



A current view of molecular dissection in autophagy machinery

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Abstract

Macroautophagy (hereafter called autophagy) is a highly conserved lysosomal pathway for catabolism of intracellular material in eukaryotic cells. Autophagy is also an essential homeostatic process through which intracellular components are recycled for reuse or energy production. The extremely regulated autophagy process begins with the formation of hallmarked double membrane bound organelles called autophagosomes which in turn fuse with lysosomes called autolysosomes and finally degrade the autophagic cargos. The multistages molecular machinery of autophagy is critically orchestrated by the action of a set of the autophagy proteins (Atg) and a supreme regulator, mTOR (mechanistic target of rapamycin). However, individual stages of autophagy are mechanistically complex and partially understood. In this review, the individual stages of autophagy are dissected, and the corresponding molecular regulation is discussed in view of current scientific knowledge of autophagy. This understanding of sequential events of autophagy machinery through this review may lead to great interest in the therapeutic potential for manipulating of autophagy in established diseases.

Keywords Molecular dissection · Beclin 1 · Connexins · ALR cycle

Introduction

Autophagy is an evolutionarily conserved cellular homeostatic process that facilitates nutrient recycling via lysosomal degradation of potentially harmful cytoplasmic entities. As a housekeeping process, autophagy constitutively executed under basal condition that controls metabolic and cellular homeostasis [2]. Based on the mechanisms by which the target substrates are delivered to lysosomes for degradation, three types of autophagy have been clarified—macroautophagy, chaperone-mediated autophagy, and microautophagy. The best-characterized variant of autophagy is macroautophagy [17]; hereafter, it simply referring as autophagy.

Key points

- Autophagy is a conserved lysosomal pathway in eukaryotic cells.
- Multistages of autophagy are orchestrated by a set of autophagy proteins.
- Regulation of individual stages of autophagy is complex to understand.
- This review may lead to the treatment options for established diseases.

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General regulation of autophagy machinery

As an adaptive catabolic process, autophagy is activated in response to a wide variety of cellular stresses, including nutrient deprivation and hypoxia [17]. To date, more than forty-one Atg proteins have been identified for the dedication and execution of autophagy [92]. Most of them identified by genetic screens in yeast have mammalian homologs suggesting that are evolutionarily conserved in mammals. The core machinery of the classical and best-characterized autophagy involves the following steps: initiation, vesicle nucleation, elongation, and enclosure for the formation of an isolation membrane (IM, previously known as phagophore), cargo recruitment in IM, completion of the double-membrane vesicles called autophagosomes (APs) in the cytosol, transport of AP and lysosomes on the microtubules (MTs), docking and fusion of the AP with lysosome to form autolysosome (AL), autophagic cargo degradation for recycling, and finally autophagic lysosome reformation (ALR) cycle (Fig. 1). In general, canonical autophagy occurs via class III phosphoinositide 3-kinase (PI3K) complex (PI3KC3)/mechanistic target of rapamycin complex 1 (mTORC1) signaling axis [2, 80] which directly regulates the autophagic activity through the phosphorylation and inhibition of Ser/Thr protein kinase, UNC51-like kinase 1 (ULK1), a part of the first protein complex involved in autophagy initiation [1].

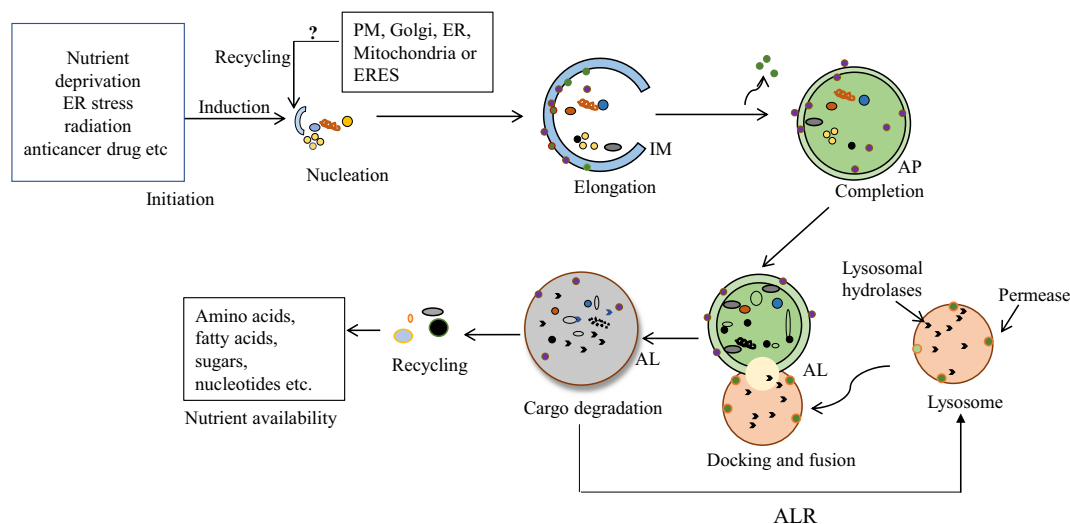


Fig. 1 A simply form of mammalian autophagy process. Cellular stress such as nutrient deprivation, ER stress, and radiation or anticancer drug are well known autophagy inducers. Autophagy initiates with IM formation (also known as phagophore). The IM appears to have several sources such as organelles-endoplasmic reticulum (ER), Golgi apparatus, mitochondria, or PM or their contact sites such as ER exit sites (ERES). Through a series of chain reactions, numerous molecular machinery

events of this IM are subsequently processed including nucleation, elongation, AP maturation, docking and fusion AP with lysosome, cargo degradation as well as ALR (autophagic lysosome reformation) cycle. Once the cargos are degraded, the degrading metabolites are exported to cytosol to be recycled for ATP production, biosynthesis new macromolecules as well as new organelles like lysosomes

