

# Autophagy: Can it become a potential therapeutic target? (Review)

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**Abstract.** Autophagy is a cellular lysosomal degradation pathway involved in proteins and organelles recycling for promoting cell survival, development and homeostasis. It is a multistep process and genetic studies have identified many proteins that participate in autophagosome formation and fusion with lysosomes, and various signaling factors that associate with the regulation of autophagy. In general, autophagy acts as a cell protector and its dysfunction is correlated with diverse pathologies, such as neurodegeneration, liver, heart and muscle diseases, cancer, inflammation and ageing. However, its role in cell death increases the complexity of the autophagic degradation system. A broad understanding of autophagy, ranging from detailed processes, including induction, formation and degradation, to function in physiology and pathology, revealed by accumulating studies, may be helpful for formulating therapeutic strategies for autophagy-associated human diseases.

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## 1. Introduction

Autophagy is a process of cellular self-digestion that is essential for survival, differentiation, development and homeostasis. Misfolded proteins, superfluous or damaged organelles, and invading microorganisms are digested by lysosomal autophagic-degradation, generating intracellular nutrients and energy for recycling. Several autophagy related genes have been identified in diverse organisms from single cell yeast to multicellular worms, plants, flies and mammals. Most of the genes in yeast are highly homologous with those in higher organisms, suggesting that autophagy is an evolutionary conserved function (1). Based on the physiological function and mechanism, there are at least three types of autophagy, microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). Microautophagy and macroautophagy deliver large structures (e.g. organelles, invasive microbes or bulk cytoplasm) to lysosomes through both selective and non-selective manner, whereas CMA only delivers unfolded substrate protein in a selective manner, chaperone-mediated degradation (2,3). Macroautophagy (hereafter referred to as autophagy) is the most studied form of autophagy, and is the principal basis of this review. Population studies show the complicated roles of autophagy in human health and diseases. A basal level of autophagy is required for cell survival and cellular homeostasis; a disruption of the basal expression level, too little or too much autophagy, may result in certain diseases. Understanding autophagy genesis and its physiological, as well as its pathological functions may provide a rationale for the treatment of autophagy-related diseases in the future and will promote further progress in this field.

## 2. How autophagy happens

*The process of autophagosome maturation and related genes.* Nutrient deficiency, hormonal factor withdrawal and/or microbial pathogen infection induces autophagy, in which the isolation membranes, autophagosomes, and autolysosomes are formed in order. Atg proteins encoded by AuTophagy-related (Atg) genes, which have been thoroughly investigated in yeast, are involved in the process of autophagy (4).

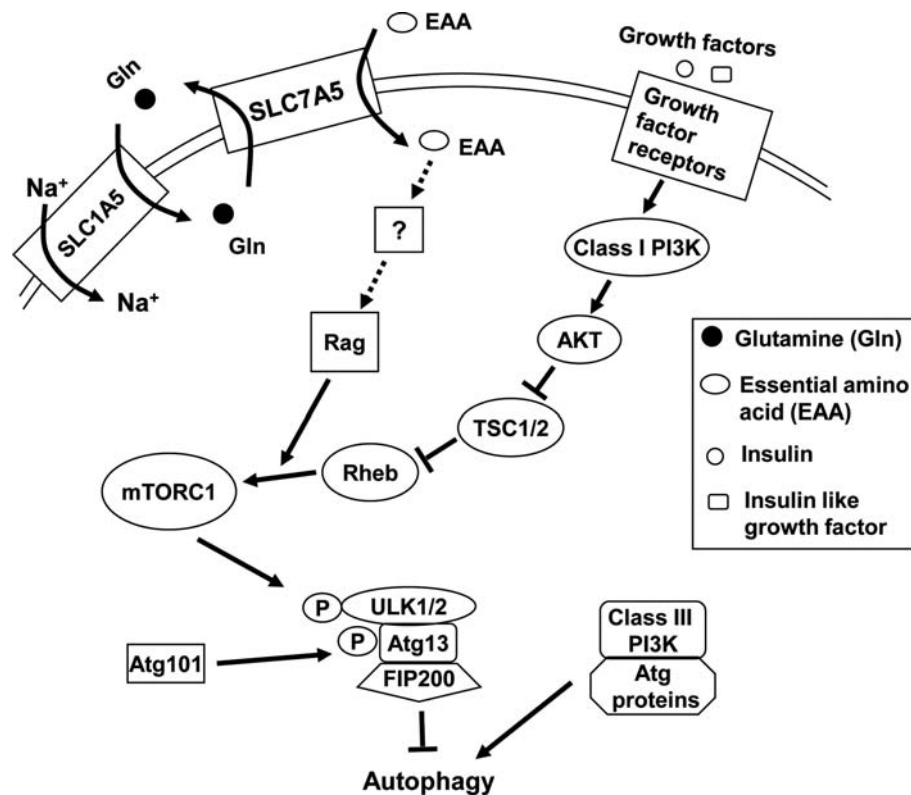


Figure 1. Regulation of autophagy induction. Basal expression of autophagy is enhanced by environmental stimuli including amino acids, growth factors and microbial pathogens. Essential amino acids are transported into the cytoplasm in a glutamine/ $\text{Na}^+$ -dependent manner. Intracellular amino acids influence unknown factors that activate mTORC1 through the Rag GTPases. Growth factors stimulate the class I PI3K-Rheb pathway and then activate mTORC1. mTORC1 inhibits autophagy via the ULK1/2-Atg13-FIP200 complex, the direct downstream target of mTORC1. Atg 101 is required for the stability and basal phosphorylation of Atg13 and ULK1. Furthermore, class III PI3K is an activator of autophagy. Dashed lines indicate possible regulatory effects.

