (*Au**T**o*phaGy related gene) that were first identified in yeast (Klionsky et al., 2003). These landmark studies have led to numerous recent breakthroughs in mammals demonstrating a critical role for autophagy in both physiological and pathological processes, including cancer initiation and progression (Mizushima et al., 2008). Bulk degradation of cellular material through autophagy allows cells to recycle both nutrients and energy during starvation and stress; in this regard, autophagy is proposed to function as a "battery" that buys cells valuable time, allowing them to survive if the stressor is removed in a timely manner (Lum et al., 2005; Roy and Debnath, 2010). This indispensable contribution of autophagy as a stress response mechanism is poignantly illustrated by studies in mice, in which the genetic deletion of critical ATGs results in neonatal lethality within a day after birth (Komatsu et al., 2005; Kuma et al., 2004). Autophagy is also activated in response to multiple stresses relevant for cancer progression, including nutrient starvation, the unfolded protein response (ER stress), and hypoxia; in addition, it is observed upon treatment of cancers with a wide spectrum of cytotoxic and targeted chemotherapeutic agents (Kondo et al., 2005). Because autophagy most often functions as a survival mechanism in response to these diverse stressors, one can speculate that autophagy functions entirely as a tumor-promoting mechanism by promoting the cellular fitness of cancer cells under various forms of duress. However, genetic evidence indicates otherwise; rather, autophagy can exert important tumor suppressive functions (Roy and Debnath, 2010). Clearly, to effectively target autophagy for therapeutic purposes against cancer, several fundamental issues must be addressed.

In this review, I will first summarize recent advances in our understanding of the mechanics of autophagy. Next, I will overview the tumor-suppressive and promoting functions of autophagy and how they both dictate oncogenic transformation in vitro and cancer progression in vivo. Though findings most germane to breast cancer will be highlighted, it is important to recognize that our current understanding in this field is largely derived from results from a broad spectrum of model systems and tumor types. Lastly, I will speculate on specific circumstances in which autophagy may be most effectively targeted to improve clinical outcomes in breast cancer.

Recent advances in the molecular regulation of autophagy

Autophagy is a multi-step process characterized ultrastructurally by portions of cytoplasm becoming engulfed by an isolation membrane, also termed the phagophore. The extension and completion of this isolation membrane results in the formation of a double-membrane organelle called the autophagosome. The outer membrane of the autophagosome subsequently fuses with a lysosome leading to the degradation of the sequestered cytosolic proteins and organelles (Figure 1). Studies conducted in yeast have revealed over 30 ATG genes involved in this process, many of whose mammalian orthologues have also been identified (Nakatogawa et al., 2009) Although the process of autophagy remains incompletely understood, rapid advances have been made in two topics— formation of the autophagosome membrane and the molecular control of selectivity. Hence, in this section, I will review these two exciting areas of research.

Autophagosome formation

Several recent studies suggest that isolation membrane nucleation in mammalian cells occurs at sites that emanate from the endoplasmic reticulum (ER); moreover, electron tomography analysis delineates direct connections between the ER and autophagosomal membranes(Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009). Several key autophagy proteins translocate to the ER at the earliest steps of the process, including the unc-51-like kinase (ULK) complex, the Class III phosphatidylinositol 3-kinase (PI3K) complex, and the WIPI/ATG18 proteins (Figure 1).

ULK complex—In mammals, autophagy induction requires ULK (orthologous to yeast ATG1), which exists in a large complex with mATG13, FIP200 and ATG101 and is regulated by mammalian target of rapamycin complex 1 (mTORC1) (Mizushima, 2010). Although it bears no structural homology to the yeast counterpart, FIP200 has been proposed to be a functional orthologue of yeast ATG17 during autophagosome induction (Hara and Mizushima, 2009). Interestingly, FIP200 truncation mutants have been described in breast cancer patients (Chano et al., 2002). At least three different ULK proteins are involved in different aspects of autophagy, among which ULK1 and ULK2 bear highest similarity to yeast ATG1. Under nutrient rich conditions, the ULK complex interacts with mTORC1 and remains inactivated by mTORC1-mediated phosphorylation of ULK1 and ULK2. However, upon nutrient deprivation, mTORC1 dissociates from the complex resulting in the dephosphorylation of inhibitory sites and concomitant autophosphorylation of activating sites in ULK1 and 2 (Chan, 2009). The kinase activation of ULK1 and 2 then results in the phosphorylation and activation of mATG13 and FIP200, leading to subsequent localization of the activated ULK complex from the cytosol to the ER (Jung et al., 2009). However, the genetic deletion of ULK1 in mice has been demonstrated to have minimal effects on the initial formation or completion of the autophagosome; this lack of phenotype may arise from compensation by other ULK isoforms (Kundu et al., 2008).

Class III phosphatidylinositol 3-kinase (PI3K) complex—The ULK complex results in both the activation and the ER recruitment of the autophagy-specific class III PI3K complex. The class III PI3K core complex is essential for phosphatidylinositol 3-phosphate (PI3P) production during the early stages of phagophore nucleation, and consists of the yeast ATG6 orthologue Beclin1 (Becn1), the PI3K protein Vps34, and p150 (Simonsen and Tooze, 2009). Though the exact mechanism is not yet clear, it is evident that the core complex localizes to the phagophore and facilitates recruitment of subsequent ATGs. Recent studies have identified various binding partners of Becn1, including UVRAG (Itakura et al., 2008; Liang et al., 2006), ATG14L/Barkor (Matsunaga et al., 2009; Zhong et al., 2009), and Ambra1 (Fimia et al., 2007), which all positively regulate Becn1 activity and regulate different steps of autophagosome formation and maturation. Notably, ATG14L plays a critical role in specifying the site of the hVps34 complex relocation and therefore phagophore nucleation(Matsunaga et al., 2009). Depletion of ATG14L restricts autophagic puncta formation, whereas overexpression of the protein leads to increased PI3P production and increased autophagosome formation. Interestingly, ATG14L and UVRAG cannot simultaneously bind Becn1(Itakura and Mizushima, 2009). UVRAG also interacts with Bif-1 (an N-BAR domain protein), which potentially leads to phagophore membrane curvature, and expedites autophagosome-lysosome fusion (Liang et al., 2008; Takahashi et al., 2007). Moreover, another molecule named Rubicon (RUN domain and cysteine-rich domain containing, Becn1-interacting protein) functions as a negative regulator of Becn1 (Zhong et al., 2009), which binds to the UVRAG-Beclin complex and regulates late stages of autophagy, more specifically, the late endosomal and lysosomal maturation process.

Overall, these studies indicate that multiple class III PI3K complexes exist concurrently within the same cell, suggesting that these proteins can exquisitely tune membrane dynamics during both autophagosome formation and maturation. Interestingly, as discussed in detail in the subsequent section, several proteins in this complex have been demonstrated to have tumor suppressive or anti-proliferative effects.

DFCP1 and WIPIs—PI3P produced by the class III PI3K complex subsequently recruits the next stage of effectors, including the double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) family proteins, the mammalian orthologues of ATG18 (Polson et al., 2010). Upon autophagy induction, DFCP1 rapidly translocates in a PtdIns(3)P-dependent manner to an ER subdomain called the omegasome, which is proposed to serve as a major autophagosome formation site (Axe et al., 2008). In support, the omegasome subsequently localizes with ATG18 orthologues (WIPI2 and WIPI1-4) and at a later stage, with ATG16 and LC3. WIPI2, the major WIPI isoform in most cell types is proposed to promote the development of omegasomes into isolation membranes and autophagosomes (Polson et al., 2010).

