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Autophagy in tumor Suppression and cancer therapy

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Abstract

Autophagy is a stress-induced cell survival program whereby cells under metabolic, proteotoxic, or other stress remove dysfunctional organelles and/or misfolded/polyubiquitylated proteins by shuttling them via specialized structures called autophagosomes to the lysosome for degradation. The end result is the release of free amino acids and metabolites for use in cell survival. For tumor cells, autophagy is a double-edged sword: autophagy genes are frequently mono-allelically deleted, silenced, or mutated in human tumors, resulting in an environment of increased oxidative stress that is conducive to DNA damage, genomic instability, and tumor progression. As such, autophagy is tumor suppressive. In contrast, it is important to note that although tumor cells have reduced levels of autophagy, they do not eliminate this pathway completely. Furthermore, the exposure of tumor cells to an environment of increased metabolic and other stresses renders them reliant on basal autophagy for survival. Therefore, autophagy inhibition is an active avenue for the identification of novel anti-cancer therapies. Not surprisingly, the field of autophagy and cancer has experienced an explosion of research in the past 10 years. This review covers the basic mechanisms of autophagy, discusses its role in tumor suppression and cancer therapy, and posits emerging questions for the future.

Keywords

autophagy; cancer; mTOR; Beclin-1; p53; oncogene; tumor suppressor; chloroquine

I. INTRODUCTION

Autophagy (from the Greek term for “self-eating”) is a catabolic process whereby cells degrade via the lysosome proteins and organelles in order to survive periods of stress, especially nutrient deprivation. There are three modes of autophagy that differ in the manner in which the “cargo” (that which will be degraded) is delivered to the lysosome. Microautophagy refers to the nonselective process whereby cytosolic proteins are sequestered by invagination of the lysosomal membrane. Chaperone-mediated autophagy is a selective process whereby proteins with defined consensus sequences are recognized by molecular chaperones, including Hsc70, and delivered to the lysosome. Finally, macroautophagy (the topic of this review, and hereafter referred to as autophagy) is the process whereby bulk proteins and organelles in the cytosol are delivered by

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autophagosomes to the lysosome for degradation. While it is generally regarded as nonselective, there can be some selectivity in autophagy; for example, mitochondria or the endoplasmic reticulum can be selectively degraded ("mitophagy" and "reticulophagy," respectively), or misfolded and/or polyubiquitylated proteins can be selectively degraded, such as following proteotoxic stress. Autophagy is a critical mechanism for the adaptation of cells to stress, and it is induced by numerous stimuli, including nutrient deprivation, hypoxia, hormone stimulation, and DNA damage.[1,2]

The process of autophagy, and the identification of genes critical for this pathway, was originally identified in the yeast *Saccharomyces cerevisiae* in the early 1990s.[3] Over 30 autophagy (Atg) genes have been described in yeast, and over 20 of these have been identified in mammalian cells.[4] Because some of the yeast genes influential in autophagy were originally cloned in the pathway involved in vesicular protein sorting, some of these genes are denoted by the acronym Vps instead of Atg. Importantly, the molecular mechanism of autophagy is conserved from yeast to mammals, and the orthologs of the majority of the yeast Atg genes can be found in mammalian cells, with identical or similar function.

II. THE PROCESS OF AUTOPHAGY

Autophagy is a multi-step process that starts with the nucleation of a membrane called the phagophore (Fig. 1). The exact source of the phagophore membrane is not known, but there is evidence that it may be derived from the endoplasmic reticulum and trans-Golgi network[5] as well as the mitochondria[6,7]; one possibility is that the derivation of the phagophore membrane depends upon the nature of the stress that induces autophagy. The phagophore expands and grows, selectively or nonselectively engulfing organelles and proteins during its expansion. At the end of this elongation step, a portion of the cytosol is sequestered into a double-membrane vesicle termed the *autophagosome*. The autophagosome then fuses with an endosome and/or the lysosome, thereby forming the autolysosome. In the autolysosome, lysosomal hydrolases degrade the protein constituents, resulting in free amino acids and macromolecules that are transported back into the cytosol for reuse.

There are at least four core subgroups of Atg complexes that play key roles in autophagy. The first is the Atg1/Unc-51-like kinase (ULK) complex, which is directly controlled by mTOR phosphorylation. The second is the class III phosphatidylinositol 3-kinase (Pt-dIns3k)/Vps34 complex. The third and fourth are two ubiquitin-like protein conjugation systems, involving the ubiquitin-like proteins Atg12 and Atg8/LC3, respectively. In addition to these four core subgroups, there are also two transmembrane proteins that deserve mention, Atg9 and VMP1.

The yeast serine/threonine kinase Atg1 is a master regulator of autophagy; Atg1 interacts with Atg13 and Atg17, and these are essential for Atg1 activity. The yeast TOR protein is a serine/threonine kinase that is evolutionarily conserved among species and plays a central role in various cellular events like cell cycle, cell growth, and autophagy.[8] The yeast TOR protein phosphorylates Atg13, reducing its ability to bind Atg1 and thereby decreasing Atg1 activity.[9,10] Inhibition of TOR causes dephosphorylation of Atg13, accumulation of Atg1-Atg13-Atg17 complexes, and activation of autophagy (Fig. 2A). In mammals, ULK1 and ULK2 (Unc-51-like kinases 1 and 2) are the homologues of yeast Atg1; these proteins also exist in complex with Atg13 (mAtg13), along with the scaffold protein FIP200 (an ortholog of yeast Atg17), and mTOR. In mammalian cells, there are two known mTOR complexes, mTORC1 and mTORC2.[11] mTORC1 is the predominant form associated with autophagy. mTORC1 is quickly dissociated from the Atg13/FIP200/ULK1/2 complex during starvation.[12–14] This dissociation is accomplished by several phosphorylation events,

including phosphorylation of mAtg13 by ULK1, ULK2, and mTORC1; phosphorylation of FIP200 by ULK1 and ULK2; and phosphorylation of ULK1 and ULK2 by mTORC1. Overall, however, the regulation of autophagy by mTORC1 follows similar principles: a decrease in mTORC1 activity leads to dephosphorylation of ULK1, ULK2, and mAtg13; this leads to activation of ULK1 and ULK2, which then phosphorylate mAtg13 and FIP200.[12,14] Therefore, while the events are seemingly more complicated in mammals, the end result in both yeast and mammals is the direct control of Atg1/ULK, the master regulator of autophagy, by the master sensor of nutritional stress, mTOR.

Nucleation and elongation of the pre-autophagosomal membrane is controlled by the class III PI3 kinase (PI3KIII) Vps34. Vps34 exists in a complex with Beclin-1 (Atg6) and p150 (a homolog of Vps15),[15, 17] along with the protein UVRAG (ultraviolet irradiation resistance-associated gene, a homolog of Vps38) and Bif-1; the latter two proteins bind and enhance the activity of PI3KIII through their interaction with Beclin-1.[16,18] Vps34 also interacts with Atg14, which directs the Vps34 complex to the pre-autophagosomal membrane.[19] In vivo, the Vps34 complex phosphorylates phosphatidylinositol to form phosphatidylinositol-3-phosphate (PI3P). PI3P serves as a docking point for proteins necessary for the formation of the autophagic vacuole (Fig. 2B).

Elongation and completion of the autophagic vacuole is mediated by two ubiquitin-like conjugation pathways (Fig. 3). The first pathway consists of the Atg12-Atg5 conjugation system, which is formed by the action of the E1- and E2-like proteins Atg7 and Atg10, respectively.[20–23] The Atg12-Atg5 conjugate then interacts noncovalently with a small coiled-coil protein, Atg16L (an ortholog of Atg16 in yeast); these oligomerize to form a large multimeric Atg12-Atg5-Atg16L complex. Atg16L directs this complex to the outer autophagosomal membrane,[24] and in turn this oligomerized complex is in part responsible for creating the concave nature of the forming autophagosome membrane.