**Autophagy induction.** Autophagy is tightly regulated during development (Fig. 1). The presence of extracellular rich nutrients (amino acids) and growth factors (i.e. insulin, insulin-like growth factor) shut off autophagy through the class I phosphatidylinositol 3-kinase (PI3K) (5) and target of rapamycin (TOR) pathways (6). TOR complex 1 (TORC1) performs a key role in mammals during autophagy and it is active in normal growth conditions as a negative regulator of autophagy. Amino acids and especially the essential amino acids are required for its activation. The pathway by which the extracellular amino acids are transported intracellularly, subsequently leading to the activation of mTORC1 has been identified by Nicklin *et al* (7). Extracellular L-glutamine, non-essential amino acid, is transported into cells in a sodium ( $\text{Na}^+$ )-dependent manner via Solute carrier family 1 member 5 (SLC1A5). Intracellular L-glutamine acting as an efflux substrate is used for regulating the import of extracellular L-leucine through the heterodimeric Solute carrier family 7 member 5/Solute carrier family 3 member 2 (SLC7A5/SLC3A2) bidirectional transporter. In cytoplasm, transported amino acids activate mTORC1 via the Rag GTPases (a family of four related small guanosine triphosphatases) to re-localize mTORC1 in the same intracellular compartment that contains its activator Ras homolog enriched in the brain (Rheb) (8). Conversely, when cells are deprived of glutamine, leucine cannot be imported, resulting in the inactivation of mTORC1 and the induction of autophagy (7). For the response of cells to growth factors, the extracellular fragments of growth factor receptors bind to growth factors, leading to the activation of

the intracellular tyrosine kinase of the receptor. The activation form of growth factor receptors induce the class I PI3K/AKT pathway following mTOR activation that inhibits autophagy (9). Compared with the inhibitory functions of class I PI3K to autophagy, class III p13K activates autophagy (5).

Although it is well known that mTOR is a key negative regulator of autophagy, the exact molecular mechanism by which mTOR regulates autophagy is still unclear. Several studies have reported the transduction of mTOR signaling associated with the ULK-Atg13-FIP200 complex (~3-MDa protein complex) (ULK, the mammalian Atg1 homologue; Atg13, the mammalian Atg13 homologue; FIP200, focal adhesion kinase family interacting protein of 200 kDa), which is an essential part of the regulatory protein complex of autophagy (10-12). In nutrient rich conditions, mTOR phosphorylates Atg13 and ULK (including ULK1 and ULK2) inhibits the kinase activity of ULK to FIP200 mediated by Atg13, resulting in the inhibition of the formation of autophagosome. In starvation conditions, however, the phosphorylation of Atg13 and ULK by mTOR is inhibited, Atg13, ULK, and FIP200 are phosphorylated by the activated kinase activity of ULK to promote autophagosome formation (11). These studies show that mTOR regulates autophagy mediated by the ULK-Atg13-FIP200 complexes in a nutrient-dependent manner. ULK-Atg13-FIP200 is a direct target of TOR in mammals. Furthermore, a novel mammalian autophagy protein Atg101 has been identified and it causes the induction of autophagy by interacting with Atg13, ULK and FIP200 (13) (Fig. 1).

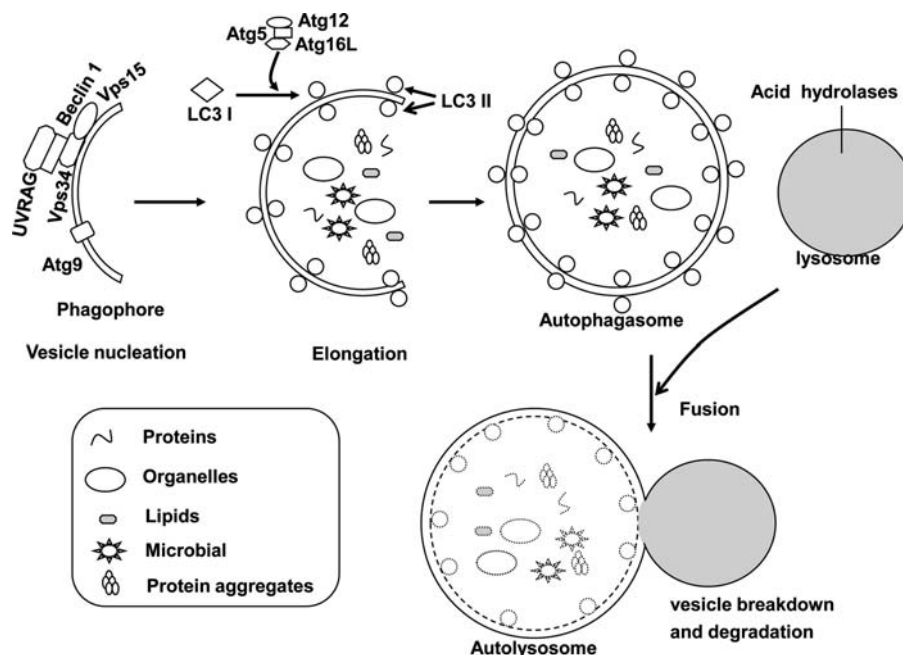


Figure 2. The process of autophagosome formation and degradation. Non-required proteins and their aggregates, organelles, lipids and microbial pathogens are the substrates of the autophagic-lysosomal digestion system. The process of autophagy consists of four stages: vesicle nucleation (initiation of the formation of the phagophore), vesicle elongation, docking and fusion (autophagosome maturation and fusion with lysosome), and final degradation. The main complex systems during autophagy are the LC3 conjugation system and the Atg12-Atg5-Atg16L complex. Also, many other proteins are involved in each step of the process of autophagy.

**Autophagosome formation and degradation.** After induction, the autophagy machinery begins to assemble. The cellular event during autophagy can be dissected into four stages: vesicle nucleation, vesicle elongation, docking and fusion, and vesicle breakdown and degradation (Fig. 2) (14). This is a comparatively perplexing process and involves many Atg and other proteins.

