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TOPIC HIGHLIGHT

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Autophagy in mammalian cells

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Abstract

Autophagy is a regulated process for the degradation of cellular components that has been well conserved in eukaryotic cells. The discovery of autophagy-regulating proteins in yeast has been important in understanding this process. Although many parallels exist between fungi and mammals in the regulation and execution of autophagy, there are some important differences. The pre-autophagosomal structure found in yeast has not been identified in mammals, and it seems that there may be multiple origins for autophagosomes, including endoplasmic reticulum, plasma membrane and mitochondrial outer membrane. The maturation of the phagophore is largely dependent on 5'-AMP activated protein kinase and other factors that lead to the dephosphorylation of mammalian target of rapamycin. Once the process is initiated, the mammalian phagophore elongates and matures into an autophagosome by processes that are similar to those in yeast. Cargo selection is dependent on the ubiquitin conjugation of protein aggregates and organelles and recognition of these conjugates by autophagosomal receptors. Lysosomal degradation of cargo produces metabolites that can be recycled during stress. Autophagy is an important cellular safeguard during starvation in all eukaryotes; however, it may have more complicated, tissue specific roles in mammals. With certain exceptions, autophagy seems to be cytoprotective, and defects in the process have been associated with human disease.

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Key words: Autophagy; Phagophore; Autophagosome; Atg proteins; Cell survival

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INTRODUCTION

Macroautophagy (called autophagy henceforth) is a catabolic process in which organelles and soluble and aggregated cellular components are enveloped in double membrane vesicles called autophagosomes, which eventually fuse with lysosomes, leading to the degradation and reuse of the vesicular contents. Autophagy occurs constitutively in all eukaryotic cells and operates as a homoeostatic mechanism. In addition, autophagy can be activated in response to various physiological and pathological stimuli to promote cell survival (e.g., starvation, oxidative stress), or to act as a mode of cell death, type Ⅱ programmed cell death (e.g., during development). The formation of the autophagosome involves several steps that are energy-dependent and orchestrated by a set of molecular ef-

fectors, the autophagy-related proteins or Atgs (Figure 1). This process has been conserved from yeast to mammals and although many of the Atgs were first discovered in yeast; mammalian homologs have also been found[1-6].

THE PHAGOPHORE

In both yeast and mammals, the formation of the autophagosome begins with a double membrane structure called the phagophore or isolation membrane (Figure 1). Briefly, the phagophore elongates and engulfs cytoplasmic "cargo". Then, the double membrane structure closes to become the autophagosome and fuses with a lysosome to become an autolysosome that digests the cargo and some of the components of the vesicle itself. In yeast, the immediate precursor of the phagophore is a small membranous organelle, the pre-autophagosomal structure (PAS). The PAS contains Atg9, one of the few integral proteins that is typical of the autophagosome. Yeast Atg9 contains 997 amino acids and spans the membrane six times. After siRNA depletion of Atg9, autophagy is impaired[7]. Since Atg9 is also found in the trans-Golgi network (TGN) and late endosomes, it seems possible that the TNG may be the origin of the PAS. In fact, this conclusion has been reached by Ohashi *et al*[8]. On the other hand, Mari *et al*[9] have reported that Atg9 containing "clusters of vesicles and tubules, which are derived from the secretory pathway", coalesce near the vacuole and may be precursors to the PAS. More recently[10], experiments with loss of function mutants have shown that the formation of these Atg9-containing structures depends on exocytic (and, perhaps, endosomal) Q/t-SNAREs (soluble NSF attachment protein receptors). It was suggested that aside from their other roles in yeast, these SNAREs may be essential for fusions that convert the Atg9-bearing structures into the PAS.