In the second ubiquitin-like pathway, LC3 (the mammalian ortholog of Atg8) is lipidated upon conjugation with phosphatidyl-ethanolamine (PE).[25] The carboxy-terminal residue of Atg8/LC3 is cleaved off by a cysteine protease, Atg4, generating the cytosolic LC3-I, which now will contain a glycine residue at the C-terminus.[26] LC3-I then conjugates to PE by the formation of an amide bond between the amino group of PE and the C-terminal glycine of LC3-I. This reaction requires the E1 protein Atg7 and the E2 protein Atg3.[25] Lipidation of Atg8/LC3 converts the soluble Atg8/LC3-I into the autophagosome associated form LC3-II. LC3-II recruits lipid molecules to expand the autophagosome membrane. When membrane elongation is completed, Atg8/LC3 is detached from PE via Atg4 and then released back to the cytosol.[26]

Recent studies suggest that these two ubiquitin-like systems regulate each other. For example, recruitment of Atg8/LC3 to the pre-autophagosomal membrane requires the Atg12-Atg5-Atg16 complex; in addition, the Atg12-Atg5 conjugate acts as an E3-like enzyme, determining the sites of Atg8/LC3 lipidation.[27,28] Conversely, the Atg8/LC3 conjugation system seems to be required for Atg16 complex formation.[29] The importance of this joint regulation is presently unclear, though it likely ensures that this self-eating survival program is tightly regulated.

There are two transmembrane proteins that are required for mammalian autophagy that aid in the function of the four complexes outlined above: mAtg9 (the mammalian homolog of Atg9 in yeast) and vacuole membrane protein 1 (VMP1). mAtg9 protein localizes to the trans-Golgi network and endosomes; during autophagy this protein localizes with GFP-LC3-positive autophagosomes.[30] In yeast, the available data suggest that Atg9 may be required for the delivery of membranes to the autophagosome.[31] In contrast, VMP1 is localized to the

plasma membrane; during autophagy this protein co-localizes with LC3 and Beclin-1, and helps recruit components of class III PtdIns3k to the phagophore.[32]

A. Sequestration of "Cargo"

Autophagy possesses both selective and non-selective degradation processes. For example, autophagy plays a role in the selective degradation of protein aggregates and polyubiquitylated proteins.[33,34] Autophagy also plays a role in the selective degradation of dysfunctional mitochondria, endoplasmic reticulum, and peroxisomes. During selective degradation, it is believed that LC3, which acts as a "receptor" at the phagophore, interacts with "adaptor" molecules on the target (e.g., mitochondria), promoting their selective delivery and degradation. The best-characterized autophagy receptor is $p62^{SQSTM1}$, a multi-functional adaptor protein that contains an LC3-interacting region (LIR) as well as a ubiquitin-association (UBA) domain, which binds to polyubiquitin molecules.[35] Mounting evidence suggests that $p62^{SQSTM1}$, along with another autophagy receptor NBR1, serve as cargo receptors for selective clearance of misfolded/polyubiquitylated proteins and damaged organelles.[35–40] The identification of such cargo receptors is ongoing, and it is highly likely that other similar receptors are yet to be identified.

B. Movement and Fusion of Autophagosomes

In yeast, successful fusion of the autophagosome with the lysosome relies on micro-tubules along with the proteins Ypt7p (the yeast homologue of Rab7),[41] Vam3p (a syntaxin homologue),[42] Sec18p (the yeast homologue of N-ethylmaleimide sensitive factor, NSF), and Vti1p (a SNARE protein).[43] Similar roles for Rab7 and Vti1p in autophagy have been demonstrated in mammalian cells.[44–46] The movement of autophagosomes in the cytosol to peri-nuclear regions, where fusion takes place, is mediated by the motor protein dynein.[47]

C. Autophagy Inducers

It is highly likely that the majority of cellular stresses induce autophagy. The best characterized inducer of autophagy is nutrient deprivation. In cultured mammalian cells, only minutes of nutrient depletion can induce autophagy, and the highest levels can be achieved when cells are cultured in the complete absence of nutrients and growth factors.[48] As mentioned briefly, the key molecular regulator of starvation-induced autophagy is the serine-threonine kinase mTOR (mammalian target of rapamycin). Depletion of insulin, insulin-like growth factor, or other growth factors is sufficient to lead to inhibition of mTOR (specifically, mTORC1, or mTOR complex 1) and induce autophagy. A key enzyme in the signaling of nutrient deprivation is AMPK, which monitors the energy status of the cell via its ability to sense the AMP:ATP ratio. Upstream kinases—such as liver kinase B1 (LKB1), calcium/calmodulin kinase kinase-β, and TGF-activated kinase-1—can activate AMPK by phosphorylation.[49] AMPK activation induces autophagy through mTORC1 inhibition, via phosphorylation of the tuberous sclerosis complex 2 (TSC2), and the regulatory associated protein of mTOR, Raptor.[50]

Although much attention has been given to autophagy regulation by mTOR, several signaling pathways seem to influence autophagy independently of mTOR. For example, Akt phosphorylates and activates another autophagy stimulator, the Forkhead box O (FoxO) transcription factor FoxO3, by an mTORC2-dependent but mTORC1-independent mechanism.[51,52] Similarly, AMPK has been shown to induce autophagy in an mTORC1-independent manner, by phosphorylating and stabilizing p27 under conditions of metabolic stress.[53] AMPK can directly facilitate autophagy by directly phosphorylating and activating ULK1.[54,55] Sirtuins are a family of NAD-dependent deacetylases that can deacetylate the key autophagy proteins Atg5, Atg7, and LC3, as well as the transcription factor Forkhead box O3a (FOXO3a).[56] Following its deacetylation, FOXO3a translocates into the nucleus

and upregulates key autophagy genes, including *ULK2*, *Beclin 1*, *VPS34*, *BNIP3*, *ATG12*, *ATG4B*, and *LC3*, thereby leading to autophagy induction.[52]

A second stress that induces autophagy is hypoxia. If oxygen concentrations fall below 5%, the HIF1 (hypoxia-inducible factor) transcriptional regulator is activated,[57,58] and this protein transactivates two key autophagy inducers, *BNIP3* and *BNIP3L* (NIX).[59] These proteins then function to activate the key autophagy complex containing the class III PI3K Vps34. Hypoxia also increases the transcription of the essential autophagy genes *LC3* and *Atg5* through the transcription factors ATF4 and CHOP, respectively.[60] Notably, there is strong evidence for co-localization of regions of hypoxia with autophagy in vivo.[61]

III. AUTOPHAGY IN NORMAL DEVELOPMENT

Recent studies indicate that transient activation of autophagy occurs directly after birth in several tissues of the mouse.[62] A critical role for autophagy in neonatal survival was shown by inactivation of the autophagy-related genes *Atg5* and *Atg7* in mice. *Atg5* and *Atg7* knockout mice were normal at birth (although they had lower body weight than controls), but they could not survive the neonatal starvation period and died within 1 day after birth. Notably, under nonsuckling conditions these mice died much earlier than wild-type mice (after 10–13 h compared to 20–22 h after birth). The concentration of amino acids in the plasma of knockout mice 10 h after birth was approximately 20% lower, compared to controls. In addition, there was no autophagosome formation in these mice.[62] The combined data suggest that autophagy is critical for survival during the neonatal starvation period when the transplacental nutrient supply is suddenly ended.

Mice deficient for either *Atg5* or *Atg7* also accumulate polyubiquitylated protein aggregates and abnormal mitochondria, indicating that this process is required for protein and organelle quality control. Interestingly, these mice also undergo neuronal degeneration with age,[63,64] suggesting that autophagy plays a role in normal aging and degeneration. Along these lines, an absence of autophagy has been hypothesized to underlie Huntington and Parkinson's diseases, due to the accumulation of misfolded proteins.[65] The liver is an organ that extensively undergoes autophagy, and the livers of conditional Atg7-deficient mice display several abnormalities, including accumulation of peroxisomes and deformed mitochondria, along with hepatomegaly.[66] In tissues of the central nervous system of *Atg7*-deficient mice, a loss of cerebral and cerebellar cortical neurons takes place and ubiquitin aggregates accumulate in axons, which leads to neurodegeneration and eventual death.[63,66] The combined data support the relevance of autophagy in nutrient deprivation, protein quality control, and aging.