Vesicle nucleation refers to the formation of phagophore or isolation membrane (a lipid bilayer structure) from a phagophore assembly site or the pre-autophagosomal structure (PAS), localized in the peri-vacuolar region, in response to starvation conditions (15). Most Atg proteins recruit to the PAS, as analyzed by fluorescence microscopy, and facilitate the formation of phagophore (16). Beclin 1 (yeast Atg6), UVRAG (yeast Vps38), Vps34 and Vps15 are assembled to form a lipid kinase signaling complex to mediate vesicle nucleation (14). Recently, mammalian Atg9, a multi-transmembrane protein, has been reported to be required for the formation of phagophore and plays a crucial role in lipid membrane transport to PAS by self-interaction (17,18). However, the origin of PAS is not well understood in mammals, or even in yeast.

The molecules to be digested are sequestered by the elongated membrane after the formation of the isolation membrane (Fig. 2). This process is called vesicle elongation. It involves two ubiquitin-like conjugation systems, mammalian LC3 (yeast Atg8) and Atg12, among which the major one is LC3 conjugated with phosphatidylethanolamine (PE) (14). The C-terminal proteolysis of LC3 is processed by Atg4, based on the residues Phe80 and Leu82 of LC3 that may be recognized by Atg4 (19), and immediately follows synthesis to yield a soluble form, LC3-I. LC3-I is converted to a membrane bound form, LC3 II, through a ubiquitin-like reaction involving Atg7, a ubiquitin-activating enzyme (E1)-like enzyme, and Atg3, a ubiquitin-conjugating enzyme (E2)-like enzyme (20).

LC3 is conjugated to Atg3 via Atg7 and then transferred from LC3-Atg3 (I form) to LC3-PE (II form) through the interaction between Atg7 and Atg12 that forms an Atg16L complex, Atg12-Atg5-Atg16L, which allows the LC3 recruitments to the membrane proximal with PE (21).

The isolation membranes elongate and curve until their leading edges merge to envelope portions of the cytoplasm and form the double membrane autophagosome. LC3s are located in both the inner and outer membrane of the forming autophagosome and may play an important role in the closure of the autophagosome (22). One possible mechanism by which LC3s are involved in closure is that lipidated LC3s on the isolation membrane may have the function of lateral diffusion, leading LC3-PE to traverse the narrowing leading edge and tether other LC3-PE to close the autophagosome (22). However, there is no direct evidence to demonstrate this hypothesis. Based on the fact that LC3-PE binds to the autophagosomal membrane during autophagy, approaches for detecting LC3 (LC3 II) (generation or localization) have been developed to detect and manipulate the autophagy pathway. Notably, when observing the localization of LC3, the punctate dots do not always reflect the autophagosome due to LC3s aggregate in an autophagy-independent manner. Therefore, the interpretation of LC3 localization should be made carefully (23). After their formation, the outer membranes of autophagosomes fuse with multivesicular lysosomes (called autolysosome), while the inner membranes and sequestered contents are subsequently digested into amino acids by lysosomal hydrolases within the lysosome lumen. Lysosomal transmembrane proteins such as lysosomal-associated membrane protein 2 (LAMP-2) (24), CLN3 (25) and Rab7 (26) (a small GTP binding protein), rather than Atg proteins, are required for this fusion. At the vesicle degradation and breakdown step, lysosomal cysteine proteases, cathepsins B,

D, and L are required for the degradation of the sequestered contents (14). LC3 and other autophagosome components are also degraded and diminished.

Taken together, the maturation and degradation of autophagosome and autolysosome undergo the complicated process (Fig. 2), regulated by many Atg and other autophagy associated proteins. Physiologically, autophagy occurs at a low basal level, which is enhanced during starvation as well as the accumulation of non-required cellular components, to execute its homeostatic mission. Pathologically, however, dysfunctions of autophagy are the cause of several diseases.

### 3. What roles does autophagy play in physiology?

*Maintenance of cellular homeostasis.* Autophagy rids the cell of misfolded protein and organelles (eg. mitochondria, peroxisomes, ER and intact intracellular microorganisms) over time, prevents the accumulation of unwanted components and, at least in part, removes intracellular pathogens (Fig. 2). Such functions maintain cellular homeostasis and probably underlie the roles of autophagy in cell survival and in protection against cancer, neurodegeneration, ageing and infection (14). Studies of the tissue-specific *Atg* gene deletion demonstrate the essential function of basal level autophagy on intracellular protein quality. In *Atg5* deleted mice, diffused and abnormal proteins accumulate and aggregate to form microscopically visible structures known as inclusions, which can disrupt the neural system (27). Some protein inclusions may protect cells by sequestering toxic protein and delaying protein degradation or alternatively may inhibit the proteasome and induce cytotoxicity under which autophagy as a compensatory mechanism degrades ubiquitin-positive abnormal protein accumulation (28,29). Autophagy may selectively turn over ubiquitin-positive inclusions. In this process, LC3 on the autophagosome may play a vital role in the selective degradation of p62/A170/SQSTM1, which is a ubiquitin-binding protein and involved in the formation of inclusions, due to its direct interaction with p62 (30).