Autophagy initiation: the activation of ULK1 complex

Autophagy is initiated by molecular network machineries that centrally involved a set of Atg proteins for modulating IM formation and AP maturation. Under cellular stress, ULK1 and PI3KC3 complexes are involved to initiate autophagy in mammalian cells [1, 2, 80]. The ULK1 complex consists of ULK1 itself and the noncatalytic subunits FIP200 (focal adhesion kinase (FAK) family kinase-interacting protein of 200 kD), Atg13 and Atg101 [84]. In starvation, ULK1 is activated by autophosphorylation at Thr180 in its kinase domain's activation loop [49]. This autophosphorylation is promoted by coassembly with other subunits such as Atg13 of the complex and by the conditions that induce autophagy. This coassembly in turn enhances the local ULK1 concentration and promotes their mutual autophosphorylation. Since Atg13 bridges ULK1 to the scaffolding protein FIP200 [35, 51], the recruitment of ULK1 to FIP200 with the help of Atg13 may be a major mechanism for ULK1 transautophosphorylation.

The lipid kinase PI3KC3 phosphorylates the lipid head group of PI (phosphatidylinositol) to generate PI3P (phosphatidylinositol 3-phosphate) [3]. Formation of PI3P is an essential early event in autophagy initiation, occurring just downstream of ULK1 [17]. Both protein complexes are regulated by phosphorylation, mainly due to mTORC1, the master regulator of cell growth and metabolism, and AMPK (AMP-activated protein kinase) in nutrient-sensing pathways [1, 2, 80] as well as ubiquitination [29]. Under available growth factors and nutrients conditions in the extracellular space, the PI3K/mTORC1 pathway is highly active; mTORC1

phosphorylates Atg13 [2] and prevents ULK1 activation by phosphorylating it at Ser757 residue. This Atg13 phosphorylation by mTORC1 inhibits the assembly of Atg13 with ULK1, by introducing steric and electrostatic repulsion into the binding sites on ULK1 [23]. However, during nutrient deprivation or limited growth factors, mTORC1 becomes inactive and can no longer repress the ULK1 complex and triggers autophagy [2, 80]. In response to energy deficits (e.g., glucose starvation or amino-acid deprivation) AMPK can also modulate ULK1 activity via fine-tuning of the phosphorylation status of ULK1 (Fig. 2). The ULK1 kinase provides proautophagic transduction signals by phosphorylating many substrate proteins [43]. The numerous substrates of ULK1 include itself and other subunits of the ULK1 complex; other elements of the core autophagy machinery, including PI3KC3 subunits and the sole conserved transmembrane protein Atg9 [43].

The source(s) of isolation membrane

Although morphological features of APs are basically common to conventional and alternative autophagy, the origin(s) of IMs has long been an enigma and debated [35], because of the key questions where does the IM membrane material come from and in turn how does it form to AP. Although many microscopic techniques such as scanning electron microscopy (SEM) and live-cell imaging provide detailed morphological data, the images are unable to reveal the autophagy dynamics, particularly where and how rapidly does the sequential completion of APs with demonstrating kinetics [27, 57]. These

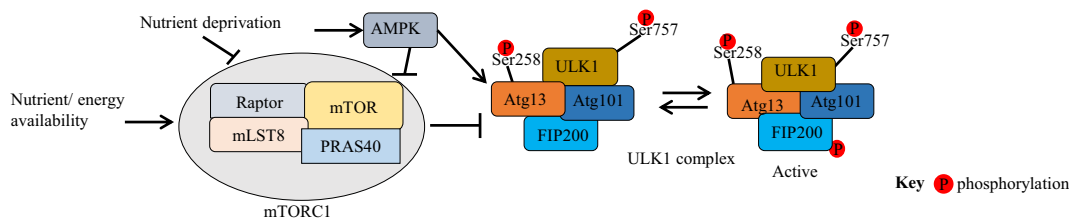


Fig. 2 Regulation of autophagy initiation by ULK1 complex. Under nutrient deprivation, autophagy is initiated by mTORC1 inhibition and AMPK activation, which in turn positively regulate ULK1 complex. Induction of ULK1 complex subsequently activates PI3KC3, which leads to PI3P synthesis in IMs. In nutrient rich conditions, mTORC1 represses autophagy activation by phosphorylation at Ser757 residue of ULK1 and at

Ser258 of Atg13. In response to nutrient deprivation, mTORC1 is released from ULK1 complex, leading to dephosphorylation of ULK1 and Atg13 and induction of autophagy. Activated ULK1 phosphorylates both FIP200 and Atg13 that events are necessary for autophagy initiation. In contrast, AMPK-dependent phosphorylation of ULK1 activates autophagy induction under energy-depleted condition

may suggest the occurrence of AP formation from multiple IM assembly sites or by effective vesicular trafficking from existing compartments and membranes. In these cases, the AP membranes appear to expand by phospholipid membranes derived from various sources rather than by de novo lipid synthesis [6]. Several organelles and their contact sites such as ER (endoplasmic reticulum) exit sites (ERES), mitochondria, ER-mitochondria contact sites, ER-Golgi intermediate compartment (ERGIC), Golgi apparatus, and plasma membrane (PM) have been suggested to serve as membrane donors for the growing IM in mammalian cells, but the exact mechanism mediating this process remains ambiguous [91]. Dependent on the induction conditions and possible contribution in autophagy stages, different membrane sources are as shown in Table 1.