IV. MONITORING AUTOPHAGY IN THE CELL

Autophagy was first detected by transmission electron microscopy. The focal degradation of cytoplasmic areas of the cell, sequestered by the phagophore (a specialized type of smooth, ribosome-free double membrane), is one of the hallmarks of autophagy. In general, the use of electron microscopy is considered an important method for the qualitative and quantitative analysis of autophagic structures, such as the phagophore, autophagosome, and the autolysosome.[67,68]

There are several methods that can be used to monitor autophagy in cells and organisms; some of these are depicted in Fig. 4. For example, the lipidated form of LC3 (LC3 II) is predominantly associated with autophagic organelles,[25,69] and can be monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with LC3 antisera, where LC3 II is the faster mobility form.[70–72] One caveat to this approach is that LC3 II is both induced and degraded during autophagy. Therefore, Western blot

detection of LC3 II must be accompanied by efforts to halt autophagic flux in the cell, such as by the use of Bafilomycin A1, hydroxychloroquine, or combinations of pepstatin A and E64d. These latter treatments inhibit the Na+/H+ pump at the lysosome, increase lysosomal pH, and inhibit the acidic lysosomal protease, respectively; all prevent the degradation of LC3 II and hence halt autophagic flux.[73]

The expression of a GFP-LC3 fusion protein, and its existence in autophagosomes, has also been used extensively to monitor autophagy in cultured cells.[69] It has recently been determined that GFP-LC3 is sensitive to acidic pH, which impedes fluorescence after autolysosome formation. For this reason, a new tandem fluorescent-tagged LC3 (tfLC3) has been developed. In this vector, LC3 is expressed tandemly fused with both GFP and RFP (red fluorescent protein); the latter is resistant to lysosomal proteolytic degradation. Co-localization of both GFP and RFP fluorescence in a cell indicates an autophagosome that has not yet fused with a lysosome, whereas vesicle-associated RFP signals denote fused autolysosomes.[74,75]

$p62^{SQSTM1}$ (sequestosome 1, and herein referred to as p62) is an adaptor molecule involved in activating autophagy that interacts with polyubiquitinated protein aggregates and targets them to autophagosomes. Because p62 is degraded along with cargo in the autolysosome, Western blotting for steady-state levels of this protein is often used as a reliable indicator of autophagy, and there is no need for cessation of autophagic flux when monitoring p62. Recent studies show that inhibition of autophagy correlates with increased levels of p62.[35,76]

Measurement of the degradation of long-lived proteins is a reliable measure of autophagic flux. In this approach, intracellular proteins are pulse-labeled for over 24 h with a radiolabeled amino acid, such as [14 C]-leucine or [14 C]-valine. Following a long (>24 h) chase period in unlabeled excess amino acid, the time-dependent release of acid-soluble radioactivity is measured by liquid scintillation counting.[77] While this assay is considered a “gold-standard” for the measurement of autophagy, the extensive time courses involved and the use of radioactivity have limited its use. Fortunately, assays that avoid the use of radioactivity have been developed, based upon the starvation-dependent accumulation of betaine homocysteine methyltransferase (BHMT).[78–81]

V. AUTOPHAGY AND CANCER

That autophagy plays a significant role in cancer initiation and progression is best evidenced by the number of proteins with roles in autophagy that also play key roles in cancer (see Fig. 5). Some of these key players in both cancer and autophagy are denoted here.

A. Beclin-1

The connection between cancer development and autophagy was not firmly established until the discovery and characterization of Beclin-1.[82–84] Beclin-1 was first identified as a Bcl-2–binding protein that is structurally similar to Atg6 in yeast and evolutionarily conserved among various species.[84–88] Beclin-1 induces autophagy by binding and activating Vps34 through an evolutionarily conserved domain between amino acids 244 and 337.[88]

Several lines of evidence indicate that Beclin-1 is a *bona fide* tumor suppressor. The *Beclin-1* locus frequently undergoes monoallelic deletion in various human malignancies, including brain tumors and ovarian, prostate, and breast cancers.[89,90] Overexpression of Beclin-1 in human breast cancer cell line MCF7 inhibits its proliferation in vitro, as well as tumorigenesis in a mouse xenograft model.[82] Moreover, in a genetically engineered mouse model with heterozygous disruption of Beclin-1, spontaneous development of lymphoma,

breast, and lung cancers, as well as hepatocellular carcinoma, occur.[87,91] The combined data firmly support the identification of Beclin-1 as a haplo-insufficient tumor suppressor (that is, that deletion of only one copy of Beclin-1 is sufficient to drive tumorigenesis). That the role of Beclin-1 in tumor suppression was related to its role in autophagy was first suggested when it was discovered that the same domain required for Beclin-1 to bind Vps34 and induce autophagy was also necessary for its tumor-suppressive activity.[88]

Several regulators of Beclin-1 have been identified to date. The two major positive regulators of Beclin-1 are UVRAG and Bax-interacting factor-1 (Bif-1). UVRAG is required for Beclin-1–mediated autophagy and enhances Beclin-1 activity by promoting its binding with Vps34.[92,93] Monoallelic deletion or mutation of UVRAG has been reported in various human cancers, including colon, gastric, and breast cancers.[94–96] Bif-1 also acts as a positive regulator of Beclin-1.[18] Similar to UVRAG, Bif-1 enhances the binding between Beclin-1 and Vps34, resulting in increased autophagy. Expression of Bif-1 is markedly reduced in human malignancies, including colon cancer, prostate cancer, urinary bladder cancer, and gastric carcinoma,[97–100] and homozygous deletion of Bif-1 was reported in some cases of mantle cell lymphoma.[101] Bif-1 knockout mice develop spontaneous tumors at a significantly higher rate compared to wild-type mice, indicating that Bif-1 is also likely to be a tumor suppressor gene. Despite the compelling nature of the evidence implicating UVRAG and Bif-1 as tumor suppressor genes, that they function in tumor suppression through autophagy has yet to be stringently proven. For example, some groups have suggested that Bif-1 suppresses tumorigenesis through its interaction with pro-apoptotic protein Bax.[102]

Two new Beclin-1 regulators have recently been identified. Rubicon and ATG14L function antagonistically upon interaction with Beclin-1.[103,104] Specifically, in a mutually exclusive manner, ATG14L induces Vps34 kinase activity and autophagy independent of UVRAG, whereas Rubicon inhibits autophagy through mediating impaired maturation of the autophagosome. Whether these regulators are altered in expression in cancer has yet to be determined.

B. Bcl2 Family

Perhaps the most important negative regulator of Beclin-1 is Bcl-2 (B-cell CLL/lymphoma-2).[105,106] Originally cloned as an anti-apoptotic gene, the role of Bcl-2 in autophagy was not realized until the identification of Beclin-1 as a Bcl-2 interacting protein. Bcl-2 inhibits Beclin-1–mediated autophagy by constitutively binding with Beclin-1 and blocking the interaction between Beclin-1 and Vps34.[107–109] For autophagy to be initiated, two possible mechanisms have been described for the release of Beclin-1 from Bcl-2. First, phosphorylation of either Beclin-1 or Bcl-2 has been shown to weaken their association and result in induced autophagy. This can occur either in the N-terminal loop of Bcl-2 or the BH3 domain of Beclin-1, mediated by c-Jun N-terminal protein kinase 1 (JNK1) or death-associated protein kinase (DAPK), respectively.[110–112] Alternatively, BH3-only proteins are hypothesized to compete with Beclin-1 for the interaction with Bcl-2, and to induce the release Beclin-1 from the inhibitory effect of Bcl-2. This latter phenomenon has been reported to occur with Bid, Bad, a BH3-mimetic compound, and the *Caenorhabditis elegans* BH3-only protein EGL-1.[109,113,114]

With the accumulation of information implicating Bcl-2 in autophagy, it was hypothesized and subsequently found that other Bcl-2 family members also play roles in this process. The Bcl-2 family includes proteins with Bcl-2 homology (BH) domains; anti-apoptotic Bcl-2 family members have four BH domains, and these include Bcl-2 and Bcl-xL. Pro-apoptotic Bcl-2 members either contain three BH domains, such as Bax and Bak, or they possess only the BH3 domain; these include Bad and Noxa. Recently, it has been reported that other anti-

apoptotic Bcl-2 family members—like Bcl-xL, Mcl-1, Bcl-w, and viral Bcl-2 homologs encoded by KSHV and γHV68—also suppress autophagy.[108,109,113–115] In addition, pro-apoptotic BH3-only Bcl-2 family members—such as Bad, Bik, Noxa, Puma, Bi-mEL, and BNIP3—all exhibit autophagy-inducing abilities.[113,116–119] Therefore, the Bcl-2 family serves as a central regulator of both the autophagy and apoptosis pathways.