The quality control of highly complex cytosolic cargo, including mitochondria, ribosomes and peroxisomes, is important to maintain proper cellular homeostasis. Because of their ability to sequester structures with varying sizes and geometries, autophagosomes can efficiently clear superfluous and damaged mitochondria, peroxisomes and ribosomes to control the proper number and quality of this cargo. Increasing evidence suggests that the degradation of organelles by autophagy may not be in a random, nonselective fashion, instead, in a selectively targeted pathway. For instance, time-lapse confocal imaging directly visualizes the progression of mitophagy (autophagy degradation of mitochondria) in hepatocytes derived from transgenic GFP-LC3 mice in response to nutrient deprivation; green-fluorescing GFP-LC3s envelope and sequester red-fluorescing mitochondrion (31). The molecular mechanism of this process is unclear. In mammals, however, a BH3-only member of the Bcl-2 family, BNIP3L/NIX, was reported to be required for selective autophagic elimination targeting mitochondria in erythroid maturation (32). Similar to mitophagy, the autophagic degradation of peroxisomes, a normally long-lived cytoplasmic organelle, is commonly termed pexophagy. When the cyto-

plasmically exposed C terminus tail of PMP34, which is a multi-transmembrane integral membrane protein of peroxisome, was linked to mono-ubiquitin moiety, the peroxisome became a selective target of autophagic degradation in mammalian cells. Further experiments showed that pexophagy requires the ubiquitin-binding protein p62, similar to ubiquitinated protein aggregates, suggesting that this process involves ubiquitin and p62 in the selective autophagy of peroxisomes (33). One recent study showed that GFP labeled ribosome proteins are targeted to the vacuole by ubiquitination during starvation, proposing an autophagy selective elimination of ribosomes (referred to as ribophagy) in yeast (34). Currently, there is no report involving the process of autophagic degradation of ribosomes in mammalian cells. Furthermore, it is reported that autophagy affects the lipid metabolism and regulates intracellular lipid stores (macro-lipophagy) by an unknown mechanism (35). More work therefore is needed to explore the process and molecular mechanism in detail.

*Autophagy acts as a cyto-protector or a cyto-killer.* It is well known that autophagy is essential for survival upon starvation in all eukaryotic organisms from yeast to mammal. In response to different types of stress, including nutrient and growth factor depletion, endoplasmic reticulum (ER) stress, hypoxia, microbial infection, and diseases characterized by the accumulation of protein aggregates, autophagy controls a stress adaption pathway that promotes cell survival (36-39). Autophagy degradation supplies amino and fatty acids that are required for recycling and cellular bioenergetics (ATP). In growth factor-deprived autophagy-inhibited cells, the addition of methylpyruvate, a tricarboxylic acid cycle (TCA) substrate, maintains ATP and cell survival, otherwise the cell viability declines and dies (40). Therefore, ATP production may be through TCA catabolism in which the raw materials are mainly supplemented by autophagy in growth factor withdrawn cells. In addition to the essential role of autophagy-dependent ATP production in cell survival, a novel biological role in the clearance of apoptotic corpses has been reported during embryonic development (41). Restoration of normal ATP levels by methylpyruvate in autophagy gene deficient (*Atg5*<sup>-/-</sup> and *beclin 1*<sup>-/-</sup>) embryos is involved in the reversal of the defects in apoptotic cell engulfment, LPC secretion and PS exposure, strongly suggesting that autophagy promotes apoptotic cell corpse clearance that associates with the generation of energy-dependent engulfment signals (41).

Consistent with experiments *in vitro*, increasing studies *in vivo* also demonstrate the indispensable cytoprotective role of autophagy. For example, autophagy is transiently increased in normal newborn mice after birth under physiological conditions. A possible explanation is that during neonatal starvation, neonates underwent self-nourishment by inducing autophagy to generate adequate metabolic substrates. Deletion of the autophagy gene (*Atg5*) results in early neonatal lethality due to amino acid insufficiency (42). A similar study was done by Komatsu *et al* (43). They established an *Atg7* conditional knockout mice model, in which the *Atg* conjugation system and autophagosome formation were impaired, also showed evidence of autophagy function in cell survival and development. *Atg7*<sup>-/-</sup>



died within 1 day after birth and under starvation (43). *SPANDIDOS* mice, died earlier than wild-types after caesarean delivery, suggesting that the inability of these mice to is due to the critical autophagy related protein *Atg7* deficiency. Based on these studies, in physiology, autophagy can promptly meet the cellular demands for metabolic substrates in response to metabolic stress by the transformation of intracellular nutrients and energy resources. Impairment of this protein recycling system might disrupt cell survival and development. This may explain the reduced protein synthesis and developmental defects in autophagy null mouse embryos during pre-implantation development, in which fertilization of autophagy-deficient (*Atg5*<sup>-/-</sup>) oocytes with autophagy-deficient (*Atg5*<sup>-/-</sup>) sperm did not develop and remained at the four- or eight-cell stage, but those with wild-type sperm developed (44).

Autophagy also regulates innate and adaptive immune systems. This functions through the killing of intracellular pathogens, antigen presentation, tolerance and lymphocyte development, type I interferon production, as well as the negative regulation of cytokine signaling and inflammation (45). Autophagy can selectively target microorganisms, including viruses, bacteria and parasites (free microbes in the cytosol or pathogen-containing phagosomes), and deliver them to lysosomes for hydrolysis. With respect to the innate defense system, a representative example of autophagy targeting intracellular microbes is the elimination of group A *Streptococci* (GAS), which escapes from endosomes into the cytoplasm and then are killed by autolysosomes in wild-type cells, but are released from autophagy-deficient *Atg5*<sup>-/-</sup> cells (46). Endogenous antigen presentation to the adaptive immune system is also performed by autophagy. For example, endogenous Epstein-Barr virus nuclear antigen 1 (EBNA1) (47) and *Mycobacterium tuberculosis* (Mtb) 85B antigen (48) are processed for MHC (major histocompatibility complex) class II presentation to CD4<sup>+</sup> T lymphocytes. BCG vaccine efficacy is enhanced by autophagy through increasing mycobacterial antigen Ag85B presentation in a mouse model further demonstrates autophagic enhancing immunity (49).