As the largest organelle in the cell, the ER is always in close contact with other endomembrane compartments and establishes membrane contact sites (MCS) for facilitating signaling events. It has been suggested that IM expansion and elongation are found to intimately association with ER membrane via physically connected to an ER subdomain called omegasome (an omega-shaped DFCP1 enriched) [5]. In mammalian cells, the IM begins at a tubular outgrowth [5] of the

PI3P-positive domain of the ER omegasome [28]. Another evidence has revealed that ERES, the specialized ER regions are important mediators in the formation of APs [78]. Multipurpose membrane-traffic factors such as coat complexes COPII and Atg9-containing cytoplasmic vesicles (Atg9 vesicles) seem to have central roles in mammal autophagy initiation [13]. After synthesis and translocation into the ER, Atg9 vesicle arrives to the Golgi possible by COPII-coated vesicles [1, 28]. From here, the Atg9 vesicle is transported via Golgi membranes into other vesicles and/or compartments that contribute to the formation of the IM. An evidence also suggests that AP is nucleated by the coalescence of Atg9 and COPII vesicles [6].

The Golgi apparatus is proposed to be a pivotal membrane source for AP growth due to the association of the Atg9 [99]. However, others argued that mammalian Atg9 is seen to associate with many other compartments, including recycling endosomes, early endosomes, and late endosomes [67]. It is possible that these organelles all participate in AP formation. In starvation, Atg9 vesicles are also mobilized to the IM by TRAPPIII complex [47, 99]. The amount of Atg9 expressed in cells appears to regulate the frequency of AP formation [38]. Phosphorylation of the N-terminal domain of Atg9 by

Table 1 The organelles and their contact sites for AP membrane

Organelle and/or contact sites	Stimulatory condition	Contributing stage of APs
Endoplasmic reticulum (ER) omegasome [5, 91]	Fasting and amino acid starvation	Expansion and elongation of IM [32]
Outer membrane of mitochondria (OMM) [98]	Deprivation of serum and/or amino acid [44].	Expansion of IM [1; 6]
Mitochondria-associated ER membrane (MAM) contact site (ERES) [24; 25]	Rapamycin	Expansion of IM [83]
Trans-Golgi network (TGN)	Fasting and nitrogen starvation	Formation and expansion of IM and maturation of APs [67]
ER-Golgi intermediate compartment (ERGIC) [26]	Nutrient starvation	Generation of IM precursor [25]
Lipids of the plasma membrane (PM)	Serum starvation	Formation of IM [75]
Endosomes	Nutrient starvation	Membrane lipids recycling for AP formation [82]

an unknown kinase initiates autophagy and recruits Atg9 to the IM [20]. Thus, mammalian Atg9 is not a bulk component of APs but rather seems to recycle out of the IM prior to closure.

The generation of COPII transport-vesicles in ERES is also spatially, physically, and functionally linked to APs. Maday et al. [55] suggested that the generation of APs at DFCP1-positive subdomains of ER in the distal end of the axon is distinct from ER exit sites in primary neurons. Hence, ERES are the core elements in ER associated with IM formation and extension in mammalian cells [78]. However, emerging contradictory data indicates that only 30% of all APs are associated with the specialized regions of the ER [32]. In addition, the ERES protein TECPR2 (tectonin β -propeller-containing protein 2) which is mutated in HSP (hereditary spastic paraplegia) connects the COPII subunit SEC24D to microtubule (MT)-associated protein 1 (MAP1) light chain 3 (LC3) in human cells [39, 83].

Membrane traffic between the ER and the Golgi is bidirectional. COPII transport vesicles emerge from ERES, and most of the output of ERES is directed to the cis-Golgi via the ERGIC [78]. The transport of ERGIC structures from ERES to the Golgi apparatus may occur with the help of MTs for cargo sorting and recycling. As the donor membrane, the ERGIC acts as a sorting station for exchanging of dynamic membrane with COPI and COPII vesicles [78]. Thus, the generation of COPII vesicles from the ERGIC may participate to a special event for mobilization of autophagy-related membrane [24, 25]. For example, ERGIC supports the LC3 family protein lipidation [25, 83]. In addition, super-resolution imaging of mammalian autophagy initiation sites shows that Atg9 vesicles and the ULK1 complex subunit Atg13 coalesce with ERGIC components COPI and COPII [12].

In starvation-induced autophagy, mitochondria play a central role for translocation of Atg5 and LC3 to puncta localized in the outer membrane of mitochondria (OMM) [44]. Moreover, ER-mitochondria contacts, also called mitochondria-associated ER membranes (MAMs) are claimed to be the origin of IM in mammalian cells [44]. MAMs spatially overlapping with omegasomes have been claimed to serve as a cradle of IM and AP vesicle formation in close to the ER [28, 32]. Upon starvation, phosphorylation of Beclin 1, one of the components in PI3KC3, promotes the local production of PI3P in omegasomes that recruit early autophagic effector proteins such as WIPI1/2 (WD-40 repeat domain containing protein 1 and 2 that interact with phosphoinositide) which are essential for the formation of LC3-positive APs [72]. The contradictory argument is that the sources of AP membrane are obscure in mitochondria due to presence of mammalian Atg9 in transGolgi network (TGN) and late endosomes, but not to mitochondria [82], as Atg9-containing vesicles are an important source of membranes for AP formation.