C. mTOR

The research on molecular pathways regulating autophagy was brought to the forefront with the identification of the role of the target of rapamycin (TOR; or mTOR in mammalian cells).[120–122] The mTOR complex mTORC1 is best known for its role in regulating protein synthesis through two of its substrates, 4E-BP1 and p70S6K. Phosphorylation of 4E-BP1 prevents its inhibitory activity on RNA cap-binding protein eIF4F, leading to the induction of cap-dependent mRNA translation.[123] Conversely, p70S6K is activated after being phosphorylated. Activation of p70S6K results in increased protein synthesis through induced expression of proteins involved in the translational apparatus, including elongation factors and ribosomal proteins.[124,125] Moreover, activated p70S6K phosphorylates and inhibits eEF-2 kinase, which blocks elongation by phosphorylating eEF-2. Therefore, inhibition of eEF-2 kinase removes the block on elongation and promotes protein synthesis.[126]

Three cancer-relevant upstream signaling pathways have been shown to connect external stimuli to mTOR regulation: these are the PI3K-Akt pathway, the ERK-RSK-DAPK pathway, and the AMPK pathway. All of these pathways regulate mTOR activity through the tuberous sclerosis complex (TSC) 1/TSC2 complex, albeit in different ways. For example, the TSC1/TSC2 complex inhibits mTOR functions by inactivating Rheb; Rheb binds and activates mTOR only when the former is in its GTP-bound form.[127] The phosphoinositide-3-kinase (PI3K)-Akt pathway activates mTOR by Akt-mediated phosphorylation of TSC2, followed by disassembly and inhibition of the TSC1/TSC2 complex.[128,129] The extracellular signal-related kinase (ERK) stimulates mTOR activation by either directly phosphorylating TSC2 or by inducing TSC2 phosphorylation via its two downstream effectors, ribosomal S6 kinase (RSK) and DAPK.[130–132] Finally, AMP-activated protein kinase (AMPK) phosphorylates TSC2 and stabilizes the TSC1/TSC2 complex, thereby inhibiting Rheb-mediated activation of mTOR.[133] AMPK can also regulate mTOR function in a TSC-independent manner by directly phosphorylating Raptor, an essential component of mTORC1, to inhibit mTOR activity.[134] Notably, TSC1 and 2, PI3K, and Akt are all subject to mutation in human tumors.

Our understanding of the role of mTOR in autophagy is still emerging. For example, it has been unclear why p70S6K, one of the key effectors of mTORC1 signaling, is actually required for autophagy.[135,136] One potential explanation for this is that p70S6K is believed to function in a negative feedback loop, by phosphorylating and inhibiting the insulin receptor and PI3K.[137–140] It has been suggested that this negative feedback pathway might enable cells to mount a rapid autophagic response upon metabolic stress, followed by a gradual loss of p70S6K activity; this gradual loss would be predicted to prevent cell death induced by excessive autophagy.[141,142]

D. PI3K

PI3K is heavily involved in autophagy regulation, both positively and negatively. In initial studies, rat hepatocytes were treated with the PI3K inhibitors wortmannin or LY294002, with the expectation that inhibition of PI3K-mTOR–mediated p70S6K phosphorylation would induce autophagy. Somewhat surprisingly, both p70S6K phosphorylation and autophagy were blocked by these inhibitors.[143] This unexpected finding prompted the subsequent identification of distinct classes of PI3K with roles in autophagy[139]; of these,

class I PI3K (PI3KI) and class III PI3K (PI3KIII) have negative and positive roles, respectively.[144]

The effects of PI3KI and PI3KIII on autophagy depend on the products of each kinase. The product of PI3KI, phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), has inhibitory effects on autophagy mainly through the regulation of the Akt-mTOR pathway, as described above. In contrast, the product of PI3KIII is phosphatidylinositol 3-phosphate (PI3P), and this molecular is essential for autophagy inhibition. In autophagy, PI3P is believed to serve as the platform for autophagosome biogenesis. Consistent with the distinctive functions between PI3KI and PI3KIII in autophagy regulation, PTEN, an exclusive PI3KI inhibitor, only functions positively in autophagy.[145]

E. p53

Since its discovery, the tumor suppressor gene p53 has become the most well-studied cancer-related gene due to the fact that it is mutated or deleted in over 50% of human malignancies. In response to various stresses like DNA damage, oxidative stress, and oncogene activation, p53 acts as the guardian of tumorigenesis by inducing cell cycle arrest or apoptosis. Several recent studies have added autophagy to the impressive résumé of molecular events regulated by p53. Interestingly, p53 has been described as both a positive and a negative regulator of autophagy, depending upon the cell type and the stresses that cells are experiencing.

Upon treatment of mouse embryo fibroblasts with the DNA damaging agent etoposide, p53 mediates the activation of AMPK and thus the inhibition of mTOR and induction of autophagy.[146] In addition, activation of p53 by genotoxic stress causes the trans-activation of two p53-target genes, Sestrin 1 and 2, both of which are negative regulators of mTOR activity.[147,148] Another p53 target gene, DRAM (damage-regulated autophagy modulator), is a lysosomal membrane protein that is also induced by p53 to facilitate autophagy upon genotoxic stress.[149]

Seemingly contradictory to the aforementioned autophagy-inducing activity of p53, this protein has also been reported to inhibit autophagy. Specifically, inhibition of p53 by chemical inhibitors, siRNA, or genetic deletion was found to increase the basal level of autophagy. In this case, evidence was found that cytosolic, and not nuclear, p53 played this negative regulatory role.[150,151] This phenomenon was also observed with certain oncogenic p53 mutants that are preferentially localized in the cytoplasm, suggesting a connection between p53-mediated inhibition of autophagy and tumorigenesis.[152] Alternatively, because the p53 tumor suppressor negatively regulates the ARF tumor suppressor, it has also been suggested that silencing or inhibiting p53 induces autophagy because this leads to increased expression of ARF.[153]

F. ARF

The ARF tumor suppressor ($p14^{ARF}$ in humans, $p19^{ARF}$ in mice) plays a positive role in the induction of autophagy. This was first found with studies of a small-molecular-weight version of ARF (termed “smARF”), which was reported to traffic to mitochondria and induce autophagy.[154] Subsequently, other groups reported that full-length ARF was likewise capable of trafficking to mitochondria and inducing autophagy.[155–157] The mechanism for ARF-induced autophagy is by virtue of its ability to directly interact with Bcl-xl, and to limit the ability of Bcl-xl to bind and inhibit Beclin-1.[156] Notably, and consistent with a role for autophagy in survival, tumors with ARF expressed at high levels were found to survive nutrient depletion in a superior manner, and ARF levels were found, in p53-null tumors, to confer increased transformed properties to tumor cells in vivo.[153]

G. Other Regulators of Autophagy With Roles in Cancer

Overexpression of DAPK, a serine/threonine kinase, has been shown to induce the formation of autophagic vesicles.[158] DAPK has also been identified as a positive autophagy mediator in *C. elegans*.[159] Mechanistically, DAPK is believed to mediate the induction of autophagy partly through releasing Beclin-1 from the inhibitory effects of Bcl-2.[112] Interestingly, DAPK has also been shown to induce mTOR signaling by phosphorylating TSC2, which would be predicted to lead to inhibition of autophagy.[132] Further studies will be required to understand how DAPK balances these two opposing effects in autophagy regulation.

The first core component of the autophagy machinery that has been identified as a tumor suppressor gene is Atg4C.[160] Atg4C is the cysteine protease that mediates the formation of the autophagosome; mice with homozygous deletion of the Atg4C gene are more vulnerable to chemical-induced fibrosarcomas, along with reduced starvation-induced autophagy, providing a connection between Atg4C's abilities to induce autophagy and to suppress tumorigenesis.[161]

VI. AUTOPHAGY IS TUMOR SUPPRESSIVE

That autophagy plays a key role in cancer is not disputed. There are, however, some controversies regarding whether autophagy is tumor suppressive or tumor promoting. The difference seems to be in the stage of cancer: autophagy clearly suppresses the initiation and development of tumors. However, it is also a key survival pathway in response to stress, and many established tumors require autophagy in order to survive. Evidence that autophagy is tumor suppressive, and that it can promote the survival of established tumors, is presented here.