However, an apparent paradox of autophagy is that it acts upon both cytoprotection and cytotoxicity. The death induced by autophagy is defined as non-apoptotic or type II programmed cell death and possesses the characteristic of the increased autophagosomes without any signature of apoptosis or necrosis (50). However, when a dying cell shows features of several types of death (e.g. autophagy and apoptosis), it is difficult to identify if the cell is dying with autophagy or through autophagy. If cell death accompanies autophagy, it means autophagy may be induced by some death events rather than being an executor of cell death. If the cell is dying through autophagy, autophagy may be a part of the apoptotic program or be in parallel with apoptosis for triggering cell death (50). The first evidence that autophagy contributes to cell death (independent of apoptosis) was that the embryonic fibroblasts (MEF) from *Bax/Bak* double knockout mice, which were resistant to apoptosis, were treated with various apoptotic stimuli, leading to non-apoptotic cell death that associated with the generation of autophagosomes/autolysosomes, the accumulation of Beclin 1 and APG5-APG12 complex, and the overexpression of *Bcl-2/Bcl-xL* (51). In that case, the antiapoptotic *Bcl-2/Bcl-xL* may promote autophagosome formation by the regulation of

beclin 1. Nevertheless, the opposite role of the *Bcl-2* family in autophagy was reported. The interaction between *Bcl-2* and beclin 1 inhibits autophagy to sustain the physiological functions of autophagy, whereas if *Bcl-2* cannot bind to beclin 1, an over-physiological range of autophagy may promote cell death. This antiautophagic function of *Bcl-2* possibly maintains autophagy at the basal level being more compatible with cell survival than cell death (52). Different stress conditions, at which the former is apoptosis-induced autophagy but the latter is starvation-induced autophagy, might be the reason for the converse roles of the *Bcl-2* family. *Bcl-2* might function more generally under starvation conditions but this still remains to be further identified.

Although autophagy plays a role in cell death in certain settings, there is no report that autophagy related genes promote programmed cell death in physiological *in vivo* or that the down-regulation of autophagy genes avoid cell death. On the contrary, *Atg5* or *beclin 1* deficiency in embryonic cells increase the apoptotic cells and the cells undergo apoptotic death during development (41). The removal of apoptotic cells (apoptotic bodies), the final step of apoptosis, may require autophagy, based on the fact that autophagy null embryoid bodies fail to generate an 'eat me' signal (phosphatidylserine exposure) and secrete low levels of 'come and get me' signal (lysophosphatidylcholine). Interestingly, the 'come and get me' signal in apoptotic cells is also expressed by sphingosine 1-phosphate (S1P) (53), which was reported to be associated with autophagy formation (54).

Many studies have shown autophagy, which primarily acts as a cell guardian, and apoptosis, which unchangeably leads to cell death, share common regulatory factors and one can be regulated by the other (39). Both apoptosis and autophagy lead to cell death via death-associated protein kinase (DAPK), BNIP3 and ceramide. Viewed from several signaling pathways that associate with autophagy and/or apoptosis: Beclin 1, BH3-only protein BAD, the lysosomal protein DRAM, the nuclear form of p53 (p53n), and Sestrin2 are the positive effectors of autophagy, whereas mTORC1, mTORC2, PKB, *Bcl-2*, *Bax/Bak*, the cytoplasmic form of p53 (p53c), the transcription factor NF- $\kappa$ B, inositol 1,4,5-trisphosphate receptor [IP(3)R] are the negative ones (see details ref 50,55,56). The factors that inhibit apoptosis also inhibit autophagy. For example, anti-apoptotic *Bcl-2* inhibits beclin 1 (52). Notably, the factors that stimulate apoptosis can also induce autophagy. For instance, pro-apoptotic BH3-only protein BAD activates autophagy by removing *Bcl-2* inhibitory interaction with beclin 1 (57). Also, more apoptosis-sensitized cells are likely to die via autophagy if autophagic protection of these cells cannot withstand unanticipated, unfavourable stress circumstances. This intricate molecular interplay between autophagy and apoptosis must be fully elucidated by further studies and might be a mirror of the complex relationship of autophagy with human disease pathogenesis.

#### 4. Autophagy and diseases

As mentioned above, a basal and constitutive level of autophagy is indispensable for intracellular homeostasis and quality control for healthy individuals. Mounting evidence has demonstrated that disruption of its physiological function is

strongly related to human diseases, including neurodegeneration, cancer, liver and heart diseases, ageing, myopathies, and infections (58). This section will discuss the current knowledge of autophagy-related diseases and their potential therapeutics.

*Autophagy-related neurodegenerative diseases.* Neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, prion diseases, Huntington's disease and spinocerebellar ataxias, have the common characteristic of protein aggregation (59). For example,  $\beta$ -amyloid and tau aggregates accumulate in Alzheimer's disease. Expanded polyglutamine (PolyQ) stretch caused by the expansion of CAG trinucleotide repeat within several gene exons seen in Huntington's disease and several types of spinocerebellar ataxias.  $\alpha$ -synuclein fibrils linked to familial Parkinson's disease self-aggregate and deposit in the brain (60,61). These protein aggregates, the neurotoxic components, are substrates of the autophagy degradation system. Boland *et al* suggested that the basal expression of autophagy is crucial in primary cortical neurons and there is no large amount of autophagosome accumulation in healthy brains because of efficient lysosomal hydrolysis of newly formed autolysosomes (62). Therefore, it is true that autophagy plays a role in the protection against neurodegeneration. However, in patients with diverse neurodegenerative diseases, the autophagic clearance system may reach saturation and thereby become unable to eliminate excess aggregate-prone mutant proteins, or dysregulation or defection may occur in the autophagy pathway. Impaired autophagic vacuoles (AVs) clearance during autophagosome transport to lysosomes or during substrate degradation within the lysosome may lead to AVs accumulation with morphologies that are observed in Alzheimer's disease (AD) (62). Contrary to the beneficial effect of basal autophagy, an excess accumulation of autophagosomes promotes neuronal cell loss under certain circumstances, such as dysfunctional endosomal sorting complex required for transport III (ESCRT-III), which is associated with frontotemporal dementia (63). This implies that either excessive autophagosome synthesis or reduced autophagosome-lysosome fusion may contribute to the pathogenesis of neurodegeneration by the dysfunctional autophagic process.