The large surface area of plasma membrane (PM) may act as a massive membrane store that allows cells to synthesize AP at much higher rates than under basic conditions [75]. Because autophagy contributes to the turnover of PM proteins such as connexins (Cx) [75] that act as an important mediator for transport of nutrient and growth factors from the extracellular environment as well as for sensing pH. The transmembrane proteins, Cx, assemble to form PM gap junctions [75]. In normal conditions, connexin 43 (Cx43) is associated with AP precursor proteins such as PI3K complex or Atg16L1 as Cx-Atg complex at the PM prior to the formation of AP [75]. Here, the Cx-Atg complex acts as a negative regulator of autophagic flux [4]. During starvation, Atg14L (autophagy-related14-like; also called BARKOR), a component of PI3KC3 and Atg9 reach to Cx43-enriched PM and activates class III PI3K kinase that triggers to neutralizes the Cx inhibitory effect through internalization of Cx-Atg complex in presence of EPS15 (epidermal growth factor receptor substrate 15), an endocytic adaptor protein and deliver the preAP components to the sites of AP formation [75]. ER-MP contact sites (ER-PMcs) are also essential for AP biogenesis. ER-PMcs can tether to extended synaptotagmins proteins which are important for PI3P synthesis at the cortical ER membrane and adjust the AP biogenesis [61]. In mammalian cells, the endosomal system is extremely dynamic and generates several structurally and functionally distinct compartments, namely recycling endosomes (REs), late endosomes, and lysosomes. REs emerge from vacuolar sorting endosomes and divert cargoes towards the cell surface, the Golgi, or lysosome-related organelles. Hence, REs may also implicate in AP formation through Atg9 trafficking from PM [67]. As a positive regulator of AP formation, synexin18 (SNX18) is also required for regulating Atg9 trafficking from REs [82].

Vesicle nucleation: the activation of class III PI3K complex

The class III PI3K complex, PI3KC3 consists of its catalytic subunit type 3 (best known as vacuolar protein sorting, VPS34 lipid kinase in yeast), Atg14L, p150 (a myristoylated Ser/Thr kinase), Beclin 1 (BECN1: coiled-coil, moesin-like BCL2-interacting protein), and DFCP1 [double FYVE (Fab1-YotB-Vac1p-EEA1) domain-containing protein 1] [3, 37]. In order to achieve complete enzymatic and biological activity, PI3KC3 has to coordinate with extraregulatory factors (Fig. 3). Here, PI3KC3 and Beclin 1, a multivalent adaptor protein form two distinct complexes, namely Beclin 1-PI3KC3 complex I and II. In Beclin 1-PI3KC3 complex I, Atg14L crosslinks between Beclin 1 and the PI3KC3-p150-NRBF2 (nuclear receptor binding factor 2)-complex for stimulation both initiation and maturation of autophagosome formation.

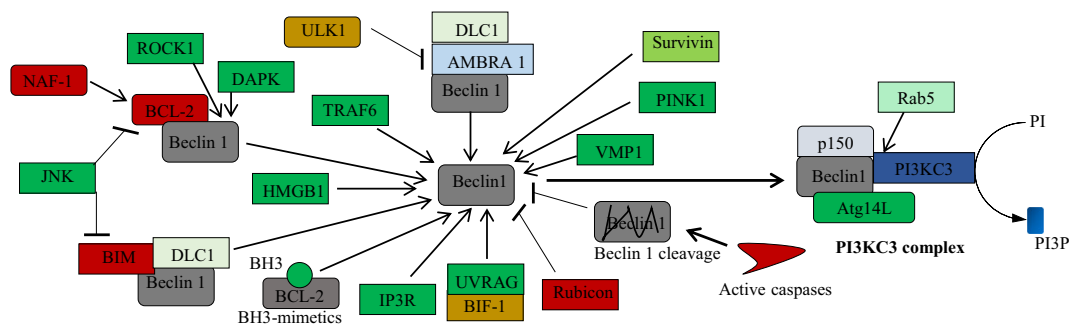


Fig. 3 Regulation of autophagy by the complex Beclin 1 network. The PI3KC3 comprises a multiprotein complex PI3KC3-p150-Beclin 1 associated with Atg14L, UVRAG, AMBRA1, or Rubicon to perform distinct roles in AP formation. Rubicon binds with PI3KC3 through UVRAG and represses AP maturation. Under nutrient rich conditions, PI3KC3 mostly associates with UVRAG involved in endosomal transport. Upon starvation, ATG14L replaces UVRAG to induce autophagy. AMBRA1 translocates from mitochondria to ER and associates with Beclin 1-PI3KC3-p150-Atg14L, which is essential for autophagy activation. Microtubules (MTs) contribute to sequestration of Beclin 1 through their association with DLC1 in the two complexes such as DLC1-Beclin 1-AMBRA1, and the other DLC1-Beclin 1-BIM. Upon stimulation of autophagy, activated ULK1 phosphorylates AMBRA1, thus releasing it from the Beclin 1 complex and allowing the recruitment of Beclin 1 into PI3KC3. BIF-1 interacts with Beclin 1 through UVRAG and functions as a positive mediator of PI3KC3 and promoter of Atg9 trafficking. Phosphorylation

of regulatory loop of BCL-2 and BIM by JNK1, releases Beclin 1. By binding BH3 domain of BCL-2, NAF-1 dissociates Beclin 1-BCL-2 complex within ER and positively regulates autophagy. Activated ROCK1 binds and phosphorylates at T119 of Beclin 1 and disrupts Beclin 1-BCL-2 interaction. DAPK phosphorylates at Thr119 in BH3 domain of Beclin 1 and promotes the dissociation of Beclin 1 from BCL-2. For dissociation of Beclin 1-BCL-2 complex, HMGB1 competes with BCL-2 for interaction with Beclin 1 and promotes ERK for phosphorylation of BCL-2. TRAF6 controls K63-linked ubiquitination of Beclin 1 to trigger autophagy. The full-length PINK1, Survivin and VMP1 interact with Beclin 1 and enhance autophagy. In addition, caspase-mediated cleavage of Beclin-1 inhibits autophagy. PI3KC3 phosphorylates PI (phosphatidylinositol) to synthesize PI3P in IMs, allowing for elongation of IMs and completion of APs. In addition, Rab5 also activates PI3KC3 for autophagy induction