Even before the association between cancer and autophagy was made, it was noted that proteolysis, one of autophagy's hallmarks, is reduced in transformed cells.[162] Autophagy was later found to be reduced in cancer tissues compared with their normal counterparts, suggesting that autophagy is tumor suppressive.[163] Later, with the identification of Beclin-1 as both a autophagy mediator and tumor suppressor,[82] the premise that autophagy is suppressive to tumor development became solidified. Since then, a number of oncogenes/oncogenic pathways and tumor suppressors have been found to play critical roles in the regulation of autophagy (Table 1). Consistent with the hypothesis that autophagy is tumor suppressive, most of the oncogenes on this list are potent inhibitors of autophagy, whereas most of the tumor suppressors positively regulate this process. In support of this premise, many core autophagy genes, including Beclin-1, UVRAG, and Bif-1, can be found mutated in human tumors.[16,18,89,91] Moreover, a recent study identified a high incidence of frameshift mutations in Atg2B, Atg5, Atg9B, and Atg12 in human gastric and colon cancer samples with microsatellite instability.[164] The combined data firmly support the role of autophagy in tumor suppression. To date, at least three mechanisms have been described to explain the role of autophagy in tumor suppression: 1) inhibition of necrosis-mediated inflammation, 2) maintenance of genome integrity, and 3) autophagy-mediated cell death and senescence.

A. Inhibition of Necrosis-Mediated Inflammation

Upon oncogene activation, a highly inflammatory microenvironment is often created that favors tumor development because of elevated oxidative stress.[165] Similarly, inhibition or inactivation of autophagy in apoptosis-deficient cancer cells was shown to induce an inflammatory response due to enhanced necrotic cell death along with increased secretion of pro-inflammatory factors, such as high mobility group 1 (HMGB1) protein; this increased inflammatory response led to increased tumor growth.[166] This hypothesis is consistent with

the findings that autophagy deficiency is associated with inflammation-related disease. For example, Atg26L1 deficiency is a risk factor for Crohn's disease, as is Atg5 deficiency.[167] Moreover, autophagy is important for the disposal of aggregated mutant alpha-1-antitrypsin, which causes chronic inflammation and diseases in the lungs and the liver due to its aggregation in the endoplasmic reticulum of hepatocytes.[168,169] This is also consistent with the finding that the loss of Beclin-1 results in failure of autophagy-mediated protein quality control and cancer promotion mediated by accumulation of p62.[61]

B. Maintenance of Genome Integrity

The heterozygous loss of Beclin-1 or loss of Atg7 in the mouse liver causes aberrant accumulation of p62 and other polyubiquitylated protein aggregates.[35] Similarly, in mice deficient for Atg5 or Atg7 in the brain, misfolded and aggregated proteins, along with dysfunctional mitochondria, accumulate.[63,64,170] White et al. have found that, prior to the onset of tumorigenesis, a major event accompanying aberrant accumulation of p62 and impaired organelle disposal in autophagy-deficient cells is DNA damage and chromosomal instability, indicated by DNA double-strand breaks, centrosome abnormalities, and/or increased DNA content.[166,171,172] The link between the failed elimination of aggregated/misfolded proteins and dysfunctional mitochondria with the loss of genomic stability has been suggested to be increased oxidative stress due to increased reactive oxygen species (ROS). In support of this premise, in Beclin-1–deficient tumor-prone cells, p62 accumulation leads to the production of high levels of ROS and DNA damage, which can be reversed by knocking down p62 or introducing ROS scavengers.[61]

C. Autophagy and Oncogene-Induced Senescence

Senescence is a potent tumor-suppressive mechanism that forces oncogene-expressing cells to permanently exit the cell cycle. Autophagy is activated during oncogene-induced senescence,[173] and a subset of autophagy-related genes are upregulated as well. Moreover, inhibition of autophagy delays the senescence phenotype.[173] The detailed mechanism of autophagy-mediated senescence is still unclear, and its significance to tumor suppression remains to be determined. Notably, autophagy-mediated cellular senescence has also been described in biliary epithelial cells and in a plant model organism, *Arabidopsis thaliana*.[174,175]

D. Autophagy-Mediated Cell Death

There are some researchers who contend that autophagy might suppress tumorigenesis by inducing cell death; in some instances this is mediated by the accumulation of ROS. This cell death can be blocked by knocking down key autophagy proteins like Atg7 or Atg8, and has been shown to be caspase independent.[176] In vivo evidence connecting autophagy and cell death can be traced back to reports of the formation of autophagic vacuoles during the destruction of larval tissues in early *Drosophila* salivary gland development.[177,178] This connection was confirmed recently, and an engulfment receptor, Draper, was identified as an important regulator to steer autophagy toward cell destruction instead of survival.[179,180] Interestingly, autophagy-mediated cell death has also been suggested to be instrumental in the progression of neurodegenerative diseases like Alzheimer's and Parkinson's disease.[181,182] It should be noted, however, that there is considerable controversy with the premise that autophagy induces cell death, and to date there is no concrete evidence to suggest that autophagy induces, as opposed to accompanies, cell death. Therefore, whether autophagy inhibits tumorigenesis by inducing programmed cell death remains a highly debated subject.[64,183]

E. Other Mechanisms for Tumor Suppression by Autophagy

Other mechanisms for autophagy-mediated tumor suppression, including autophagy-regulated immunosurveillance and autophagy-inhibited angiogenesis, have also been suggested. It was known early on that autophagy can be induced by immune factors like interferon gamma (IFN-γ), and autophagy-mediated innate immunity is important for protection against infection of viral and bacterial pathogens.[83,184–187] It was only recently that autophagy was found to also play an important role in modulating adaptive immunity, which has more implications in preventing tumorigenesis by increasing tumor antigen presentation. Autophagy has been shown to facilitate both MHC class I and class II antigen presentation.[188,189] More importantly, a recent study showed that autophagy is essential for cross-presentation of melanoma-cell antigens by dendritic cells in vitro and in vivo, with autophagosome as the antigen carrier.[190] This is the first direct evidence supporting the potential of autophagy to induce immunosurveillance against tumor cells. Interestingly, autophagy was also found to enhance MHC class II presentation of Epstein-Barr virus antigen-1 (EBNA1), an essential protein for the latent infection of Epstein-Barr virus (EBV), a prominent oncogenic virus.[191] This finding presents an unconventional way for autophagy to suppress tumorigenesis by controlling the infection of tumor-inducing viruses.

It has also been proposed that autophagy can inhibit cancer development by blocking angiogenesis. Neuropilin 1, a positive regulator of vascular endothelial growth factor (VEGF) signaling, is degraded by autophagy under metabolic stress.[192] Inhibition of angiogenesis and tumor growth was also observed in vitro and in vivo with the treatment of mTOR inhibitors, which are known to induce autophagy.[193,194]

VII. AUTOPHAGY CAN PROMOTE TUMORIGENESIS

Whereas accumulated data suggest that autophagy can inhibit tumorigenesis, there are also data indicating that it can promote cellular transformation and that low levels of autophagy may be necessary for tumor cell survival. Recently, it was found that autophagy promotes Ras-induced transformation. Specifically, autophagy-deficient mouse embryo fibroblasts were found to display decreased soft agar colony formation in response to transformation with Ha-Ras (V12); similar findings were made in immortalized mouse kidney cells.[195,196] Intriguingly, autophagy deficiency did not affect the proliferation of nontransformed cells, suggesting that autophagy may possess a unique role in facilitating the proliferation of Ras-transformed cells. These researchers found that autophagy enhanced the glycolytic capacity of Ras-transformed cells[195]; similarly, other groups showed that autophagy was necessary for efficient oxygen consumption and metabolism in cancer cells.[196] In summary, basal levels of autophagy were absolutely required in Ras-transformed cells for efficient metabolism and survival. Therefore, while reducing the levels of autophagy in a nontransformed cell may make the cell conducive for transformation, basal levels of autophagy seem to be required for the proliferation and survival of cancer cells.