Owing to the protective role of autophagy in neuron systems, it may be a therapeutic target against neurodegenerative diseases. Upregulation of autophagy may improve the clearance of neurotoxic aggregate-prone proteins. The inhibition of TOR, a negative regulator of autophagy, by rapamycin or its analogue CCI-779 is protective in *Drosophila* and mouse models of Huntington disease (64). mTOR is required for various cellular events, including protein synthesis (e.g. the initiation of mRNA translation, ribosome biogenesis, transcription), cell growth and proliferation, and cytoskeletal reorganization (65), but its inhibition by rapamycin causes some adverse effects such as cell cycle arrest, poor wound healing, and mouth ulcers (66). Small molecule enhancers of rapamycin (SMERs), however, induce autophagy in an mTOR independent mechanism. They enhance the elimination of mutant huntingtin and  $\alpha$ -synuclein, and protect against neurodegeneration in the Huntington's disease model of *Drosophila* (67). Recently, autophagy induction by a group of new drugs, L-type  $\text{Ca}^{2+}$  channel antagonists,  $\text{K}^{+}$  ATP channel opener

minoxidil, and Gi signaling activator clonidine in an mTOR independent pathway were reported in Huntington's disease mammalian cell, fly and zebrafish models (68).

Nevertheless, it is important to understand the pathology underlined by autophagy in different neurodegenerative diseases. Pharmacologically enhancing autophagosome formation in AD where the autophagosome clearance system is defective may exacerbate the accumulation of the unmaturing autophagosomes, some of which may generate toxic proteolytic product  $\text{A}\beta$  and/or others (62,69). Therefore, improvement in the late stages of the autophagy process may be the best therapeutic modulation of autophagy in AD. In addition, the inhibition of autophagy by small molecules, such as 3-MA, might be promising therapeutic strategies for the early stages of some forms of FTD or other neurodegenerative diseases where excess produces autophagosomes (63).

*Autophagy in liver, heart and muscle diseases.* The basal level of autophagy plays an important role in protein and organelle quality control in several tissues such as the liver, heart and muscles. Once absent of autophagy activation, the tissues induced are to form certain protein aggregates that are physiologically cytotoxic and thereby lead to occurrence of diseases. In  $\alpha$ 1-antitrypsin deficiency, which causes severe chronic liver injury and hepatocellular carcinoma, mutant  $\alpha$ 1-antitrypsin Z ( $\alpha$ 1-ATZ) proteins accumulate to form aggregates within the endoplasmic reticulum (ER) of the liver cells (70). Deletion of *Atg5* *in vitro* hampered the degradation of  $\alpha$ 1-ATZ, especially inclusions of  $\alpha$ 1-ATZ accumulated; transgenic liver-specific expression of  $\alpha$ 1-ATZ *in vivo* is sufficient to induce mouse hepatocyte autophagy, suggesting that the protein quality control function of autophagy is important in  $\alpha$ 1-ATZ and its degradation function which protects liver cells from the cytotoxicity of  $\alpha$ 1-ATZ accumulation. Based on this, autophagy augmentation by pharmacological agents would likely be beneficial in this setting.

Similar to the neuron and liver, basal autophagy is also required for protein and organelle control in normal cardiac function and invalidity in this process results in cardiac dysfunction and heart failure. Abnormal protein aggregation and ubiquitinated protein accumulation can be detected in human hearts with idiopathic or ischemic cardiomyopathies (71-73). In a mouse model of load-induced heart failure, autophagy, probably triggered by protein aggregation, sequestered a high density of ubiquitinated protein aggregates (74). Also, knockout of cardiac-specific *Atg5* in adulthood results in heart hypertrophy and contractile dysfunction that is accompanied by elevated ubiquitinated proteins, sarcomere and abnormal mitochondria accumulation. Thus, stress-induced upregulation of autophagy provides sufficient energy substrates and enhances ventricular remodeling, playing an adaptive role (75). However, opposite to this adaptive role, cardio-myocyte autophagy is maladaptive in certain settings based on the experimental outcome where contractile function was maintained and not handicapped in a beclin 1<sup>-/-</sup> mice model in a pressure overload setting (76). Perhaps, it is necessary to clarify whether the contradictory effects of cardiomyocyte autophagy are induced by different types of protein aggregates that form and/or the associated autophagic response (77).



athogenesis of several muscle diseases like Danon and Pompe disease may be associated with an impairment in autophagosome-lysosome fusion. Danon disease, an extremely rare X-linked dominant disorder characterized by cardiomyopathy, myopathy, and variable mental retardation, is caused by the mutation of LAMP-2 (78). The failure of autophagosome to fuse with lysosome leads to large amount of autophagosomes accumulating in the muscles of LAMP-2-deficient mice and patients. The impairment of autophagosomal-lysosomal fusion also contributes to the accumulation of autophagic substrates, including potentially toxic aggregate-prone ubiquitinated proteins (e.g. glycogen), in the core of the Pompe myofibers that occur in Pompe disease, and the deficiency of glycogen-degrading lysosomal enzyme acid  $\alpha$ -glucosidase (GAA) (79). Ramachandran *et al* recently uncovered the molecular mechanism of X-linked myopathy with excessive autophagy (XMEA) that is characterized by progressive vacuolation and atrophy of skeletal muscle (80). *VMA21* gene deficiency is the associated with autophagic myopathy and it reduces lysosome's ability of degradation and inhibits autophagy by increasing lysosomal pH. This leads to an accumulation of large and ineffective autolysosomes and finally vacuolates the cell and atrophies the tissue. These data suggest that regardless of the diseases from the liver, heart, or muscle, aggregate-prone proteins that are mainly cleared by autophagy are involved in the pathology of these diseases, and thereby autophagy upregulation is helpful for toxic protein clearance.