ATG12 and ATG8 conjugation pathways—The final stage of autophagosome formation, the elongation of the phagophore membrane, requires two ubiquitin-like systems (Nakatogawa et al., 2009). The first involves the conjugation of ATG5 to ubiquitin-like ATG12 via E1 and E2-like activities of ATG7 and ATG10, respectively. The ATG5-ATG12 complex binds ATG16 and forms a large multimeric complex called the ATG16L complex, which localizes on the outer surface of the extending autophagosomal membrane. The second conjugation system involves cleavage of the ubiquitin-like molecule, ATG8, by the protease ATG4 to expose a C-terminal glycine residue required for subsequent activation and conjugation reactions. Ultimately, ATG8 is conjugated to the lipid phosphotidylethanolamine (PE) via ATG7 and E2-like ATG3 and is subsequently recruited to both the outer and inner surfaces of the autophagosomal membrane. Several mammalian orthologues to ATG8 have been identified, of which the best characterized is microtubule associated protein light chain 3 (MAPLC3 or LC3) (Weidberg et al., 2010). Importantly, the ATG16L complex with ATG5-ATG12 has been demonstrated to provide an E3-like activity that promotes the lipidation of ATG8/LC3 (Fujita et al., 2008; Hanada et al., 2007). Because these early ATG complexes are proposed to be uniquely devoted to membrane biogenesis during the formation of isolation membranes and/or autophagosomes, they appear attractive as specific drug targets to potentially modulate autophagy in cancer (Rubinsztein et al., 2007). Nonetheless, it is important to recognize that any of these ATGs may serve cellular functions distinct from their canonical roles in autophagy. For example, recent work has identified a new conjugate between ATG12 and ATG3; interestingly, ATG12-ATG3 has novel roles in mitochondrial homeostasis, but in contrast to either individual ATG, this complex does not regulate autophagosome formation (Radoshevich et al., 2010).

Alternative membrane sources—Although the aforementioned studies point to vital role for the ER in autophagosome formation, other studies demonstrate that additional membrane sources, such as mitochondria and the plasma membrane, may exist (Hailey et al., 2010; Ravikumar et al., 2010). Most notably, mitochondria have been proposed as an alternative route of autophagosome membrane generation during nutrient starvation. In this model, mitofusin 2 (Mfn 2), a GTPase that regulates mitochondrial dynamics, is required for autophagosome generation. Mfn2 mediates mitochondrial-ER interconnections that facilitate the transfer of phosphatidylserine (PS) from the ER to mitochondria. Subsequently, in mitochondria, PS then gets processed to PE which serves an essential component of the lipidation reactions required for autophagy; accordingly, during nutrient starvation, both Atg5 and LC3 are found to localize at the outer membrane of mitochondria, in association

with phagophore development (Hailey et al., 2010). Most likely, multiple membrane sources contribute to autophagosome formation in mammalian cells, which are dependent on both cell type and activating stimulus; regardless, a rapidly expanding body of literature now supports that the molecular control of the early events in mammalian autophagy are very different from those in yeast, in which the initial pre-autophagosomal structure (PAS) is believed to be assembled *de novo* (Nakatogawa et al., 2009).

Mediators of selective autophagy

Though autophagy was originally described as a non-selective process, recent evidence clearly supports that autophagy can degrade both cytosolic substrates and organelles in a selective manner (Johansen and Lamark, 2011). Interestingly, when considered in total, these studies point to the involvement of ubiquitin as a specificity factor for various forms of selective autophagy (Dikic et al., 2010; Kirkin et al., 2009).

Autophagy cargo receptors—Several ubiquitin-binding proteins are proposed to serve as cargo receptors for autophagy substrates, among which the prototype is p62/SQSTM1 (Bjorkoy et al., 2005). p62 contains a carboxy-terminal ubiquitin-associated (UBA) domain (Figure 2A) and has been demonstrated to act as an adaptor between ubiquitin-containing protein aggregates and the autophagic machinery(Pankiv et al., 2007) (Figure 2B). Since the discovery of p62/SQSTM, several additional proteins with analogous functions have been identified, including NBR1 (neighbor of Brca1 gene) and NDP52, all of which are proposed to serve as cargo receptors for the degradation of ubiquitinated substrates by autophagy. Similar to p62, these proteins possess ubiquitin-binding domains; moreover, they are selectively targeted for autophagic degradation, which requires the ability of these adaptors to physically bind LC3 (and other ATG8 orthologues) via a well-conserved linear amino acid motif, called the LIR (LC3-interating region) (Figure 2A) (Johansen and Lamark, 2011). Interestingly, the LIR consensus sequence has been uncovered in a growing number of proteins, suggesting that the repertoire of LC3-interacting proteins that serving as cargo receptors for selective autophagy may be expansive. In support, a large-scale proteomic study demonstrates that the mammalian ATG8 family has 67 high confidence interactions with other cellular proteins (Behrends et al., 2010).

Mitophagy—In addition, the selective autophagic degradation of organelles, namely mitochondria, also involves ubiquitination in certain instances. A landmark study identified the ubiquitin E3 ligase, Parkin, as a critical mediator of the autophagic degradation of mitochondria (mitophagy) in response to mitochondrial depolarization (Narendra et al., 2008). In this pathway, which is viewed as a critical mitochondrial quality control mechanism, the specific recruitment of Parkin to depolarized mitochondria, and the subsequent ubiquitination of various mitochondrial substrates, is crucial for the elimination of mitochondria (Figure 2B) (Chan et al., 2011; Yoshii et al., 2011). Furthermore, the cargo receptor p62/SQSTM has been implicated in Parkin-mediated mitophagy(Geisler et al., 2010). The other major pathway implicated in mitophagy involves the two related BH3 family proteins, BNIP3 and NIX/BNIP3L, which promote mitochondrial clearance during reticulocyte development (Novak et al., 2010; Sandoval et al., 2008; Schweers et al., 2007), and during hypoxia (Papandreou et al., 2008; Tracy et al., 2007; Zhang et al., 2008; Zhang and Ney, 2009). These proteins are resident components of the outer mitochondrial membrane and do not bind ubiquitin; nevertheless, NIX has been demonstrated to be a bona fide cargo receptor during mitophagy. NIX possesses a LIR motif and physically binds ATG8 orthologues, including LC3 and GABARAP, independently of ubiquitin (Figure 2A) (Novak et al., 2010).

Selective autophagy and cancer—The proper control of selective autophagy has important implications for neoplasia, because in mammalian cells, this process is fundamental for the removal of long-lived proteins and damaged organelles, as well as in mitigating oxidative stress. As described in detail below, defective autophagy in normal tissue leads to the accumulation of ubiquitinated protein aggregates and damaged mitochondria, and has been implicated in neoplastic transformation, not only due to unchecked proteotoxic and genotoxic stress, but also because of deregulated cellular signaling (Dikic et al., 2010). For example, because p62/SQSTM serves as a scaffold protein in multiple signaling pathways, such as NF-κB activation, its accumulation in autophagy defective cells leads to deregulation of downstream pathways (Mathew et al., 2009).