VIII. AUTOPHAGY INHIBITION LEADS TO CELL DEATH

Autophagy is frequently down-regulated in many tumors, primarily due to the fact that the PI3K-Akt-mTOR axis is constitutively activated in most tumors. This renders tumor cells with critically low levels of this pathway, and hence increased sensitivity to metabolic stress and/or chemical inhibitor of autophagy. Along these lines, the loss of autophagy promotes sensitivity to metabolic stress in apoptosis-impaired tumor cells.[166,197] In addition, inhibition of autophagy is known to promote cell death. In the liver, the induction of cell death in response to autophagy inhibition was shown to rely on the accumulation of the cargo sequestration protein p62, as the silencing of p62 rescued the toxic effects of autophagy inhibition.[35] When analyzed further, it was found that autophagy-deficient cells

accumulate aggregates of p62; these aggregates bind and inactivate the protein Keap1, which normally targets the transcription factor Nrf2 for degradation. Inactivation of Keap1 frees the Nrf2 transcription factor to transactivate genes involved in the oxidative stress response. Notably, silencing of Nrf2 protected these autophagy-deficient cells from death.[198] A similar study showed that aggregated p62 can inactivate the pro-inflammatory and pro-survival transcription factor nuclear factor-kappa B (NF-κB).[61] Therefore, while autophagy is tumor suppressive, this pathway remains at low levels in human tumors, rendering these cells very sensitive to inhibitors of the autophagy pathway.

There are considerable data indicating that tumor cells use autophagy for survival in response to cytotoxic agents. Increases in autophagy have been observed after many anti-neoplastic treatments, including the DNA alkylating agent temozolomide,[199] the selective estrogen receptor modulator tamoxifen,[200] and radiation.[201] Autophagy in these instances is considered a tumor protective mechanism, as it allows recycling of protein and damaged cellular components during the cancer treatment, ultimately yielding tumor survival. Indeed, inhibition of autophagy seems to be a viable treatment strategy for tumors that have escaped the cytotoxic effects of anti-apoptotic therapies.[197,201,202] In addition, autophagy inhibitors could target the specific subpopulations of tumor cells that are most difficult to treat: the populations within the hypoxic and nutrient-scarce regions of the tumor that are particularly resistant to radiation and chemotherapeutic treatments and most likely to be associated with recurrence or metastasis. The best data implicating autophagy as a survival pathway for tumor cells, particularly in response to treatment with cytotoxic agents, are that inhibitors of autophagy frequently synergize with these agents.

A. Autophagy Inhibition for Cancer Therapy: Chloroquine

Chloroquine (CQ) and its derivative hydroxychloroquine impair lysosomal function by altering lysosomal pH; these drugs thereby inhibit the lysosome-dependent degradation of autophagosomes.[203,204] Thompson et al. were the first to demonstrate that CQ inhibited tumor growth and prolonged the tumor refractory period in a B-cell lymphoma model in the mouse.[205] The mechanism by which CQ inhibited the tumor growth was inhibition of autophagy, which was confirmed by observation of accumulating autophagosomes by electron microscopy (EM) and by genetic knockout of the essential autophagy gene, *Atg5*. Notably, combining CQ with the DNA alkylating agent cyclophosphamide significantly increased the rate of tumor regression and, more importantly, significantly delayed tumor recurrence.[205] Coincidentally, CQ prophylaxis against malaria in Africa was found to result in a significant reduction in the number of Burkitt's lymphoma cases.[206]

Inhibition of autophagy with CQ enhances the apoptotic activity of the histone deacetylase inhibitor, SAHA.[207] The combination of autophagy inhibition with SAHA treatments was effective in murine B-cell lymphoma models and in primary cells of chronic myeloid leukemia (CML) patients that were refractory to imatinib. Importantly, the combination treatment of CQ and SAHA was selective for malignant cells even when p53 function was compromised. The ability of CQ to enhance the anti-tumor activity of SAHA was linked to generation of ROS, modulation of cathepsin D, and down-regulation of thio-redoxin, ultimately leading to ROS-mediated cell death.[207]

More recently, a phase III clinical trial of glioblastoma multiforme utilized hydroxychloroquine (HCQ) treatments in adjuvant settings, following surgery, chemotherapy, and radiation treatments. In a small cohort, patients receiving HCQ had a markedly better median survival compared to placebo-treated patients.[208] Currently, there is a number of clinical trials that utilize CQ, either as a single or combination treatment for various malignancies (see Table 2).

B. Other Autophagy Inhibitors

Heat shock proteins (HSPs) function as molecular chaperones to regulate protein folding, intracellular protein trafficking, and re-folding of misfolded proteins. Elevated levels of these chaperones have been found in a number of cancers including gastric, breast, endometrial, ovarian, colon, lung, and prostate.[209] While not considered *bona fide* oncoproteins, overexpression of Hsp70 contributes to oncogenesis in vitro[210] and in vivo.[211] Pharmacological inhibition of Hsp70 by the small molecule inhibitor 2-phenylethynesulfonamide (PES) is selectively cytotoxic to tumor cells, due to the fact that they overexpress Hsp70 and generally experience increased proteotoxic stress.[212] PES-mediated toxicity is associated with protein aggregation and impairment of lysosomal function, resulting in inhibition of autophagy. Notably, like CQ, PES is effective at inhibiting lymphomagenesis in a preclinical mouse model of Burkitt's lymphoma.[212]

IX. AUTOPHAGY INDUCERS IN CANCER THERAPY

While the general consensus is that autophagy inhibition is an effective strategy for cancer therapy, some drugs that are being used in the clinic induce autophagy. In most cases, however, it has not been proven that these drugs induce death via the autophagy pathway. Indeed, for many of these drugs it is hypothesized that combining them with autophagy inhibitors may improve efficacy. A list of chemotherapeutic agents that induce autophagy is provided in Table 3.

Rapamycin is a macrolide antibiotic that inhibits the mTOR complex by binding to FK-binding protein 12. Although initially approved by the US Food and Drug Administration as an immunosuppressant, rapamycin and its derivatives are currently being used in clinical trials for the treatment of various malignancies. Initially, the effectiveness of mTOR inhibitors was solely attributed to inhibition of the PI3K-Akt-mTOR axis; however, some reports suggest that the autophagy pathway may be involved in cell death induced by mTOR inhibitors. Specifically, the mTOR inhibitor RAD001 was found to sensitize prostate tumor cells to cell death by radiation, and this increase in cell death was associated with the induction of autophagy.[213]

Arsenic trioxide is an efficient agent for the treatment of newly diagnosed or relapsed acute promyelocytic leukemia (APL) and it can be used as a single agent with minimal myelotoxicity.[214] Treatment of tumor cells with this drug leads to autophagy.[215] In malignant glioma models, arsenic trioxide induced autophagy followed by non-apoptotic cell death.[216,217] Recently, a study investigated the synergistic effects of ionizing radiation and arsenic trioxide in human osteosarcoma models. The combination of radiation with arsenic trioxide increased G2/M arrest with subsequent cell death.[218] It should be noted that it is not clear if cell death by arsenic trioxide occurs via, or is accompanied by, autophagy.

2-Deoxy-glucose (2-DG) is an agent commonly used in cancer imaging that resembles glucose but cannot undergo glycolysis in the cell. Recently it was found that 2-DG induces autophagy in prostate cancer cell lines and in the cells of patients treated with this compound[219,220]; in tumor cell lines this autophagy was dependent on Beclin-1. Interestingly, rather than enhancing cell death by 2-DG, autophagy was found to function as a survival mechanism in treated cells. Therefore, the use of 2-DG for cancer therapy, possibly in combination with autophagy inhibitors, is currently being investigated.

X. SUMMARY

Autophagy is a critical pathway for the survival of tumor cells. However, whether some tumor types are more sensitive to autophagy inhibition remains to be determined. It will be

especially important to determine if autophagy inhibitors are more effective when tumor cells suffer particular oncogenic insults; for example, if autophagy inhibition is cytotoxic for Ras-transformed tumor cells, but not Myc-transformed cells. The search for genes or single nucleotide polymorphisms that impact the sensitivity of tumor cells to autophagy inhibitors should start with the NCI 60 panel of cell lines; such data could provide researchers with clues as to which cancer(s) could and should be treated with autophagy inhibitors. In addition, it will be important to determine if autophagy inhibitors synergize with all, or some, conventional cytotoxic anti-cancer drugs.