*Autophagy in cancer.* Cancer is genetically linked to autophagy defection and several Atg genes or genes that regulate/execute the autophagy pathway play a role in tumor suppression or oncogenesis (81). The monoallelic deleted autophagic gene *beclin 1* was identified in human breast, ovarian, prostate cancers and brain tumors, suggesting the importance of this event in tumorigenesis (82,83). Mice with heterozygous disruption *beclin 1* had a reduction of autophagy and the development of spontaneous tumors including lymphomas, lung carcinomas, hepatocellular carcinomas, and mammary precancerous lesions (84,85). Transplantation of immortalized kidney and mammary epithelial cells derived from *beclin 1* heterozygous-deficient mice were more tumorigenic compared with that of those derived from wild-type mice (82). These studies provide compelling evidence that *beclin 1* is a haploin sufficient tumor suppressor. Other essential autophagy mediators such as ultraviolet irradiation resistance-associated gene (UVRAG), Bif-1 and Ambra1, which interact with Bcl-2 to activate Vps34 (the class III phosphatidylinositol-3-kinase), may also inhibit cell proliferation and/or tumorigenesis (81). More recently, the frameshift mutation in which Atg genes with mononucleotide repeat including Atg2B, Atg5, Atg9B and Atg12 were at a high frequency in gastric and colorectal cancers with microsatellite instability, revealing that these mutations possibly contribute to cancer development. This means that these genes have an effect on tumor suppression (86). To further confirm this function of Atg genes, it might be better to establish an animal model with Atg knockdown or knockout. *Atg4c* encoding a cysteine proteinase involved in the processing and delipidation of LC3 has been proposed to contribute to tumor suppression according to the experimental results that

fibrosarcomas increased by chemical agent in *Atg4C<sup>-/-</sup>* mice (87). Therefore, it is rational that genes at different steps of autophagy also function in tumor suppression. However, some autophagy regulator genes are known as oncogenes. Oncogenes encoding class I PI3K, AKT, mTOR, and Bcl-2 are the negative regulator of autophagy, whereas tumor suppressor genes encoding p53, PTEN, DAPk, TSC1/ TSC2, BH3 - only proteins and drs are the positive regulators of it (88,89).

The molecular mechanism by which autophagy acts as a tumor suppressor is not so clear. However, more recently, Mathew *et al* have reported that p62 may be the key mediator in autophagic suppression of tumors (90). Sustaining the expression of p62 is a common feature of tumor cells in which autophagy is deficient and it is associated with oxidative stress activation through NF- $\kappa$ B and tumorigenesis. The failure to eliminate P62 in autophagy-defective *beclin 1<sup>+/-</sup>* and *Atg5<sup>-/-</sup>* iBMK cells also leads to increased DNA damage and genomic instability. In other words, under metabolic stress, autophagy may promptly recycle p62 preventing p62-induced genomic instability and tumorigenesis. This may strongly validate one of the hypotheses, proposed by White and colleagues, that explained that the loss of autophagy, a cell survival pathway, promotes oncogenesis possibly due to genomic instability in metabolically stressed cells (91). Therefore, autophagy promotion for the efficient clearance of p62 might be a strategy for cancer chemoprevention in patients with hepatocellular carcinomas where p62 is commonly upregulated.

One complicated aspect of autophagy targeting cancer therapy is that autophagy inhibition, rather than autophagy activation, may enhance the tumor cell sensitization to cytotoxic agents (88). In this situation, the pro-survival effect of autophagy, rather than the tumor suppressor effect, contributes to tumor cells preventing death induced by chemotherapy or radiotherapy. For example, autophagy induction via ATF4 may be an important resistance mechanism in Bortezomib-treated breast cancer cells (92). Autophagy activation may protect HER2-overexpressing breast cancer cells from trastuzumab toxicity (93). Pharmacological and genomic inhibition of autophagy have been utilized in several experimental cancers, such as colon cancer sensitized to chloroquine (94), breast cancer killed by the estrogen receptor antagonist tamoxifen (95), pharyngeal, breast, and lung cancers, cervical squamous cell, and rectum carcinomas sensitized to  $\gamma$ -irradiation (96). Taken together, at the early stages of the tumor, autophagy functions as a tumor suppressor, under which autophagy induction may be effective in preventing tumor formation and progression. During tumor progression, however, autophagy acts as a tumor growth promoter, under which autophagy inhibition may enhance the cytotoxic agents efficacy and tumor regression.

*Autophagy and diseases in the immune system.* Autophagy can directly eliminate pathogens, protect infected cells, and also mediate trafficking events required for innate and adaptive immunity (45). Although many pathogens such as bacteria and viruses are removed by autophagy, it is unavoidable that some pathogens have developed strategies to outsmart autophagy. There are three possible mechanisms by which bacteria escape: inhibiting autophagosome fusion with lysosomes, blocking compartments degradation after fusion with lyso-

somes, or subverting autophagosomes to their own replicative advantage. The bacterium *Shigella flexneri* escapes from autophagosome engulfment through secreting IcsB protein which blocks *S. flexneri* recognition by the autophagic machinery (97). *Listeria monocytogenes* can replicate in spacious Listeria-containing phagosomes (SLAPs), which are non-acidic and nondegradative compartments and formed in an autophagy-dependent manner. Bacterial Listeriolysin O, a pore-forming toxin essential for *L. monocytogenes* virulence, is required for SLAP formation. These suggest that listeriolysin O may inhibit autophagosome acidification by creating pores and therefore prevent lysosomal degradation in SLAPs (98). In some settings, microviral invasion may be important for microbial pathogenesis. Studies on herpesviruses provide the most compelling evidence for this presumption. The  $\alpha$  herpes simplex virus type 1 (HSV-1) encodes the neurovirulence protein ICP34.5 that binds to *Beclin 1* and inhibits autophagosome initiation through this interaction (99). The  $\gamma$  herpes viruses, Kaposi sarcoma-associated herpesvirus and murine  $\gamma$ -herpesvirus 68, however, inhibit autophagy at the initiation step by expressing the viral Bcl-2 (v-Bcl-2) that also blocks the function of beclin 1 via binding (100). In these settings, it may be feasible in antimicrobial therapy to selectively disrupt the interactions between microbial virulence factors and their target in host autophagy proteins.