A structure similar to the yeast PAS has not been described in mammalian cells, and it is possible that there may be multiple origins for phagophores in these cells[1,2]. The endoplasmic reticulum (ER), outer membranes of the mitochondria (OMs) and the plasma membrane (PM) are among the candidates for the origin of the phagophore. Of these, the evidence for the ER is the most comprehensive. Double FYVE-containing protein 1 (DFCP1), an early marker for autophagosomes (see below), has an ER-targeting signal and is also found in cup-shaped ER structures called "omegasomes". There is evidence that autophagosomes are associated with the omegasomes[11]. Two groups have used electron tomography to show that the ER/omegasome and the nascent autophagosome are connected by narrow extensions in rat kidney cells[12] and NIH 3T3 cells[13]. These findings have led to the hypothesis that the phagophore emerges from the omegasome and elongates to engulf a portion of the ER membrane prior to fusing into a mature autophagosome[13]. It seems likely that some autophagosomes originate in the ER; however, other origins have not been ruled out. Hailey *et al*[14] have presented strong evidence that the OM is an origin for autophagosomes in a rat kidney cell line. Both a fusion of the yellow fluorescent protein and the signal peptide for the mitochondrial isoform of cytochrome b5 and an analog of phosphatidylethanolamine (PE) have been shown to move from the mitochondria to autophagosomes during starvation. This and the identification of patches of OM that contain autophagosome markers indicate an OM origin for the autophagosome. Ravikumar *et al*[15] have shown that Atg16L, an early autophagosomal precursor (see below), interacts with the heavy chain of clathrin and initiates autophagosome formation in vesicles derived from the PM. According to this model, the endosomal pathway is hijacked to produce phagophores.

The abundance of points of origin in mammalian cells raises the question, why? It may be that there are different autophagosomes for different cargos; that is, the ER may be recycled by vesicles arising from the ER, and so forth. It has been suggested that the PM may serve as a reservoir for membranes when demand exceeds the capacity of other origins[15]. It is also possible that the preferred source for autophagosomes is determined by the tissue. Heart muscle, for example, has a small and very specialized ER but is rich in mitochondria. It may be that the OM is preferentially used for autophagosome formation in cardiomyocytes.

CONTROL OF AUTOPHAGY

Autophagy is under the control of multiple signaling pathways, most of which converge on mammalian target of rapamycin (mTOR) (Figure 2). Although a full description of the regulation of autophagy is beyond the scope of this review, the role of the mammalian Atg1 complex and 5'-AMP activated protein kinase (AMPK) in the initiation of autophagy is warranted. In mammals, the first steps in autophagy involve a stable cytosolic complex consisting of unc-51-like kinase (ULK) 1 or ULK 2, Atg13, Atg101 and FIP200 (focal adhesion kinase family interacting protein of 200 kDa). Except for Atg101, all of these proteins are orthologs of the components of the similar Atg1 complex in yeast. Under resting conditions, phosphorylated (active) mTOR binds to and phosphorylates a serine (S757) in ULK 1[16]. Under certain conditions that cause compromised energy production, such as glucose deprivation or hypoxia, AMP accumulates as ATP is depleted. AMPK is a heterotrimer (α, β and γ subunits) that senses the level of AMP in cells. When two AMP molecules are bound to its γ subunit, AMPK undergoes a conformational change that exposes a threonine (T172) on the α subunit for phosphorylation and activation of AMPK by AMPK kinase (AMPKK). The activated AMPK initiates a cascade including upregulation of glucose transport, fatty acid oxidation and other energy-producing pathways. AMPK also promotes autophagy by phosphorylating Rheb and Raptor leading to dephosphorylation of mTOR (Figure 2). When mTOR is dephosphorylated, it dissociates from the complex and ULK 1 is dephosphorylated. Activated AMPK can bind to dephosphorylated ULK 1 and activate it by phosphor-