The role of autophagy in tumor cell death remains unclear; specifically, at present it is not clear if autophagy accompanies, or induces, cell death by anti-cancer agents. And if tumor cells treated with autophagy inducers like mTOR inhibitors do not require autophagy for cell death, it will be important to determine what pathway they die from: necrosis, necroptosis, or some other pathway. Addressing the issue of whether cells die from autophagy will require the use of more sophisticated markers of autophagy than the mere presence of autophagosomes. Finally, there is dire need for new inhibitors of autophagy; the side effects associated with CQ may prohibit its use. Because autophagy pathways use several enzymes unique to this process, including ULK1, PI3K III, and Atg4, it seems likely that identifying inhibitors of these enzymes will prove productive for future anti-cancer therapies.

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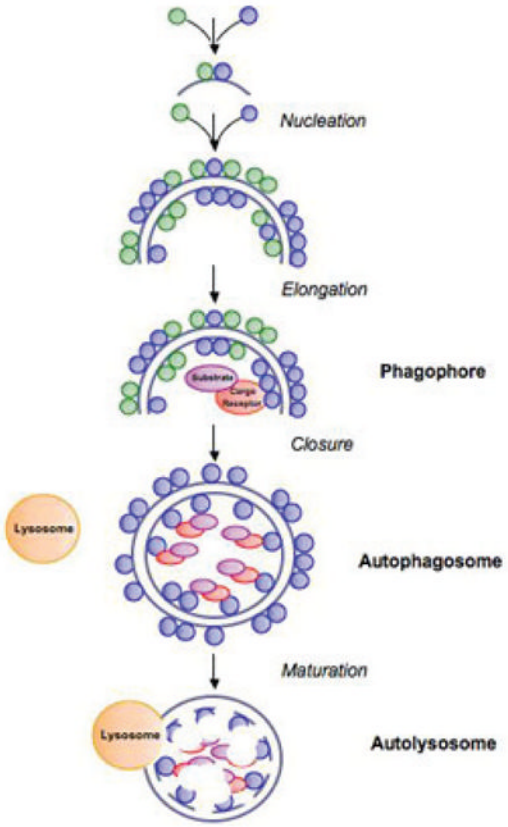

FIGURE 1.

Schematic depiction of the steps of autophagy in mammalian cells. Mammalian autophagy is initiated by the formation of the phagophore (at the nucleation step). ULK1 and class III PtdIns3K complexes are required for this step. During elongation and expansion of the phagophore membrane, Atg12-Atg5 conjugates interact noncovalently with Atg16L, and this complex acts as an E3-like enzyme, determining the sites of Atg8/LC3 lipidation. Substrates are recruited to the inner surface of the growing phagophore by binding to the cargo receptor. At the end of the elongation step, a portion of the cytosol is sequestered into a double-membrane vesicle, termed the autophagosome. The autophagosome fuses with the lysosome, thereby forming the autolysosome, where the substrates are degraded by acid hydrolases.

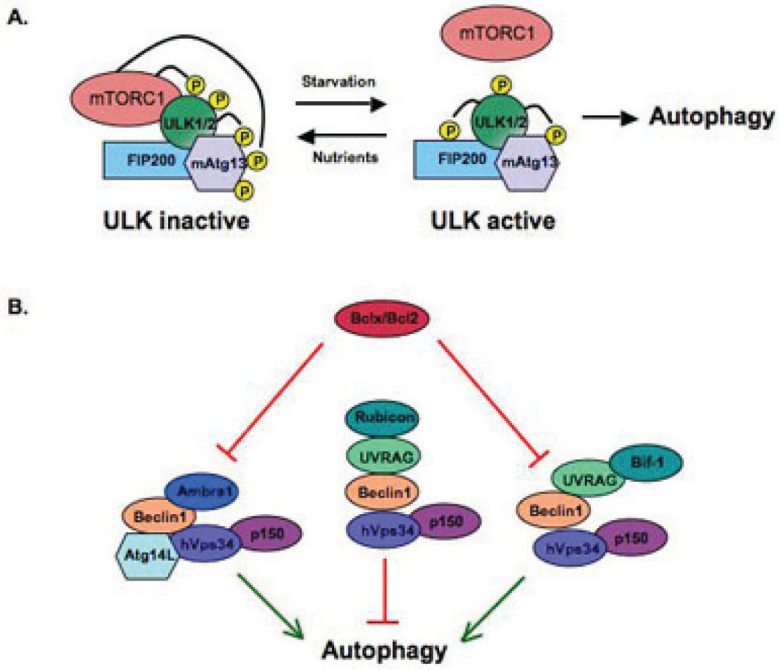

FIGURE 2.

Master molecular regulators of autophagy. (A) The mammalian complex ULK1/2-mAtg13-FIP200 is required for autophagy. mTORC1 acts as a negative regulator of autophagy by phosphorylating Atg13 and ULK1/2. During starvation, mTORC1 is released from this complex resulting in dephosphorylation of the components and activation of ULK1 and ULK2. (B) The Beclin-1/Vps34/Atg14L complex. This complex has three forms. The Atg14L (Atg14L–Beclin1–hVps34–p150) and UVRAG-Bif-1 (UVRAG–Beclin1–hVps34–p150) complexes induce autophagy. In contrast, the Rubicon complex (Rubicon–UVRAG–Beclin1–hVps34–p150) negatively regulates autophagy. Bcl-xl/Bcl-2 binds to Beclin-1, disrupting its association with hVps34, thereby inhibiting autophagy.

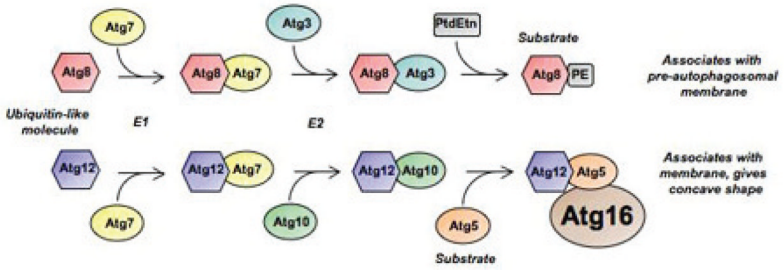

FIGURE 3.

Ubiquitin-like conjugation systems that play roles in the early steps of autophagy. Early steps in autophagosome formation are regulated by two ubiquitin-like conjugation systems. In both cases, a ubiquitin-like protein (Atg8 and Atg12) is conjugated to an E1-like enzyme (Atg7), and then to an E2-like enzyme (Atg3 and Atg10). These are then used to form phosphatidylethanolamine (PE) conjugates of Atg8 (LC3 II) as well as protein conjugates of Atg5/12/16.

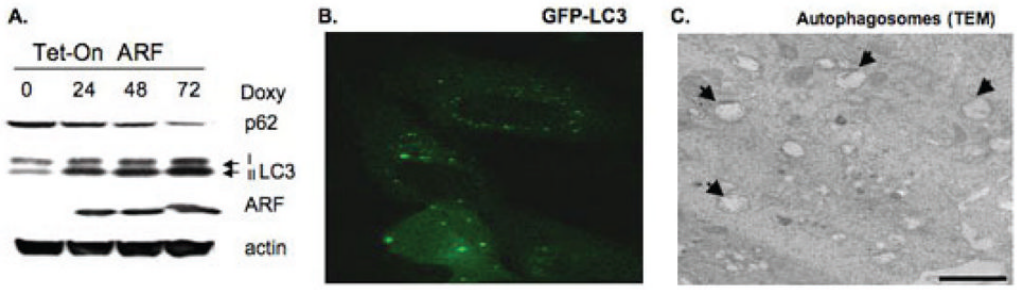

FIGURE 4.

Assays for autophagy. (A) Western blot analysis of tetracycline-inducible p19ARF cells treated with doxycycline for 24 h. The upper band represents p62, which is degraded following ARF-induced autophagy. The bottom LC3 band (LC3 II) represents the form that accumulates during autophagy. (B) Tetracycline-inducible p19ARF cells were transiently transfected with GFP-LC3 for 24 h and treated with doxycycline for 24 h, followed by confocal microscopy to detect GFP-labeled LC3, which is localized to vacuoles (autophagosomes). (C) Transmission electron microscopy of serum-starved H1299 cells. Arrows depict double membrane autophagosomes. The scale bar is 500 nm.