The putative protein kinase p150 associates PI3KC3 with membranes via the interaction of its protein kinase domain to PI3KC3. Beclin 1 also interacts with a growing number of identified proteins in relatively unstable, transient or specific conditions (Table 2 and Fig. 3) for the localization and activity of PI3KC3-catalyzed PI3P production. The nucleation machinery of initial IM is critically dependent on local production of PI3P which is enriched in the inner surface of IM and marked by its binding protein, DFCEP1 in the PI3KC3. This production of PI3P in the IM in turn engages the recruitment of scaffold proteins such as WIPI1/2 and becomes a membrane platform for accumulation of AP proteins and subsequent IM elongation and completion [18]. The functioning of PI3KC3 in autophagy requires its translocation to the IM, which is driven by its unique Atg14L subunit [33, 37]. Upon induction of autophagy, the ULK1 complex localizes to the IM site for regulation of the vesicle nucleation machinery (Fig. 3). The PI3KC3 catalytic subunit contains a major ULK1 phosphorylation site at Ser249 [51]. The ULK1 kinase transduces proautophagic signals by phosphorylating many substrate proteins including ULK1 itself and other subunits of ULK1 complex as well as the elements of core autophagy machinery subunits of PI3KC3 [43]. The PI3KC3-associated IDR (intrinsically disordered region) protein AMBRA1 (activating molecule in Beclin 1-regulated autophagy) acts as a ULK1 substrate [15]. Upon activation of autophagy, the kinase ULK1 phosphorylates AMBRA1 which is proposed to activate PI3KC3 by releasing Beclin 1 from its MTs [15, 54]. However, in Beclin 1-PI3KC3 complex II, Beclin 1 and the

PI3KC3-p150 complex are bridged by UVRAG (UV radiation resistance-associated gene) for regulation of autophagy [58]. In addition, the small guanosine triphosphatase (GTPase) Rab5 also binds to and activates PI3KC3 for autophagy induction [75].

Elongation: two ubiquitin-like conjugation systems

The elongation step of the IM is controlled by two evolutionary conserved ubiquitin-like conjugation systems. The first one involves Atg12-Atg5-Atg16 complex (called Atg12 conjugation system) and the other includes LC3-phosphatidylethanolamine (LC3-II) conjugation system. In Atg12 conjugation system, Atg12 is activated by Atg7 (an E1-like ubiquitin-activating enzyme), transferred to Atg10 (an E2-like ubiquitin-conjugating enzyme). Atg7 binds to the C-terminal glycine of Atg12 through its active cysteine site to form an intermediate complex via a thioester bond. Subsequently, Atg12 is induced by ATP hydrolysis and transferred to Atg10. Finally, the C-terminal glycine of Atg12 covalently binds to an internal lysine residue of Atg5 to form Atg12-Atg5 conjugate which is constitutive and irreversible process [43]. Then Atg12-Atg5 conjugation further interacts noncovalently with a coiled coil protein Atg16 to form Atg12-Atg5-Atg16 complex into a tetramer structure by self-oligomerization. This Atg12 complex coordinates with the formation of APs when LC3-phosphatidylethanolamine (PE) insertion is necessary in the membrane. The LC3-II conjugation system involves the cleavage and lipidation of MAP1-LC3