Similarly, the oxidative stress response is profoundly affected by p62/SQSTM accumulation in autophagy deficient cells (Komatsu et al., 2010). The transcription factor Nrf2 (nuclear regulatory factor 2) regulates the expression of a wide range of genes that promote the oxidative stress response and facilitates cell survival. Nrf2 is critically inhibited by the E3 ligase, Keap1, which ubiquitinates and degrades Nrf2 under normal conditions (Figure 2C). During oxidative stress, the activity of the E3 ligase is inhibited through the modification of cysteine residues in Keap1 (Padmanabhan et al., 2006). Recent data shows that accumulating p62/SQSTM1 in autophagy-deficient cells, directly binds to Keap1, thereby disrupting Keap1-mediated degradation of Nrf2 and promoting the aberrant upregulation of Nrf2 and it downstream transcriptional targets (Komatsu et al., 2010). Notably, the Nrf2 pathway, due to inactivating somatic mutations in Keap1, has been implicated as a survival pathway in non-small cell lung carcinomas (Singh et al., 2006). Based on this result, one can speculate that the aberrant activation of Nrf2 in autophagy deficient cells promotes tumor cell survival by amplifying the oxidative stress response; at the same time, these cells are predisposed to the deleterious accumulation of damaged organelles and toxic proteins. Indeed, this unfortunate convergence of events has recently been implicated in the spontaneous tumorigenesis of autophagy-defective liver cells (Inami et al., 2011; Takamura et al., 2011).

Autophagy and tumor suppression

Because scientific evidence supports both tumor promoting and suppressive functions for autophagy, these paradoxical effects are reconciled through a model in which the exact role of autophagy during cancer progression depends on tumor type, context and stage (Figure 3). Based on genetic studies, the tumor suppressive functions of autophagy are most apparent during tumor initiation. In contrast, the requirement for autophagy becomes more apparent in later stages as tumor cells cope with micro-environmental stresses encountered during progression and metastasis (Roy and Debnath, 2010).

ATGs as tumor suppressors

Genetic evidence supporting that autophagy can prevent tumor formation was first broached through genetic studies of Becn1/ATG6 (Liang et al., 1999). Becn1 was originally identified due to its interaction with Bcl-2 (Kihara et al., 2001; Pattingre et al., 2005). Remarkably, Bcl-2 binds to Becn1 and inhibits its autophagic activity by blocking its interaction with Vps34. Subsequently, *BECN1* was mapped to a tumor susceptibility locus that is allelically deleted in a high percentage of human breast, ovarian, and prostate cancers (Liang et al., 1999). In addition, although mice homozygously deleted for *becn1* die during embryogenesis, those lacking a single copy of *becn1* (*becn1+/−*) develop spontaneous tumors, including lymphoma, hepatocellular carcinoma, lung adenocarcinomas and mammary hyperplasia (Qu et al., 2003; Yue et al., 2003). Notably, the loss of the second allele of *becn1* did not occur in these tumors, which strikingly resembled the allelic losses

originally found in human patients. These reports provide the first direct genetic evidence that *becn1* is a haploinsufficient tumor suppressor.

Furthermore, analysis of human tissue samples revealed decreased Becn1 expression in human breast carcinomas compared to normal breast tissue (Liang et al., 1999). Complementary studies demonstrate that ectopic overexpression of Becn1 in MCF7 cells, which exhibit partial deficiency in Becn1 expression, causes reduced cancer cell proliferation in vitro and decreased tumorigenic potential in vivo. These studies further support a role for this autophagy regulator in tumor suppression in an established cell culture model for hormone sensitive (luminal A subtype) breast cancer (Liang et al., 1999).

In addition, multiple Becn1 interacting partners have been implicated as tumor suppressors. UV irradiation Resistance-Associated Gene (UVRAG), a Becn1 interacting protein that positively regulates autophagy, is allelically deleted in human colon carcinoma (Liang et al., 2006) (Liang et al., 2007a). Moreover, frameshift mutations in the polyadenine tract of the UVRAG gene are present in gastric carcinomas; gastric cancer cells harboring these mutations exhibit decreased autophagy (Kim et al., 2008). In addition, mice lacking Bif 1, which interacts with Becn1 via UVRAG, exhibit significantly higher rate of spontaneous tumors (Takahashi et al., 2007); furthermore, reduced Bif1 expression is observed in gastric carcinoma, which correlates with decreased autophagy (Lee et al., 2006).

While the complete genetic deletion of *becn1* is lethal during early embryonic development, mice lacking several other ATGs (e.g. *atg3, atg5, atg7* and *atg16*) actually survive until birth, ultimately succumbing to metabolic deficiencies during the neonatal starvation period (Komatsu et al., 2005; Kuma et al., 2004; Saitoh et al., 2008; Sou et al., 2008). These phenotypic differences indicate that Becn1 may have multiple functions beyond autophagy; hence, one question that has persisted over the last decade is whether the tumor suppressor functions downstream of Becn1 and its interacting partners are actually due to a broader effect on class III PI3K activity and organelle biogenesis. However, recent work more clearly implicates a genetic role for autophagy as a suppressor of spontaneous tumorigenesis. Mice with systemic mosaic deletion of *atg5* and liver-specific $atg7^{-/-}$ mice develop liver adenomas; notably, in these models, autophagy-deficient hepatocytes exhibit p62 accumulation as well as oxidative and genotoxic stress. Moreover, the concomitant deletion of p62 partially suppresses tumor progression in ATG7-deficient liver, supporting a role for p62 accumulation in liver tumor progression. Importantly, the spontaneous tumors that arise in these models are confined to a single tissue type and they are uniformly benign adenomas; the tumors fail to exhibit any invasive behavior or distant metastasis (Inami et al., 2011; Takamura et al., 2011). In humans, less than 10% of benign hepatic adenomas undergo malignant transformation, and the exact role of autophagy in this process remains unknown (Shanbhogue et al., 2011). Nonetheless, these benign lesions are completely autophagy deficient, these results are consistent with the hypothesis that autophagy is required for advanced tumor progression, retaining the allure of autophagy inhibition as a therapeutic target in more established cancers (Amaravadi et al., 2011).

In addition to these elegant studies, ATG4c knockout mice exhibit a higher susceptibility to fibrosarcomas upon exposure to chemical carcinogens. Lastly, frameshift mutations in ATG2B, ATG5, and ATG9B have been reported in gastric and colorectal carcinomas with high microsatellite instability, further insinuating tumor suppressor functions for the core autophagic machinery in human cancers (Kang et al., 2009).

Mechanisms of tumor suppression by autophagy

Given the prototypic functions of autophagy as a survival pathway, its role as a potential tumor suppressor mechanism seems counterintuitive. However, a mounting body of work

demonstrates an important cell-autonomous function for autophagy in protecting cells from genotoxic stress and maintaining genome integrity. This specific tumor suppressor function of autophagy has thus far been best characterized in the context of metabolic stress, a condition typically observed in tumors due to hypoxia and inadequate glucose supply coupled with increased energy demands of the rapidly proliferating cells, which leads to extensive cellular damage. Apoptosis acts as the first line of defense to remove these damaged cells. However, when apoptosis is reduced or inactivated, as commonly occurs during tumorigenesis, the cells rely significantly on autophagy for ATP maintenance and cellular fitness. In Bcl-2-overexpressing immortalized mouse mammary epithelial (iMMEC) cells and immortalized baby mouse kidney epithelial (iBMK) cells, loss of one copy of *becn1* significantly sensitizes cells to metabolic stress (Karantza-Wadsworth et al., 2007; Mathew et al., 2007). Paradoxically, in spite of increased survival, *becn1*+/+ cells are less tumorigenic than *becn1*+/− cells. This intriguing outcome is due to the fact that in autophagy-defective cells, metabolic stress induces significantly higher DNA double strand breaks and gene amplification as well as the accumulation of damaged mitochondria and ER chaperones, compared to their wild type counterparts. Moreover, p62 serves as a critical link between defective autophagy and tumorigenesis (Mathew et al., 2009). The aberrant accumulation of damaged mitochondria and protein aggregates in autophagy-defective cells leads to elevated ROS levels, which causes DNA damage as well as p62 accumulation. Again, p62 accumulation upon metabolic stress leads to ROS generation and consequent deregulation of the NF-κB pathway, thereby creating a positive feedback loop. Thus by keeping in check the intracellular ROS levels, autophagy serves as a tumor suppressor function. Remarkably, these experiments have been conducted in cells harboring multiple genetic abnormalities, including the inactivation of the tumor suppressor p53. Nonetheless, a similar DNA damage response has been observed in the spontaneous liver tumors arising in mice with mosaic *atg5* or liver-specific *atg7* deletion (Takamura et al., 2011). Fascinatingly, in a small cohort of breast cancer patients, a significant association was found between the loss of *BECN1* and amplification of *HER2/NEU*, both of which are located on chromosome 17q21. In this study, the authors also noted associations between *BECN1* loss and mutations in other tumor suppressors, including *p53* and *PTEN*, consistent with the idea that *BECN1* loss and defective autophagy facilitates DNA damage and genomic instability in HER2+ breast cancers(Negri et al., 2010). Overall, these results support the hypothesis that defective autophagy functions as a modifier, and possibly a fundamental driver, of genomic damage during tumor progression.