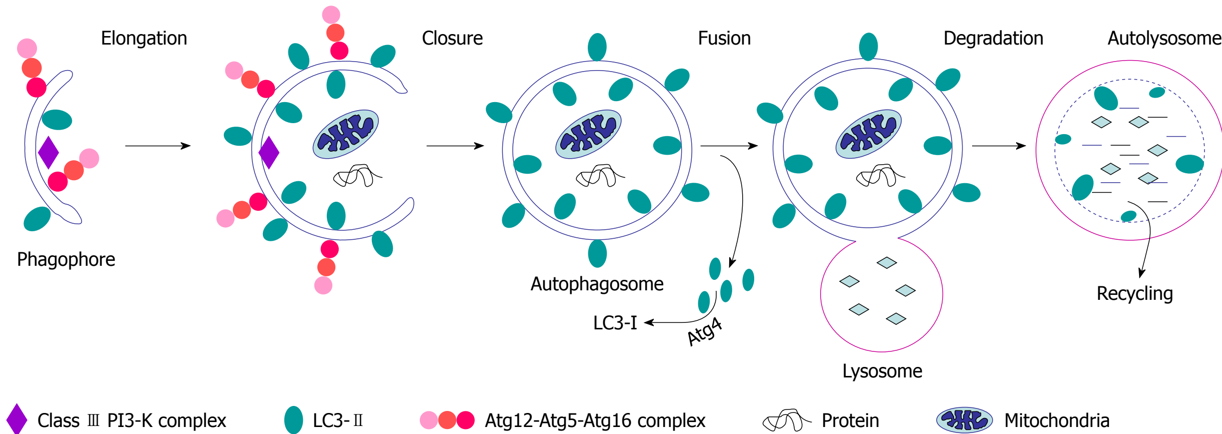

Figure 1 The process of autophagy. Autophagy is initiated with the formation of the phagophore, mediated by the class Ⅲ PI3-K complex that includes Vps34, Vps15, Atg14 and Beclin 1, and progresses through a succession of steps: elongation of the phagophore and engulfment of cytoplasmic material targeted for degradation; formation of the autophagosome, with delipidation of LC3-Ⅱ by Atg4; fusion of the autophagosome with the lysosome to form the autolysosome; degradation of the vesicle content by lysosomal hydrolases; and recycling of the degradation products (amino acids, lipids and sugars) for ATP production. The autophagy machinery consists of two conjugation systems required for the elongation and extension of the phagophore: Atg5-Atg12, which subsequently oligomerizes with Atg16, and LC3-PE, LC3-Ⅱ. LC3-Ⅱ is formed as a result of the Atg4-mediated cleavage of cytosolic LC3. The resulting form of LC3, LC3-I is subsequently conjugated to a single PE molecule to form LC3-Ⅱ, a reaction mediated by Atg3 and Atg7.