Table 2 Regulation of autophagy by Beclin 1 complexes

Molecules	Regulatory mechanisms of autophagy
Positive regulators of Beclin 1	
AMBRA1	AMBRA 1 binds to the DLC1 and concentrates Beclin 1 at the dynein motor complex. During the initiation of autophagy, phosphorylated AMBRA 1 by ULK1 is dissociated from its motor complex and transported to the ER. Thus, PI3K-III complex assembly occurs in the ER and forms AP [15]. It also regulates posttranslational modification of Beclin 1 with ubiquitin. [54]
UVRAG and BIF-1	Beclin 1 interacts with UVRAG and BIF-1. BIF-1 forms a complex with Beclin 1 through UVRAG to facilitate the activation of the PI3KC3 and initiates autophagy. UVRAG also acts on AP maturation independently from its interaction with Beclin 1 [37]
PINK1	Mitochondrial full-length PINK1 interacts with Beclin 1 and enhances mitophagy [42].
Positive regulators of Beclin 1-BH3-BCL-2 complex	
DAPK	DAPK phosphorylates BH3 domain of Beclin-1 at Thr119, isolates free Beclin 1 from its complex for AP formation [98].
JNK	JNK1 directly phosphorylates the regulatory loop of BCL-2 to release free Beclin 1 from the ER associated BCL-2/Beclin 1 complex [54].
AMPK and MK2	Phosphorylation of Beclin 1 by MK2 at Ser90 [91] as well as AMPK at Ser93 and Ser96 enhances AP formation [81].
BH3-only Proteins	BH3-only proteins such as Bad/Bax as well as BH3-mimetics interact with BH3-binding groove of BCL-2 or BCL-X _L . This binding provokes AP formation by disrupting competitively the inhibitory interaction between Beclin 1 and BCL-2/BCL-X _L and liberating Beclin 1 [54, 98].
HMGB1	HMGB1 competes with BCL-2 for interaction with Beclin 1 at the c23 and c45 vicinal sites of HMGB1. It also activates ERK1/2 that phosphorylates BCL-2 and dissociates the complex [77].
CaMKII	CaMKII directly phosphorylates Beclin 1 at Ser90 and AP formation [94].
ROCK1	Activated ROCK1 binds and phosphorylates Beclin 1 at T119; and finally disrupts and dissociates Beclin1-BCL-2 complex [21]
TRAF6	TRAF6 catalyzes the initiation of K ⁶³ poly-ubiquitin (Ub) chains on Lys in the BH3 domain of Beclin 1. This Ub chains impede the BCL-2 binding to Beclin 1, thereby it triggers Beclin 1 mediated AP formation. A20 hydrolyses the K ⁶³ poly-Ub chain and binds it with BCL-2 [1, 85].
XBP-1	XBP-1 mRNA splicing triggers autophagy through transcriptional activation of Beclin 1 [21].
miR-30b	miR-30b directly binds to Beclin 1 and greatly impacts on IM nucleation [97].
Others	VMP1 and Survivin have also been known as Beclin 1-binding proteins but the mechanisms are not clear
Negative regulators of Beclin 1	
UVRAG-Rubicon	Rubicon forms a complex with UVRAG when associated with Beclin 1. Rubicon also binds to the PI3K-III complex and this binding with Beclin 1 impairs AP formation [99].
BCL-2	Binding of BCL-2 and Beclin 1 inhibits autophagy by disrupting PI3KC3 binding to Atg14L (or UVRAG). BCL-2 also promotes Beclin 1 homodimerization, which prevents heterodimerization with Atg14L or UVRAG. [21]
IP3R	It increases the proximity to both Beclin 1 and BCL-2 separately and finally form a triparental IP3R/Beclin 1/BCL-2 complex that decreases the free Beclin 1 available to induce autophagy.
NAF-1	NAF-1 selectively binds to BCL-2 and stabilizes the BCL-2-Beclin 1 interaction at the ER
BIM	BIM sequesters with Beclin 1 onto MTs, which inhibits AP formation. In parallel, phosphorylated BIM by activated JNK releases Beclin 1, allowing Beclin 1 to be recruited to omegasome membranes. [53]
AKT/EGFR	AKT phosphorylates Beclin 1 at Ser234 and Ser295 to form Beclin 1/14-3-3/vimentin complex, thereby inhibiting autophagy [91]. Phosphorylation Beclin 1 at Tyr229, Tyr233 and Tyr352 by activated EGFR promotes Beclin 1 homodimerization and Rubicon binding.
Active caspases	Active caspases e.g., caspase-3 cleave Beclin 1 and destroy its ability to induce autophagy [85].
Calpain	Active calpain cleaves Atg5 [96] and Beclin 1 and this proteolysis inhibits AP formation [85].
mTORC1	mTORC1 inhibitory phosphorylation of Atg14L at Ser3, Ser223, Ser233, Ser383, and Ser440 positions in the C-terminal and ULK1 at Ser757 controls autophagy [2].
miR-216b/miR-376b	miR-216b [8] and miR-376b [97] attenuate autophagy by downregulating Beclin 1 level.

DLC1: dynein light chain 1; *BIF-1*: Bax-interacting factor 1; *Rubicon*: RUN domain and cysteine-rich domain-containing Beclin 1-interacting protein; *BH3*: BCL-2-homology domain 3; *BCL-2*: BCL-2, B cell CLL/lymphoma 2 (apoptosis regulator); *BCL-X_L*: B cell lymphoma-extra-large; *DAPK*: death associated protein kinase; *JNK*: c-Jun-N^{terminal} kinase 1; *AMPK*: AMP-activated protein kinase; *MK2*: MAPKAPK2; *Bad*: BCL-2 associated agonist of cell death; *HMGB1*: High mobility group protein B1; *MAPK*: mitogen-activated protein kinase; *CaMKII*: Calcium/calmodulin-dependent protein kinase II; *ROCK1*: Rho-associated, coiled-coil-containing protein kinase 1; *PINK1*: PTEN-induced putative kinase 1; *TRAF6*: Tumor necrosis factor receptor-associated factor 6; *NAF-1*: Nutrient-deprivation autophagy factor-1; *IP3R*: Inositol 1,4,5-trisphosphate receptor; *VMP1*: Vacuole membrane protein 1; *BIM*: BCL-2 interacting mediator of cell death; *XBP-1*: X box-binding protein 1; *miRNA*: microRNA; *EGFR*: Epidermal growth factor receptor; *BARA*: Beta-alpha repeated autophagy-specific; *A20*: deubiquitinating enzyme

[43, 86]. In mammalian cells, LC3 is first processed by cleaving near the C-terminal glycine residue with cysteine protease, Atg4 to form the cytosolic soluble LC3-I. Atg7 activates LC3-I and delivers it to the E2-like Atg3. LC3-I is eventually cleaved and converted to a membrane-associated form LC3-II through covalent conjugating with the target lipid PE (known as “lipidation”) via an amide bond. Although LC3 primarily functions in the cytoplasm at the sites of autophagosome and autolysosome formation, several groups have reported that a distinct pool of LC3 also resides in the nucleus [63]. This nuclear LC3 serves as a reserve for the cytoplasmic pool and shifts to the cytosol when soluble cytoplasmic LC3 is transformed to the lipidated type and incorporated into autophagic membranes. During starvation, a nuclear pool of LC3 is deacetylated at K49 and K51 by the activated nuclear histone deacetylase SIRT1 [34] leading to its redistribution to the cytoplasm. This deacetylated LC3 interacts with TP53INP2 (tumor protein p53-inducible nuclear protein 2), also known as DOR (diabetes- and obesity-regulated gene), a nuclear cofactor of thyroid hormone receptors and returns to the cytoplasm with TP53INP2, where it is able to interact directly with ATG7 to form a LC3/TP53INP2/ATG7 complex in the cytoplasm and undergo PE conjugation to autophagosome formation [52, 95]. Amazingly, TP53INP2 regulates adiposity by promoting the sequestering of glycogen synthase kinase 3 β (GSK3 β) via an autophagy-dependent and endosomal sorting complexes required for transport (ESCRT) machinery pathway-dependent manner [76]. LC3-II, the cleaved product of LC3-I, is specifically inserted within the inner and outer elongating AP membrane and remains on completed APs until fusion with the lysosomes (Fig. 4). As a result, LC3 regulates the size of the AP due to its ability to determine membrane curvature. LC3-II on the cytoplasmic face of ALs can be delipidated by Atg4 and recycled [43].