In addition, defective autophagy may contribute to the development of breast cancer in a manner independent of genotoxic stress and genomic instability through the induction of ER stress. This study also reveals a role for autophagy in p62-dependent keratin 8 (K8) homeostasis in mammary epithelial cells and correlates low Becn1 protein levels with phospho(Ser73)-K8 accumulation in human breast tumors (Kongara et al., 2010).

Autophagy also promotes oncogene-induced senescence (OIS), which is viewed as a major barrier to cellular transformation. OIS induces a permanent cell cycle arrest in response to the mitotic burst and metabolic stress generated by oncogenic transformation. Recent studies demonstrates that the induction and maintenance of OIS is mediated by an inflammatory network comprised of IL-6 and IL-8 both of which function in a cell-autonomous manner (Kuilman et al., 2008). Interestingly, autophagy is induced during OIS in an inducible Ras cell culture system; accordingly, autophagy inhibition via ATG knockdown results in a significant bypass of senescence as well as inhibition of the secretion of IL6 and IL8 (Young et al., 2009). Mechanistically, intracellular recycling associated with autophagy may facilitate OIS by providing the amino acids for the synthesis of the secretory proteins. Thus, a basal level of autophagy appears to restrict cell proliferation during OIS, and thus, potentially precludes further tumor progression.

Another potential tumor suppression mechanism of autophagy is via the inhibition of necrosis in apoptosis-resistant cells during metabolic stress. Though decreased viability in the combined absence of autophagy and apoptosis should negatively affect tumorigenicity, necrotic cell death causes macrophage infiltration and proinflammatory cytokine production and thereby facilitates tumor growth (Degenhardt et al., 2006). Remarkably, inflammatory cells infiltrate tumor sites in response to necrosis resulting from hypoxia and metabolic stress, both of which commonly affect solid tumors. Although certain inflammatory cells, such as cytotoxic T cells and NK cells are anti-metastatic, chronic tumor inflammation associated with severe hypoxia and metabolic stress generally favors pro-tumor immunity (DeNardo et al., 2008). Importantly, infiltration of pro-tumor inflammatory mediators, like macrophages, correlates with poor clinical prognosis, underscoring the importance of understanding the biological mechanisms by which tumor cells tip the balance in favor of a pro-proliferative immune response (Bingle et al., 2002). Thus, by limiting tumor cell necrosis, autophagy may actually suppress tumor growth by preventing leukocyte infiltration of the primary tumor site.

Based on exciting work in other inflammatory disorders, it is also tempting to speculate that perturbations in autophagy can initiate inflammation in other circumstances, thereby creating a pro-tumorigenic environment (White et al., 2010). Genome-wide association studies identified ATG16L as a susceptibility gene in Crohn's disease, a chronic inflammatory disorder of the gastrointestinal tract (Barrett et al., 2008). Importantly, the chronic inflammation in both Crohn's disease and a closely related inflammatory bowel disease, ulcerative colitis, are considered to be a major risk factor for the development of colorectal cancer. In mouse models hypomorphic for ATG16L, or lacking ATG5 or ATG7 in the intestine, the intestinal Paneth cells severe abnormalities that strikingly resemble the changes seen in human Crohn's disease patients carrying the ATG16L1 risk allele. Moreover, these cellular changes are associated with altered inflammatory gene transcription profiles in Paneth cells (Cadwell et al., 2008; Cadwell et al., 2010). In addition, ATG16L1 deficient macrophages produce drastically elevated levels of the inflammatory cytokines IL-1β and IL-18, again intimating that defective autophagy can promote a pro-tumor inflammatory state, and in this case, independently of tumor necrosis (Saitoh et al., 2008).

Tumor promoting functions for autophagy

Although reduced autophagy is believed to promote tumor development, a minimal level appears to be necessary for the survival and fitness of cancer cells. Moreover, increased autophagy is observed in transformed cells when exposed to diverse stresses. Thus, it is increasingly accepted that autophagy provides cancer cells with certain selective advantages to cope with stress, both in the primary tumor microenvironment as well as during metastatic progression (Figure 3). The following sections overview several potential mechanisms through which autophagy may promote tumor progression.

Hypoxia

Tumor hypoxia, resulting from inadequate tumor vasculature, is associated with a more malignant phenotype, higher predisposition for metastasis, and poor prognosis. Hypoxic stress selects for cells that are resistant to apoptosis as well as poses a major barrier to chemotherapy and radiotherapy. White and colleagues first showed that autophagy is induced specifically in the hypoxic core of tumors where it promotes survival (Degenhardt et al., 2006). Further studies have unveiled the molecular connections between hypoxia and the induction of autophagy. Hypoxia-inducible factor 1α (HIF-1α), a key transcription factor regulating a plethora of genes responsible for altered metabolism, angiogenesis, invasion, metastasis, therapy-resistance in hypoxic tumors (Bertout et al., 2008), is a positive regulator of autophagy. BNIP3, a BH3-only protein, is a downstream target of HIF1α and was shown

to induce mitophagy and thereby control ROS production in response to hypoxia (Zhang et al., 2008). Further mechanistic studies revealed that induction of BNIP3 and BNIP3L in hypoxic cells disrupts the Becn1-Bcl-2 complex, thereby releasing Becn1 to induce autophagy (Bellot et al., 2009). Though induction of BNIP3, a proapoptotic protein, was initially implicated in driving autophagic cell death (Tracy et al., 2007), subsequent studies have clearly revealed that BNIP3-induced autophagy is an adaptive survival response during prolonged hypoxia (Bellot et al., 2009). In addition, there is emerging evidence that various HIF1α independent cellular stress response pathways, like AMPK and unfolded protein response (UPR), can also mediate hypoxia-induced autophagy (Papandreou et al., 2008; Rouschop et al.).

As the role of autophagy as a key mediator of survival of hypoxic cells is emerging, the exact mechanisms underlying this phenotype remain unclear. Because chronic hypoxia leads to major metabolic perturbations in tumor tissues, one can postulate that by recycling basic cellular components, autophagy helps stressed cells cope with the increased metabolic demand (Rabinowitz and White, 2010). Further studies are needed to validate this hypothesis and unveil the interconnections between hypoxia-driven tumor metabolism and autophagy.