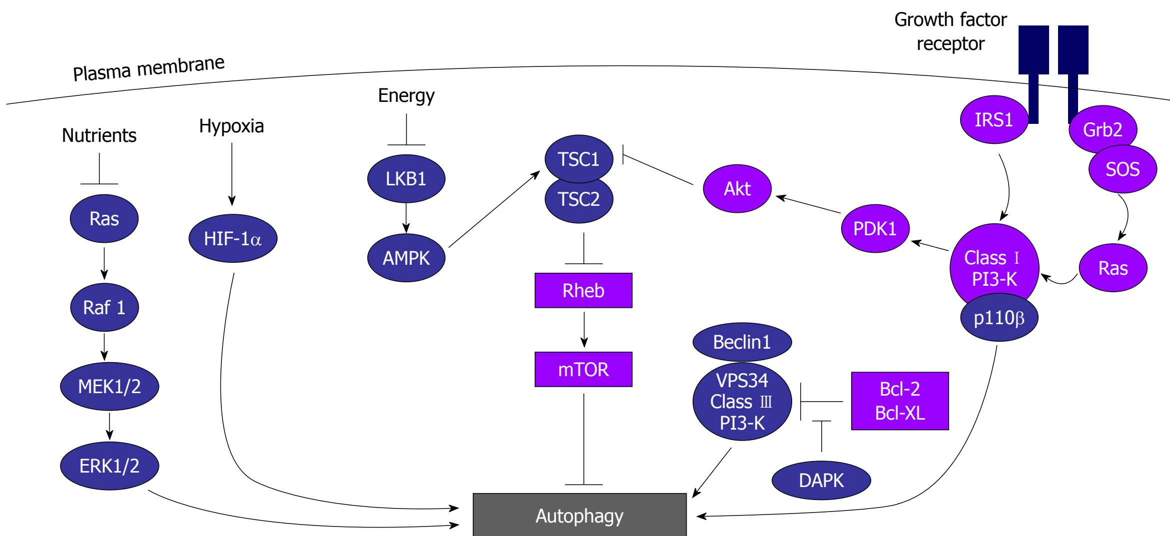

Figure 2 The regulation of autophagy. Autophagy is regulated by multiple signaling pathways. In response to growth factors, mTOR is activated by the class Ⅰ PI3-K and Akt, which inhibits tuberous sclerosis protein (TSC)1/TSC2. The activation of this cascade leads to the inhibition of autophagy. mTOR activity is inhibited by AMPK, a kinase activated in response to elevated intracellular AMP/ATP ratio. In addition, the p110-β catalytic subunit of the class Ⅰ PI3-K can directly stimulate autophagy during starvation, independently of Akt activation, through its association with the class Ⅲ PI3-K complex. The growth-factor-mediated activation of Ras induces antagonistic effects on autophagy depending on its downstream target, while the activation of the class Ⅰ PI3-K cascade represses autophagy, and the stimulation of the Raf-1-MEK1/2-ERK1/2 signaling cascade in response to amino acid depletion promotes autophagy. Hypoxia induces autophagy *via* activation of hypoxia-inducible factor 1α. Finally, autophagy is also regulated by death-associated protein kinase, which promotes the initiation of autophagy through the release of Beclin 1 from the Bcl-2/Bcl-XL complex. The DAPk-related protein kinase 1 has also been found to be necessary for the induction of autophagy.

ylating at least two serines[16]. The activated complex then becomes associated with the phagophore and initiates formation of the autophagosome[17-19].

ELONGATION AND MATURATION OF THE AUTOPHAGOSOME

An early event downstream from mTOR is the association of the vesicular sorting protein 34 complex/phosphatidylinositol-3-phosphate kinase Ⅲ (Vps34/PI3PⅢ) with the phagophore. Vps34 has roles in both the endocytic and autophagic pathways. In autophagy, Vps34 associates with the phagophore membrane *via* Vps15 (also called p150) that is anchored to the phagophore membrane by myristic acid. The third component of this complex, beclin-1, is essential for phospholipid kinase activity. Beclin-1 was first identified as a binding partner of the antiapoptotic factor Bcl2. Bcl2 inhibits autophagy by competing with Vps34 for beclin-1 binding. Two other factors, beclin-1-associated autophagy related key regulator (Barkor) and UV radiation-associated resistance gene

(UVRAG) are thought to stabilize the beclin-1/Vps34 association[20,21]. The generation of PI3P by the beclin-1/Vps34 PI3PⅢ kinase is crucial for the recruitment of factors essential for the formation of the autophagosome. This is demonstrated by the fact that autophagy is arrested by inhibitors of PI3PⅢ kinases such as 3-methyladenine and wortmannin. As PI3P appears in the phagophore membrane, other effectors begin to appear. These include WIPI-1 and -2 and DFCP1, which bind to PI3P *via* WD repeats and FYVE domains, respectively. As already mentioned, DFCP1 is also found in the ER[11]. The role of these proteins is, at present, unclear.

The maturation of the autophagosome involves two ubiquitin-like conjugations. The first of these is the covalent linkage of Atg12 to Atg5. Atg12 is activated in an E1-like reaction by Atg7 and transferred to Atg5 in an E2-like reaction by Atg10. The Atg12-Atg5 conjugate complexes with Atg16L; this complex dimerizes and associates with the exterior membrane of the phagophore[22,23]. The Atg12-Atg5-Atg16L dimer is essential for the formation of the autophagosome. In fact, Atg16L modified to contain a prenylation signal was shown to be targeted to the plasma membrane and was sufficient to direct a green-fluorescent-protein-tagged LC3-I to the PM where it was converted to LC3-Ⅱ by Atg3[24]. Recent work[25] using knockdowns of various SNAREs in HeLa cells has been interpreted to mean that certain SNAREs (VAMP7, syntaxin 7 and syntaxin 9) in conjunction with Atg16L may recruit vesicles from the PM (and perhaps, other membranes) for phagophore formation. The homotypic fusion of these vesicles leads to membrane expansion that facilitates the maturation of the autophagosome (i.e. recruitment of LC3-Ⅱ and dissociation of the Atg16L complex).

Atg7 acts as an E1 in a second ubiquitin-like conjugation that involves the orthologs of yeast Atg8, LC3 (microtubule-associated protein 1 light chain 3) and related proteins, γ-aminobutyric acid receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE-16)[23]. These soluble proteins are cleaved by one of a family of Atg4 cysteine proteases to reveal a C-terminal glycine. In the case of LC3, the cleaved protein, now called LC3-I, is activated by Atg7. The activated LC3-I is handed off to a specific E2-like enzyme, Atg3 and conjugated to the amino group of PE to produce the autophagosome bound lipoprotein, LC3-Ⅱ. There is evidence that the Atg12-Atg5-Atg16L dimer may act as an E3-like enzyme or at least, direct the location of the formation of LC3-Ⅱ.