Cargo recruitment in autophagosomes

In response to starvation, nonselective bulk autophagy degrades the cytosolic materials such as damaged organelles or aggregated proteins by sequestering the specific cargo with autophagy receptors in IM (Fig. 5). These receptors are able to bind specifically to cargos tagged with autophagy degradation signals through their LIR (LC3 interacting regions) leading to the engulfment of cargo by the AP membrane [100]. In general, LIR motifs can interact with autophagy regulatory proteins of the LC3/GABARAP family [22]. In mammalian cells, more than twenty autophagy receptors have been identified, and p62 [also known as sequestosome-1 (SQSTM1)] is one of the most common autophagy receptors [14]. p62 promotes the degradation of autophagic cargos in at least three ways: interacting with LC3 via its LIR motif, associating with early AP formation sites by recruiting Atg proteins through its PB1 domain, and binding to ubiquitinated proteins via its UBA (ubiquitin-binding domain) to form protein aggregates [11, 70]. Moreover, p62 may also provide ubiquitinated cargo recruitment to the AP via its interaction with both ubiquitin and LC3 for selective degradation [70]. Interestingly, some other proteins that have LIR and UBA domain such as FYCO1 (FYVE-coiled coil domain containing protein 1), a member of the RUFY (RUN) and FYVE domain-containing protein) family, HDAC6 (histone deacetylase 6), and ALFY (autophagy linked FYVE protein) interact with p62 [60, 65]. As a nuclear scaffold protein, the FYVE domain on ALFY may also recognize PI3P membranes and Atg12 complex and play a role in the assembly of IM recruitment. In addition, another p62 interacting partner, NBR1 (neighbor of BRCA1 gene 1 protein) is also needed ubiquitinated protein for constitutive autophagic degradation [36, 41].

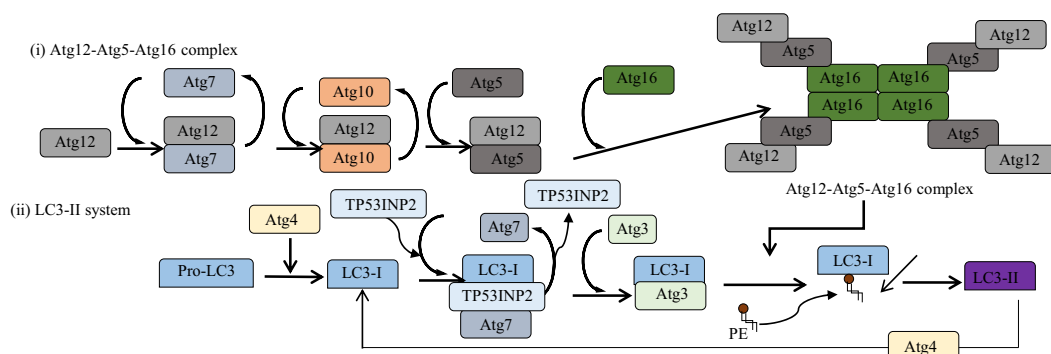
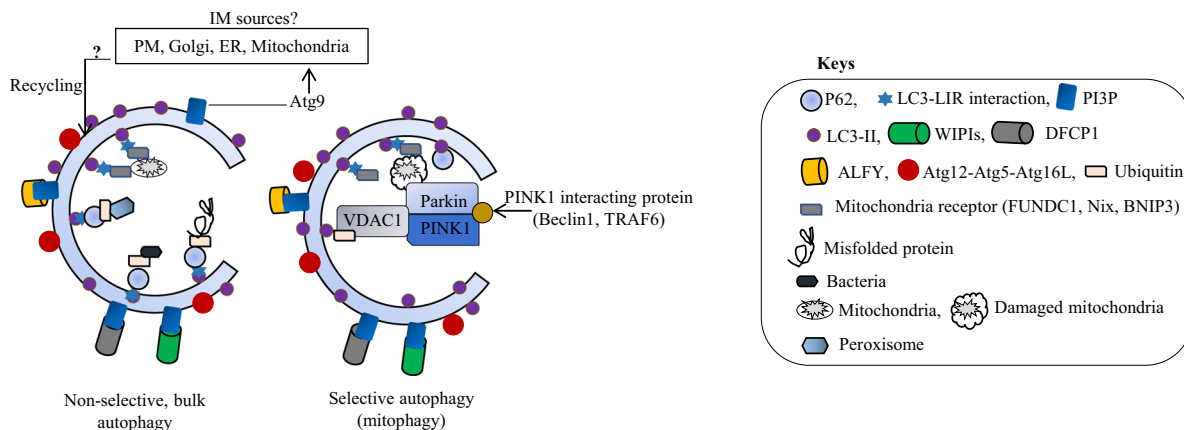