Extracellular matrix detachment

Anoikis, or detachment-induced cell death, serves the homeostatic function of killing cells that have lost contact with the basement membrane. Autophagy is induced in both non-transformed and oncogene-transformed cells following matrix detachment, which protects these cells from anoikis (Fung et al., 2008; Lock et al., 2011). Similarly, in three-dimensional (3D) epithelial cell culture models, autophagy is significantly induced in the detached luminal cells and its inhibition resulted in accelerated luminal clearance (Fung et al., 2008; Karantza-Wadsworth et al., 2007). These studies intimate that autophagy is instrumental in anoikis resistance, a process exploited by disseminating tumor cells to survive after detachment from the primary site as well as while migrating to distant metastatic sites (Kenific et al., 2010). Nonetheless, the ability of autophagy to promote tumor dissemination and metastasis by preventing anoikis must still be established in relevant in vivo models.

Metabolic fitness during oncogenic transformation

As a key pathway that sustains core metabolic functions during starvation, the requirement for a minimal level of autophagy (termed basal autophagy) during oncogenic transformation is becoming increasing appreciated (Rabinowitz and White, 2010). Autophagy is particularly important in the context of strong oncogenic insults, namely Ras activation, because they coordinately drive tumor cell proliferation and alter metabolic pathways within the cancer cell to enhance energy levels and biosynthetic demands (Levine and Puzio-Kuter, 2010). In fact, recent studies demonstrate autophagy deficient cells expressing activated H-Ras or K-Ras displayed decreased adhesion-independent growth (Lock et al., 2011). These results have been corroborated by multiple groups demonstrating that autophagy inhibition in human mammary epithelial cells or immortalized mouse kidney cells expressing activated Ras elicits decreased soft agar colony formation and decreased xenograft and allograft tumor growth in immunodeficient mice (Guo et al., 2011; Kim et al., 2011). Overall, these results support that autophagy is critical for oncogenic transformation via activated Ras.

Remarkably, these studies also reveal that the requirement for autophagy during Ras transformation is due to its ability to facilitate proliferation, rather than promoting cell survival (Guo et al., 2011; Lock et al., 2011). These findings have particular relevance for pancreatic ductal adenocarcinoma (PDAC), a uniformly lethal cancer where activating K-

Ras mutations are present in greater than 90% of tumors. Recent seminal work confirms elevated basal autophagy in both primary PDAC tumors and cell lines; the genetic or pharmacologic inhibition of autophagy in PDAC cells potently suppresses proliferation in vitro and elicits robust tumor regression and prolonged survival in pancreatic cancer xenografts and genetic mouse models. Thus, autophagy is required for tumorigenic growth and expansion of pancreatic cancers (Yang et al., 2011).

Intriguingly, autophagy deficiency has minimal impact on the proliferation of non-transformed cells (Lock et al., 2011). Because strong oncogenic insults, such as activated Ras, are marked by profound metabolic alterations that promote energy production and support the biosynthesis of macromolecules needed for rapid proliferation, one can speculate that autophagy maintains these key metabolic pathways in Ras transformed cells. Indeed, in certain Ras-transformed cells, autophagy facilitates effective glucose uptake and glycolytic flux, intimating a crucial role for autophagy in the "Warburg effect." (Lock and Debnath, 2011; Lock et al., 2011). Other studies demonstrate that the loss of autophagy during Ras transformation is associated with reduced oxygen consumption and decreased levels of tricarboxylic acid (TCA) cycle intermediates (Guo et al., 2011; Yang et al., 2011). Although the precise biochemical mechanisms may be cell type and context dependent, the above studies all point a critical role for basal autophagy in supporting the rapid proliferation of tumor cells, in part by enhancing metabolic capacity. These data also suggest the pro-tumor functions for autophagy are not restricted to its well-known ability to promote the survival of stressed tumor cells; rather, autophagy drives the metabolic fitness of the entire tumor population (Lock and Debnath, 2011). Though breast cancers do not commonly exhibit oncogenic Ras mutations, other oncogene pathways activated in breast tumors, including HER2/Neu, Myc and activated PI3K, produce metabolic alterations similar to Ras, which are required to maintain the transformed phenotype. Thus, an important area for future research is delineating how autophagy impacts the metabolic fitness of breast cancer cells and tissues harboring these common genetic alterations.

Autophagy restricts chemotherapeutic efficacy

The high levels of autophagy observed in tumor cells following virtually every anti-cancer treatment is now recognized to represent a common adaptive stress response that enables tumor cells to survive these therapeutic insults (Figure 3) (Kondo et al., 2005). This has motivated significant interest in combining autophagy inhibition with other chemotherapies to synergistically eliminate cancer cells. Abundant recent work supports this notion in multiple tumor types and in response to diverse chemotherapeutic agents, highlighting the possibility of targeting autophagy as a combination strategy for cancer. Readers are referred to several recent reviews for additional information (Amaravadi et al., 2011; Eisenberg-Lerner and Kimchi, 2009; Hoyer-Hansen and Jaattela, 2008). With regard to the rapid translation of autophagy inhibitors into the clinical setting, the lysosomal inhibitor hydroxychloroquine (HCQ) and its derivatives have gained special attention because of their long history of use as anti-malarial agents and in diseases such as rheumatoid arthritis (Amaravadi et al., 2011); thus, multiple clinical trials using HCQ as a sensitizing reagent in combination with standard cancer therapies are under evaluation in different tumor types (http://clinicaltrials.gov). However, in evaluating these studies, an important caveat is that the cytotoxic effects of HCQ and similar agents are likely to involve processes other than autophagy. To date, studies dissecting the precise contributions of autophagy toward the efficacy of these anti-malarials in diverse clinical settings have been conspicuously absent.

Targeting autophagy in breast cancer

In breast cancer treatment, a growing number of functional studies support that autophagy inhibition can be combined with established therapies in breast cancer to improve clinical

outcome. Since the early days of autophagy research, the anti-estrogen tamoxifen has been known as a potent inducer of autophagy in a variety of breast cancer cells (Bursch et al., 1996) Originally autophagy was postulated as a nonapoptotic cell death mechanism; however, recent functional studies indicate that autophagy inhibition, which was achieved either by pharmacological means or RNAi-mediated silencing of ATGs, actually sensitizes hormone receptor positive breast cancer cells to tamoxifen, thereby promoting cytotoxicity and preventing the development of anti-estrogen resistance (Bursch et al., 1996; Qadir et al., 2008; Samaddar et al., 2008; Schoenlein et al., 2009). Given the prevalence of resistance to tamoxifen (and similar agents) in ER+ breast cancers, autophagy inhibition may be useful as a combination strategy in this subset of breast cancer patients.

Similarly, in various breast cancer cell culture models, autophagy inhibition appears to possess utility as a sensitizer in the setting of radiation as well as to decrease the resistance of HER2+ positive breast cancer cells to the anti-HER2 monoclonal antibody trastuzimab (Apel et al., 2008; Vazquez-Martin et al., 2009). Lastly, in a small cohort of breast cancer patients with *HER2/NEU* amplification, the concomitant loss of *BECN1* correlated with improved clinical response to trastuzimab, leading the authors to speculate that impaired autophagy in *BECN1* deficient breast cancers promotes cell death in response to this highly-utilized targeted therapy (Negri et al., 2010). Such studies undoubtedly hold promise, but given the aforementioned tumor suppressive functions of autophagy, a certain degree of caution should be exercised in rapidly translating autophagy inhibitors as an all-purpose treatment for breast cancer. In the following section, we concentrate on two circumstances in which autophagy inhibition may be particularly attractive as a therapeutic target in certain stages of breast cancer.