In the autophagosome, LC3-Ⅱ is distributed to both the exterior and the lumen of the vesicle. Superficial LC3-Ⅱ is removed by cleavage of the PE by Atg4, while the luminal LC3-Ⅱ is digested along with the cargo[26]. Luminal LC3-Ⅱ can be preserved by inhibiting its lysosomal degradation with protease inhibitors or the proton pump inhibitor, bafilomycin A1. LC3-Ⅱ is often used as a marker for the autophagosome and as an index of autophagy. Although LC3-Ⅱ is essential for autophagosome biosynthesis, its precise role is not known. It has been suggested that it is involved in elongation of the organelle, whereas GATE-16 and GABARAP are involved further downstream[27]. It has also been suggested that LC3-Ⅱ may be involved in membrane closure[3]. It is clear that LC3-Ⅱ recruits the cargo adaptor proteins p62 (also called sequestosome 1, SQSTM1) and Nbr-1 and as such, participates in cargo selection (see below).

The final stages of the biogenesis of the autophagosome include the closure to form a double membrane vesicle. The yeast homolog of LC3-Ⅱ, Atg8, has been reported to have properties that make it a candidate for membrane closure[28]. However, it has recently been argued that the fusogenic activity of Atg8/LC3-Ⅱ depends on unphysiologically high concentrations of PE in the involved membranes[10]. The fusion of the autophagosome and lysosome is likely to be catalyzed by SNAREs. There is evidence that, in yeast, Vti1 is required for fusion of the vacuole and autophagosome and may have a similar role in mammals[29-31]; however, as has been pointed out by others[3], no factors that are specific for either closure or fusion have been identified yet. Since the SNAREs that have been proposed to be involved in membrane fusions during autophagy have been identified by loss or reduction of function and all of these SNAREs have other important cellular functions, the assignment of a specific role to a specific SNARE must be viewed cautiously.

CARGO SELECTION

Autophagy is a major cellular catabolic process for cytosolic proteins and is responsible for the degradation of ER, peroxisomes and mitochondria. The degradation of cytosolic proteins is not a random process. Instead, it is directed in part by the ubiquitination of candidate proteins and mediated by p62[32]. This multifunctional protein contains a ubiquitin-associated UBA domain, a LC3-interacting region (LIR) and a multimerization PBI domain. These domains allow p62 to capture ubiquitinated proteins and secure them to LC3-Ⅱ in the autophagosome. Because p62 can oligomerize, a larger number of ubiquitinated proteins can be tethered to a single molecule of LC3-Ⅱ. In addition, p62 can secure aggregates of superoxide dismutase (and probably other aggregates) independent of ubiquitination. Nbr-1 is an ortholog of the yeast cargo binding protein, Atg19. Nbr-1 binds ubiquitin and contains two LIR motifs and can act in concert or independently of p62. Alfy, also an ubiquitin binding protein, secures these proteins in the autophagosome by binding to PI3P and Atg5 as well as p62. The interplay of LC3-Ⅱ, p62, Nbr-1 and Alfy permits the selective autophagy of soluble ubiquitinated and aggregated proteins.

Pexophagy or the autophagic degradation of peroxisomes has been well described in yeast[1] and is known to occur in mammals[33]. However, except that the process involves p62, little is known about the mammalian process.

Both fungal and mammalian mitochondria undergo autophagic degradation a process referred to as mitophagy. In mammals, the E3 ligase parkin is recruited to mitochondria by PINK1 and plays an important role