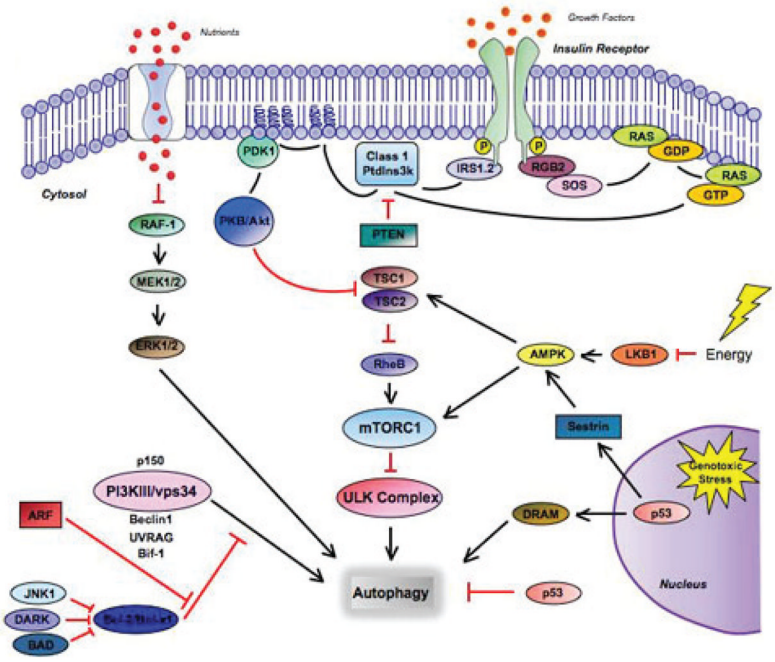

FIGURE 5.

Signaling pathways that regulate mammalian autophagy. Autophagy is regulated by a complex signaling network of various stimulatory (arrowheads) and inhibitory (bars) inputs. Stimulation of the class I PtdIns3K complex and small GTPase Ras (via activation of growth factor/insulin receptors) leads to activation of the PtdIns3K-PKB-TOR pathway. PKB phosphorylates and inhibits the tuberous sclerosis complex TSC1/TSC2, which activates mTORC1 through Rheb-GTPase stabilization, causing inhibition of autophagy. Amino acids inhibit the Raf-1-MEK1/2-ERK1/2 signaling pathway, acting as a negative regulator of autophagy. Metabolic stress, such as high AMP/ATP ratios, leads to phosphorylation and activation of AMP-activated protein kinase (AMPK) by LKB1. AMPK then activates TSC1/TSC2, leading to mTORC1 inactivation and autophagy induction. Following genotoxic stress, nuclear p53 causes autophagy induction via transactivation of AMPK subunits or upregulation of the lysosomal autophagy protein DRAM. Cytosolic p53 has an inhibitory effect on autophagy. JNK1, DAPK, BAD, and p19ARF all function to disrupt the association between BCL-2/Bcl-xl and Beclin-1, facilitating Beclin-1-class III PtdIns3K complex formation and thereby promoting autophagy.

TABLE 1

Oncogenes and Tumor Suppressor Genes That Impact Autophagy

Genes	Oncogenic or tumor suppressive	Autophagy inducer or inhibitor	Reference
Beclin 1	Tumor suppressive	Inducer	82–84, 87–91
UVRAG	Tumor suppressive	Inducer	92–96
Bif-1	Tumor suppressive	Inducer	18, 97–102
BH3-only Bcl-2 family	Tumor suppressive	Inducer	109,113,114,116–119
DRAM	Tumor suppressive	Inducer	149
LKB1	Tumor suppressive	Inducer	49,133
PTEN	Tumor suppressive	Inducer	145
TSC1/TSC2	Tumor suppressive	Inducer	127–129
ATG4C	Tumor suppressive	Inducer	160,161
ARF	Tumor suppressive	Inducer	153–157
P53	Tumor suppressive	Inducer/inhibitor	146–152
DAPK	Tumor suppressive	Inducer/inhibitor	112,132,158,159
Antiapoptotic Bcl-2 family	Oncogenic	Inhibitor	108,109,113–115
mTORC1	Oncogenic	Inhibitor	8–14,120–126,135
PI3KI	Oncogenic	Inhibitor	128,129,139,143,144
Akt	Oncogenic	Inhibitor	128,129
ERK	Oncogenic	Inhibitor	130–132
Bcl-2	Oncogenic	Inhibitor	105–109

TABLE 2

Clinical Trials Utilizing Chloroquine (CQ) and Hydroxychloroquine (HCQ)

Clinical trial.gov identifier	Condition	Drug	Phase	Sponsor
NCT00933803	Non-small cell lung cancer	HCQ, paclitaxel, carboplatin, bevacizumab	I/II	University of Medicine and Dentistry, New Jersey (UMDNJ); Cancer Institute of New Jersey (CINJ)
NCT01266057	Advanced cancer	HCQ, sirolimus, vorinostat	I	MD Anderson Cancer Center
NCT01273805	Pancreatic cancer	HCQ	II	Dana Farber Cancer Institute, Brigham and Women's Hospital, Massachusetts General Hospital
NCT01006369	Colorectal cancer	HCQ, bevacizumab XELOX regimen, capecitabine Oxaliplatin	II	CINJ, National Cancer Institute (NCI)
NCT00809237	Non-small cell lung cancer	HCQ, gefitinib	I/II	National University Hospital-Singapore, Massachusetts General Hospital, AstraZeneca
NCT00909831	Unspecified; adult solid tumor, protocol specific	HCQ, temsirolimus	I	University of Pennsylvania, NCI
NCT00714181	Unspecified; adult solid tumor, protocol specific	HCQ, temozolomide	I	University of Pennsylvania, NCI
NCT00786682	Prostate cancer	HCQ, docetaxel	II	UMDNJ, NCI
NCT01206530	Rectal, colon, metastasis, adenocarcinoma	HCQ, oxaliplatin, leucovorin, 5-FU, bevacizumab	I/II	Abramson Cancer Center of the University of Pennsylvania
NCT00726596	Prostate cancer	HCQ	II	UMDNJ, NCI
NCT01013737	Advanced solid tumors	HCQ, vorinostat	I	The University of Texas Health Sciences Center at San Antonio, Merck
NCT00977470	Non-small cell lung cancer	HCQ, erlotinib	II	Massachusetts General Hospital, Dana Farber Cancer Institute, Beth Israel Deaconess Medical Center, Genentech
NCT00486603	Brain and central nervous system tumors	HCQ, temozolomide	I/II	NCI
NCT01128296	Pancreatic cancer	HCQ, gemcitabine	I	University of Pittsburgh, NIH
NCT00568880	Multiple myeloma and plasma cell neoplasm	HCQ, bortezomib	I/II	University of Pennsylvania, NCI
NCT01144169	Renal cell carcinoma	HCQ	I	NIH
NCT01292408	Breast cancer	HCQ	II	Radboud University
NCT00962845	Melanoma	HCQ	I	CINJ, NCI
NCT01227135	Leukemia	HCQ, imatinib mesylate	II	University of Glasgow
NCT00771056	B-cell chronic lymphocytic leukemia	HCQ	II	North Shore Long Island Jewish Health System
NCT00969306	Small cell lung cancer	CQ, A-CQ 100	I/II	Maastricht Radiation Oncology, Maastricht University Medical Center, NCI
NCT01023477	Ductal carcinoma in situ (DCIS)	CQ tamoxifen	I/II	Inova Health Care Services George Mason University, Department of Defense, US Army Medical Research and Material Command

TABLE 3

Clinical Trials Utilizing Drugs That Impact Autophagy”

Agent	Target
Tamoxifen	Estrogen receptor
Temozolomide	DNA
γ-irradiation	DNA
SAHA	HDAC
Hyperthermia	Unknown
Arsenic trioxide	Mitochondria
Resveratrol	Multiple
Rapamycin	mTOR
Temsirolimus	mTOR
Everolimus	mTOR
2-deoxy-D-glucose	Glycolysis
Endostatin	Angiogenesis
Sorafenib	TKI, VEGFR
Lonafarnib	Farnesyltransferase