Chemoprevention in pre-invasive lesions

The frequency of pre-invasive lesions (in which the tumor does not invade the basement membrane or myoepithelial layer), diagnosed in patients has significantly increased due to radiological screening by mammography. Ductal carcinoma in situ (DCIS) is the most common type of pre-invasive breast cancer, and women diagnosed with DCIS remain at significantly increased risk for subsequent development of invasive breast carcinoma. Thus, the ability to both effectively diagnose these early lesions, and confidently predict future outcome for these patients, has assumed profound significance in breast cancer diagnosis and treatment (Espina and Liotta, 2011). Recent elegant work using the ex vivo culture of surgically removed primary DCIS specimens demonstrates that a subpopulation of DCIS cells possesses the unique ability to survive in the intra-ductal microenvironment. These pre-invasive cells exhibit high levels of genetic instability, resistance to treatment, and the propensity for invasive behavior in vitro, rendering them prime suspects as the malignant progenitor cells that ultimately give rise to invasive tumors in certain patients (Espina et al., 2010).

Importantly, this study also demonstrates that autophagy is required for the ability of these malignant progenitor cells to survive in the intra-ductal niche. Several stresses in the intra-ductal microenvironment predispose DCIS cells to undergo autophagy, many of which have been described in greater detail in the previous section (Figure 4). These include: 1) hypoxia and nutrient deprivation due to reduced vascular access; 2) ECM detachment; and 3) increased intracellular calcium, both insoluble (calcium phosphate deposition) and soluble, that is associated with the micro-calcifications commonly observed in DCIS lesions (Espina and Liotta, 2011; Espina et al., 2010). Notably, although these stresses impact the entire population, only a subset of cells within these lesions exhibits detectable autophagosome formation; presumably, these correspond to malignant progenitor cells lurking within the intra-ductal niche. Upon treatment with chloroquine, the outgrowth of these malignant progenitor cells from ex vivo DCIS cultures is robustly suppressed; accordingly, the authors

propose autophagy as an attractive chemoprevention target for the treatment of DCIS patients. Indeed, the exciting findings in this powerful preclinical model have already motivated the use of HCQ in a neo-adjuvant clinical trial for DCIS (Espina and Liotta, 2011; Espina et al., 2010). One can speculate that using HCQ (and more specific autophagy inhibitors in the future) as a chemoprevention strategy to eradicate malignant progenitors cells from pre-invasive breast lesions appears most appropriate for shortterm use in either neo-adjuvant or adjuvant settings. In contrast, the sustained use of these agents may be unwise if chronic autophagy inhibition predisposes residual malignant progenitor cells to both genotoxic stress and the effects of pro-tumorigenic inflammation. Ultimately, rigorous clinical trails are required to delineate whether autophagy inhibition is tenable as a chemoprevention strategy in DCIS.

Breast cancer late recurrence

Breast carcinoma is well known for its propensity to relapse after a long disease-free period, often decades after initial treatment (Aguirre-Ghiso, 2007). Unfortunately, recurrent disease is highly resistant to available treatments and commonly metastatic; thus, late recurrence remains a principal cause of lethality in breast cancer patients. Especially troublesome is that many patients are diagnosed at an early stage, with small tumors and no evidence of lymph node metastases, yet they exhibit recurrence levels in excess of 25% when followed over 10 to 15 years (Brackstone et al., 2007). This is presumably due to a small population of tumor cells that escape therapy and exist as dormant, micro-metastatic cells without any clinical manifestation. Though viable, these cells are not proliferative and are thus resistant to conventional chemotherapy that typically targets rapidly growing cells (Aguirre-Ghiso, 2007). Thus, a better understanding of the molecular events leading to tumor dormancy as well as the development of clinically relevant in vivo models are imperative for identifying suitable treatment options.

A potential role for autophagy in dormancy was originally broached in *C. elegans*, where self-eating was found to be essential for survival during dauer diapause, a stress-induced, dormancy-like state that occurs when larvae are exposed to hostile environments (Melendez et al., 2003). Recently, autophagy has been shown to be crucial for the survival of dormant cells in models of ovarian cancer and gastrointestinal stromal tumor (GIST) (Lu et al., 2008) (Gupta et al., 2010). In xenograft ovarian tumors, autophagy supports the survival of a subset of cells in the face of the cytotoxic effects of the tumor suppressor aplasia Ras homolog member I (ARHI). Upon return of more favorable conditions due to ARHI suppression, as observed in clinical ovarian carcinoma, the tumor regains proliferative potential and led to rapid re-growth. A more convincing demonstration of autophagy as a survival pathway in quiescent cells comes from studies of GIST, the first solid tumor to be treated successfully with the small molecule tyrosine kinase inhibitor imatinib mesylate (Gleevec) (Gupta et al., 2010). However, less than 5% of GISTs regress significantly upon Gleevac treatment; rather, in the vast majority of patients, tumor cells indefinitely remain in a dormant, quiescent state in the presence of imatinib. Recent work indicates that this dormant state, termed stable disease, is closely associated with the induction of autophagy in response to imatinib. Upon inhibiting autophagy using RNAi-mediated ATG depletion or antimalarials such as quinacrine, GIST cells undergo high levels of apoptosis both in vitro and in vivo. Thus, autophagy appears critical for the establishment of a dormant state in which GIST cells can survive indefinitely (Gupta et al., 2010). Moreover, these results in GIST broach the exciting idea that autophagy can be more widely exploited to kill or prevent the expansion of quiescent or dormant cancer cells, which are notorious for their resistance to both conventional and targeted therapies (Rubin and Debnath, 2010).

Tumor dormancy is also postulated to be a stress management mechanism adopted by disseminated tumor cells to cope with the unfavorable microenvironment by completely

withdrawing from the cell cycle (Aguirre-Ghiso, 2007). $p27^{Kip1}$, the cyclin dependent kinase inhibitor involved in G0/G1 cell cycle arrest, was identified as a downstream target of the energy-sensing LKB1-AMPK pathway as well as shown to induce autophagy and facilitate cell survival in response to growth factor withdrawal and metabolic stress (Liang et al., 2007b). Thus, disseminated tumor cells (DTCs) may depend on p27-mediated autophagy for survival in an inhospitable microenvironment and to resist chemotherapy. Moreover, studies in breast cancer models suggest that decreased mitogenic signaling resulting from impaired integrin and growth factor signaling may lead to tumor dormancy (Korah et al., 2004; White et al., 2004). Because β1-integrin signaling blockade is a potent inducer of autophagy in ECM detached cells (Fung et al., 2008), one can hypothesize that disrupted integrin signaling-mediated autophagy induction in DTCs can support and maintain tumor dormancy (Figure 3). These results motivate future studies, especially those using in vivo preclinical models, to assess how autophagy influences the survival and biological behavior of dormant breast cancer cells and specifically, whether autophagy inhibition can be exploited to prevent late recurrence in breast cancer patients.

Concluding remarks

The current evidence from preclinical models indicates that autophagy can suppress the early stages of tumor initiation in certain circumstances; on the other hand, it clearly promotes the survival and metabolic fitness of more advanced tumors during cancer progression and in response to chemotherapy. Ongoing clinical trials in a variety of cancers will provide our first genuine insight into whether and how to manipulate autophagy in breast cancer. Most likely, the efficacy of lysosomal agents to inhibit autophagy, namely hydroxychloroquine, will be highly context specific. In addition, it remains unclear if autophagy inhibition will elicit untoward side effects in breast cancer patients over the long term. Hence, in evaluating forthcoming data from these clinical trials, both the anti- and pro-tumor functions mediated by autophagy must be carefully considered.