in mitophagy[34]. Parkin directs the polyubiquitination of several proteins in the OM of uncoupled mitochondria and initiates the recruitment of p62 and HDAC6 (histone deacetylase 6)[35]. HDAC6 binds to polyubiquitin tracts and dynein motors and is involved in the formation of protein-organellar "aggresomes". Inclusion in perinuclear aggresomes may be preliminary to autophagy of mitochondria. VDAC1 (voltage-dependent anion-selective channel protein 1), one of three isoforms of an OM ion channel, is one of the OM proteins ubiquitinated by parkin. The importance of this conjugation is unclear. Geisler *et al*[36] have presented evidence that ubiquitination of VDAC1 is required for p62 binding and mitophagy. On the other hand, Narendra *et al*[37] have provided evidence, that although VDAC1 (but not the less common isoform, VDAC2) is ubiquitinated, this conjugation is unnecessary for either aggresome formation or mitophagy. Other OM proteins besides VDACs are ubiquitinated by parkin[35] and it is possible that they may be involved in VDAC-mediated mitophagy. Certain parkin mutations have been implicated in familial autosomal recessive Parkinson's disease (jPD), and several laboratories have found that mutations that are linked to the disease also fail to ubiquitinate OM proteins[35-37]. These observations have fueled interest in a link between mitophagy and jPD.

THE ROLE OF AUTOPHAGY

Autophagy occurs constitutively in all eukaryotic cells where it operates as a metabolic homeostatic mechanism[38]. Autophagy can be further activated in response to various physiological and pathological stimuli to either promote cell survival (e.g., starvation, oxidative stress)[4], or to act as a mode of cell death, type Ⅱ programmed cell death (e.g., during development)[39]. Defects in autophagy have been associated with various human diseases[40], and it is not possible to cover all the roles of autophagy in this review. Instead, the role of autophagy will be examined in myocardial ischemia (MI). Two factors affect the severity of MI injury: oxygen deprivation and depletion of metabolic substrates. Both are caused by occlusion of a coronary artery. HL-1 cells are an immortalized line of mouse atrial cardiomyocytes and are a convenient model for the study of MI. Recent studies indicate that autophagy has a cardioprotective role in these cells during ischemia[41]. HL-1 cells that are anoxic and deprived of glucose for 2 h were unable to carry out autophagy, and about 50% of the cells died[41]. However, autophagy (as judged by LC3-Ⅱ accumulation measured in presence of bafilomycin A1) continued at control levels and cell death was four times (only about 15%) that of the oxygenated controls. If the hypoxic cells were nourished with glucose, ATP levels increased to about 50% of controls, the accumulation of LC3-Ⅱ increased to about 120% of controls and cell survival approximated to control levels. On the other hand, inhibition of autophagy by 3-methyladenine (PI3K inhibitor) increased cell death to about 15%. That autophagy persisted after 85% of the cell's ATP was depleted suggests that it performs an important function, and the fact that the level of autophagy is inversely related to the amount of cell death suggests a protective role during MI. Similar results were obtained during reoxygenation after anoxia[41]. Three inhibitors of autophagy, 3-methyladenine, wortmannin, and a dominant negative Atg5 mutant, all reduced survival. The disease that the HL-1 cell ischemia/reoxygenation model is intended to mimic, acute MI, is much more complicated in that it also involves endothelial cells, smooth muscle cells and fibroblasts. Nonetheless, at least the cardiomyocytes seem to survive better when they can mount an autophagic response.

CONCLUSION

The precursor membrane to mammalian autophagosomes, the phagophore, appears to arise from multiple sources including the ER, OM and PM. Activation of appropriate signaling pathways leads to the initial step in the maturation of the autophagosome, inactivation of mTOR and its dissociation from a complex analogous to the yeast Atg1 complex. Subsequently, the Vps34/Vps15/beclin1 PI3P kinase assembles and associates with the phagophore membrane. The production of PI3P attracts a number of components of unknown function (e.g., WIPI-1, WIPI-2 and DFCP1) as well as components that lead to two ubiquitin-like conjugations. One results in the conjugation of Atg12 and Atg5. This conjugate subsequently forms a complex with Atg16L. The second ubiquitin-like conjugation involves the cleavage of LC3 to LC3-I and its subsequent conjugation to PE to form LC3-Ⅱ in the autophagosomal membrane. LC3-Ⅱ binds the adaptor proteins, p62, Nbr-1 and Alfy and their associated ubiquitinated protein or organellar cargo. The autophagosome then fuses into a vesicle by unknown processes, and the vesicle fuses with a lysosome causing the degradation of the cargo as well as LC3-Ⅱ and its adaptor proteins. Autophagy is critical for cellular maintenance and appears to be important in cell survival during MI and reperfusion as well as other cellular stresses.

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