Several autophagic genes have been reported to associate with inflammatory diseases. Functional knockdown of Atg16L1 that is expressed in intestinal epithelial cell lines abolished autophagy of *Salmonella typhimurium* (101). The distinct expression patterns of *IRGM* gene encoding autophagy-stimulatory immunity-related GTPase were identified in Crohn's disease, an inflammatory bowel disease (102). Reduced expression of *IRGM* significantly compromised the efficiency of anti-bacterial autophagy, but a modest overexpression of *IRGM* protein enhanced the autophagy of *Salmonella*. These studies support a role of Atg16L1 and *IRGM* in anti-bacteria autophagy. The genome-wide association scan studies in Crohn's disease demonstrated single-nucleotide polymorphisms in Atg16L1 and *IRGM* and were strongly associated with the susceptibility to this disease (101,103,104). This suggests that the dysregulation of autophagy may contribute to the pathogenesis of Crohn's disease. However, there is no evidence that these mutations result in autophagy impairment. At least the T300A Atg16L1 variant is not a loss-of-function mutant. Therefore, it might be possible that these polymorphisms disrupt specific autophagic innate defensive system in gut commensals, causing inflammation after microbion invasion, or compromise autophagic tolerance through self-antigen presentation on MHC class II molecules on the mucosal surface, which is then easily broken by proinflammatory microbial stimuli at this site (45).


**Autophagy in ageing.** A common feature of all aged cells is the intracellular accumulation of damaged proteins and organelles that leads to the decreased ability of the organism to survive (105). The decreased protein degradation plays an important role in ageing by the reduced breakdown of altered proteins and life-span prolongation of proteins in cells that consequently is post-translationally altered. During this process, the defection of the autophagic/lysosomal proteolytic system may be the

main cause of reduced protein degradation, since the proteolytic system - proteasome can not digest large molecules such as impaired organelles. Indeed, there is evidence of a gradual reduction in autophagy with age (105,106). Autophagy deficiency can lead to neurodegenerative diseases (27) and heart dysfunction (75) in experimental models, and hence it is supposed that decreased autophagic activity with age contributes to the functional deterioration of ageing organisms. Therefore, autophagy activation may protect organisms from ageing due to the increased ability to get rid of damaged proteins and organelles. Caloric restriction, the only intervention for delaying ageing and expanding life span, reverses autophagy decline with ageing possibly because of reduced insulin/IGF-1 signaling that inhibits autophagy (107). Fasting can promote longevity, but it may cause potentially adverse effects of caloric restriction on human health and hence currently, alternative approaches are being studied that mimic the benefit effects of caloric restriction. The application of antilipolytic drugs that increase autophagy and extend longevity is a good example (108,109).

The molecular basis for age-related decline in the autophagic proteolytic system remains to be clarified. However, the factors that regulate the ageing process are notably interrelated with the autophagy pathway (reviewed in ref. 110). For instance, insulin/IGF-1 signaling, in which the IGF-1 receptor may be a central regulator of longevity in mammals (111), inhibits autophagy by PI3K and mTOR signaling. The forkhead family transcription factor FoxO, a key downstream target of insulin, growth factor, nutrient, and oxidative stress stimuli, plays a role in longevity (112) and also enhances the autophagy function in protein degradation by increasing the transcription of several autophagy-related genes, including Atg8/MAP-LC3, Atg6, Atg12 and Vps34 (113). The tumor suppressor P53 not only induces autophagy in a damage-mediated autophagy modulator (DRAM)-dependent manner that is essential for P53-mediated programmed cell death-apoptosis (114), but also slows down ageing and reinforces cancer resistance through coordinating with the alternative reading frame protein of Cdkn2a locus (Arf) in p53 and arf increased mice (115). Furthermore, the reactive oxygen species (ROS), which induces oxidative damage of cytosolic materials, regulates both ageing and autophagy (110). Thus, the question to be solved in longevity and autophagy that displays multiple signaling crosstalk concentrates on how to handle unneeded macromolecules and organelles, a determinant of ageing.

## 5. Conclusion

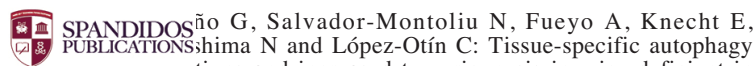
Recent studies have made great progress in uncovering autophagy, which affects both cell survival and cell death. Many proteins that are assembled to form the autophagic machinery have been identified, whereas the complicated regulatory system of autophagy controlled by multiple inhibitory and stimulatory factors involved in many signaling pathway is far from being fully understood. From current knowledge we know that both constructive or basal levels and induced autophagy are important for maintaining cellular metabolism and promoting mammalian health. However, in certain settings, autophagy's protective role may contribute to protecting cells that should die, or its over-activity may result in the death of

 SPANDIDOS should live. The impairment of autophagy clearance of cellular proteins and organelles is associated with the pathogenesis of several diseases, such as neurodegeneration, Parkinson's diseases and ageing. Therefore, targeting autophagy may be a potential strategy for the treatment of these diseases. Yet, in the manipulation of autophagy for therapeutic interventions, we should consider the type and progression of the diseases because autophagy may act as a stimulator or a suppressor, such as in cancer. Also, because of the dual functions in cytoprotection and cell death, further research is needed to clarify a more detailed understanding of how autophagy influences different types of human diseases.

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