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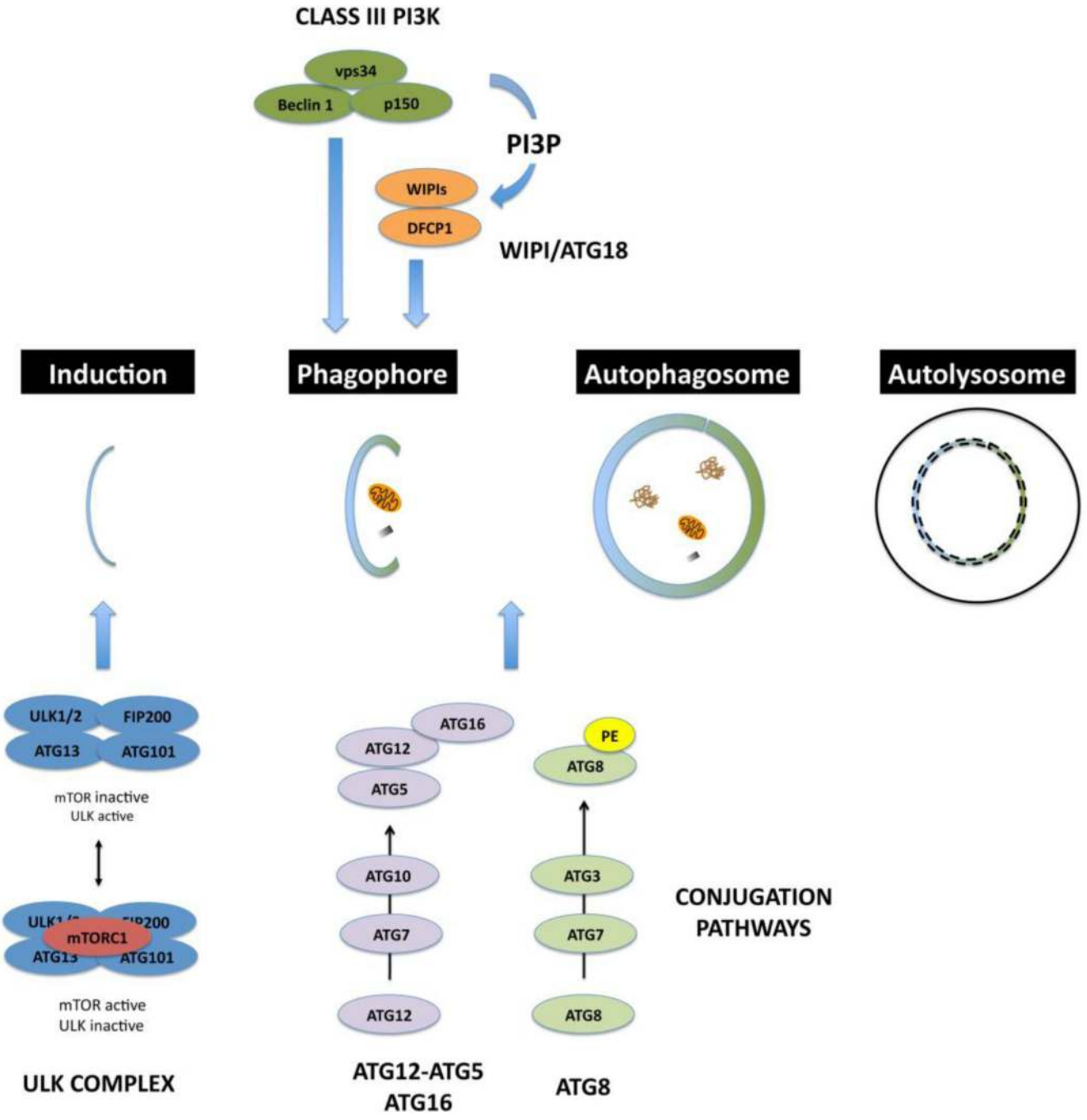

Figure 1. Molecular regulation of autophagy

Autophagy is a multistep process characterized by the induction of a phagophore by the ULK complex. Further nucleation of the phagophore is regulated by the Class III PI3K complex and WIPI/ATG18 proteins. Membrane elongation and autophagosome completion requires two ubiquitin-like conjugation systems to form the ATG12-ATG5/ATG16 complex and phosphotidylethanolamine (PE)-conjugated ATG8. The autophagosome, along with its sequestered cargo, ultimately fuses with the lysosome. The resulting autolysosome is a single membrane-bound acidic vesicle where the contents are digested by lysosomal enzymes and recycled. Details provided in the text.

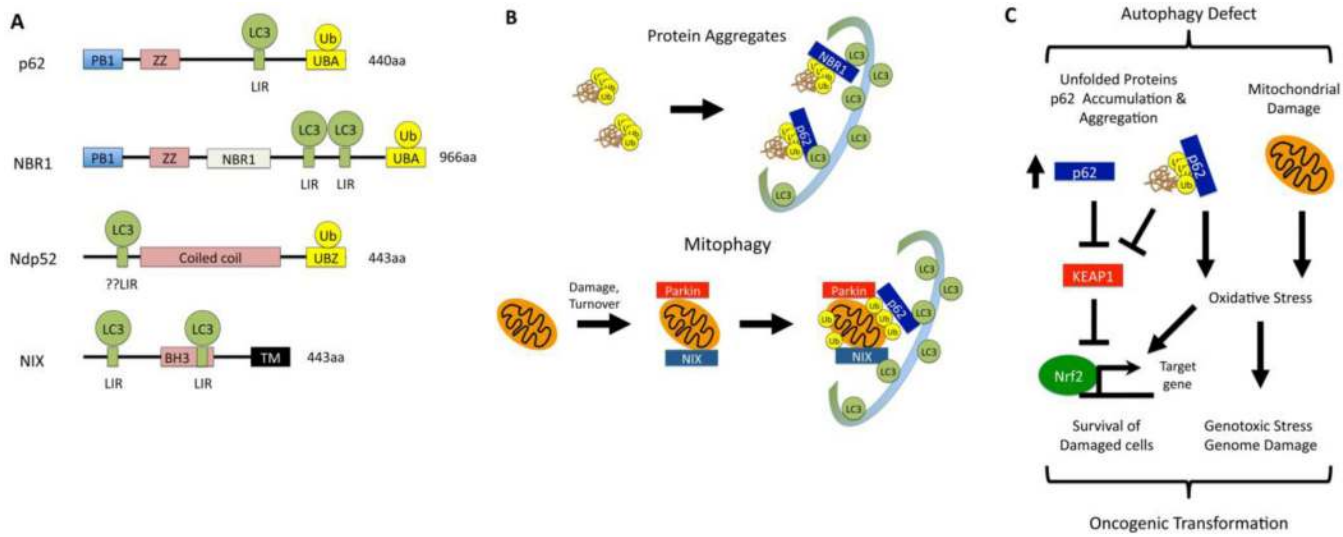

Figure 2. Selective autophagy

(A) Domain structure of the mammalian autophagy cargo receptors p62, NBR1, Ndp52, and NIX. Most of these proteins possess ubiquitin-binding domains (UBA or UBZ) and the LC3-interacting region (LIR) a linear amino acid motif required to bind LC3 and other ATG8 orthologues. (B) Misfolded and aggregated proteins are marked ubiquitin,, which is recognized by ubiquitin-binding domains of p62 and NBR1. These cargo receptors bind to LC3 (or another ATG8 orthologue) and target the substrate for autophagy. During mitophagy, damaged mitochondria are also ubiquitinated due to the recruitment of Parkin, an E3 ligase, which targets them for mitophagy. In a parallel pathway, the BH3 protein NIX is induced during mitophagy, interacts with LC3, and contributes to the recognition of mitochondria by autophagic membranes. (C) Autophagy inhibition can lead to the accumulation of damaged proteins and mitochondria, resulting in oxidative stress. At the same time, p62 accumulation and aggregation activates the Nrf2 pathway, which can promote the survival during oxidative stress, predisposing cells to genotoxic stress and oncogenic transformation.

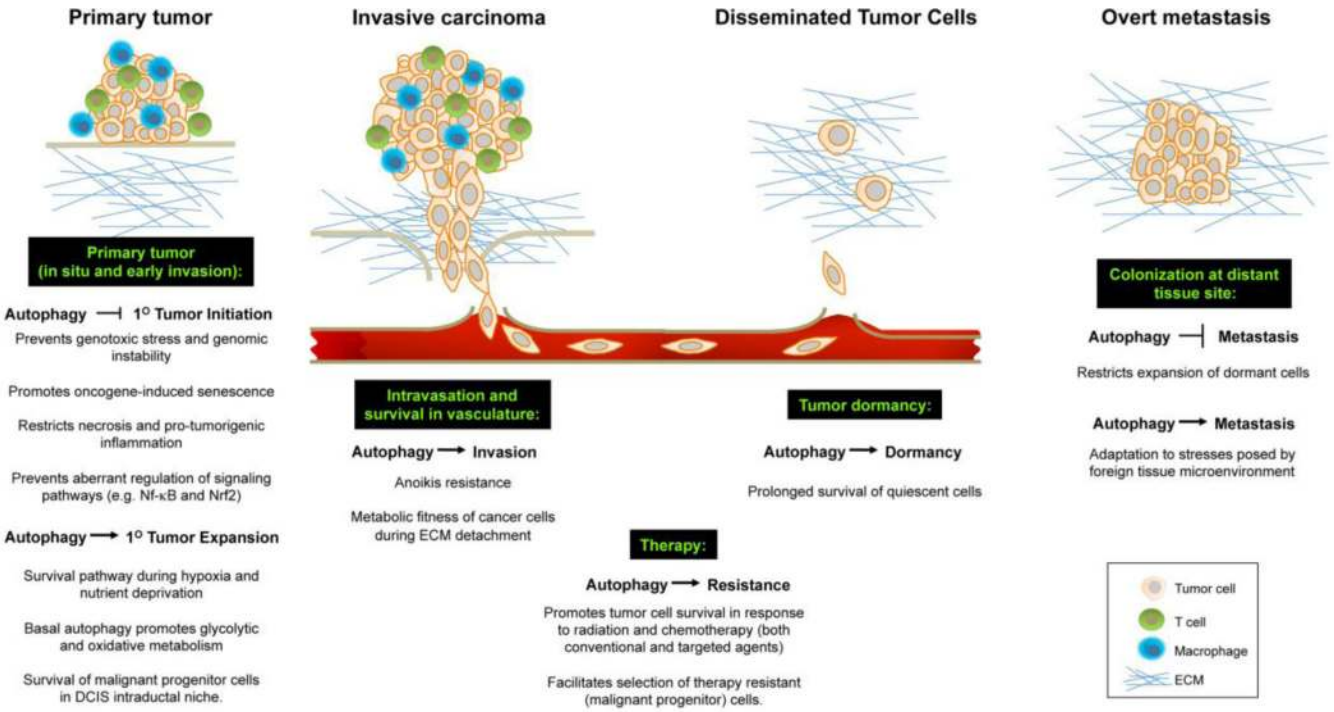

Figure 3. Overview of the multiple functions of autophagy during breast cancer initiation and progression

Details provided in the text. Adapted with permission from (Kenific et al., 2010).

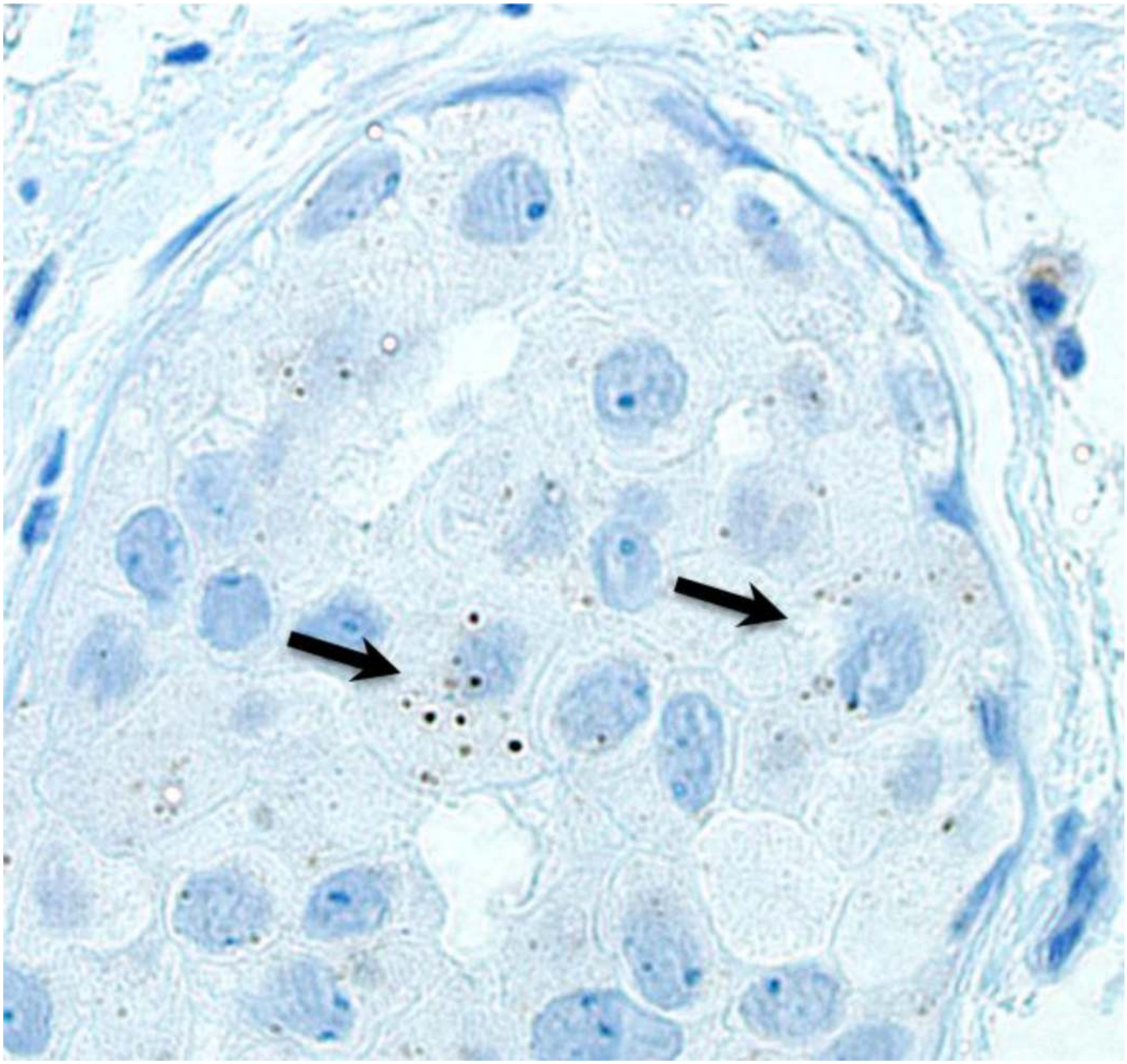

Figure 4. Autophagy in Ductal Carcinoma In Situ (DCIS)

α-LC3 IHC performed on a solid DCIS lesion reveals punctate staining in pre-neoplastic cells, indicative of autophagosme formation. Espina and Liotta propose that a subpopulation of cells within DCIS, corresponding to progenitor cells with enhance malignant potential, induce autophagy as a survival pathway in the intra-ductal niche (Espina and Liotta, 2011; Espina et al., 2010).