Fig. 4 Involvement of two ubiquitin-like conjugation systems in elongation of IM. In Atg12-Atg5-Atg16L1 conjugation system, Atg7 (an E1 enzyme) and Atg10 (an E2 enzyme) are responsible for conjugating Atg12 onto the acceptor lysine of Atg5. The conjugate of Atg12-Atg5 forms a complex with self-oligomerized Atg16L1 resulting in a formation of multimer Atg12-Atg5-Atg16L1 complex. In second conjugation

system, LC3 is synthesized as a pro-LC3 and processed to cytosolic form LC3-I. LC3-I is activated by Atg7 and TP53INP2, transferred it to Atg3 (an E2 enzyme) and finally conjugated with PE, generating membrane bound form LC3-PE (LC3-II). Atg4 (cysteine protease) can convert LC3-II into LC3-I for its recycling

a Cargo recruitment in IM



b Autophagy cargo receptors

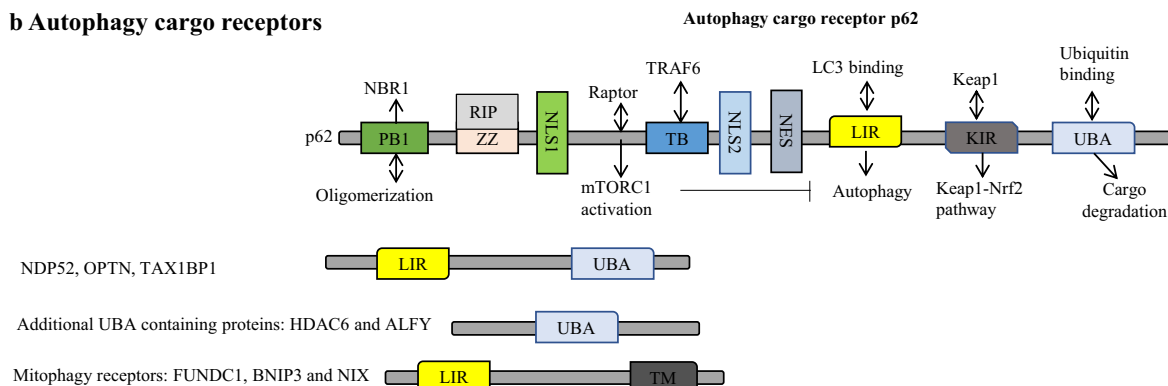


Fig. 5 Cargo recruitment in IM and autophagy receptors. Although the sources of IM are contradicted, different membrane pools may contribute to the formation of IM. Atg9 vesicles dynamically traffic to and from IM. PI3P generated by PI3KC3 at IM can be sensed by WIP1/2 and DFCP1 for regulation of Atg9 localization. Autophagy cargo receptors contain specific ligand-binding domain (e.g., UBA) for polyubiquitination and a short LIR sequence responsible for LC3 binding. Polyubiquitinated proteins are recognized by the receptor p62 that binds to LC3. ALFY sequesters the specific cargo by bridging the cargo-receptor complex to core Atg proteins. By PI3P binding, ALFY associate with p62 and Atg5. In Atg-12-Atg5-Atg16L1 complex bound, ALFY stimulates lipidation of LC3 to LC3-II. Selective autophagy can distinguish and direct specific cargos to the lysosome. Autophagy receptors contain a short LIR sequence responsible for LC3 binding. Several cargo receptors have been described for mammalian mitophagy. For example, NIX acts as a

mitophagy receptor; it has a LIR motif but lacks an UBA domain and is localized with in the OMM. Mitochondria depolarization promotes to PINK1 activation and phosphorylation of ubiquitin and Parkin. Receptors link mitochondria targeted for degradation to the IM. Domain architecture of autophagy receptors containing distinct ubiquitin-binding domains (light blue) and LC3-interacting motifs (LIR, yellow). The SLRs (sequestosome-1-like receptors) constitute of p62, NBR1, NDP52, TAX1BP, and OPTN (optineurin) as well as the known mitophagy receptors FUNDC1, BNIP3, and NIX (BNIP3L) in mammals. Here PB1, Phox and Bem1 domain; RIP, receptor-interacting protein; ZZ, ZZ-type zinc finger domain; NLS1 and NLS2, nuclear localization signals 1 and 2; NES, nuclear export signal; LIR, LC3 interacting region; KIR, Keap1 interacting region; UBA, ubiquitin-associated domain; TB, TRAF6-binding domain and TM, transmembrane domain

In case of selective autophagy, p62 is dependent on its PB1-domain-driven polymerization; thus, ubiquitin interactions with other proteins are also important for the delivery of p62-mediated cargos [46]. Following discovery of p62 as a sequestosome-1-like receptor (SLR) for selective autophagy, the NBR1 [41], the nuclear dot protein 52 kDa (NDP52), optineurin, and Tax1-binding protein 1 (TAX1BP1) [88] are also important selective autophagy receptors. These autophagy receptors use LIR-motif-dependent interactions to target their cargos for autophagic degradation (Fig. 5). Although selective autophagy receptors obscure a clear specialization, they often cooperate with each other in selecting a specific cargo, e.g., NBR1 interacts with p62 and plays an essential

role in p62-dependent sequestration and degradation of aggregated proteins [41].

For higher organisms, mitophagy receptors and their mediated mechanisms are probably the best studied so far. When mitochondria are depolarized by uncouplers such as CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone), PINK1 (PTEN-induced putative kinase 1) is activated and accumulated at the outer mitochondrial membrane (OMM) [48, 66]. The activated PINK1 recruits Parkin (ubiquitin E3 ligase) from the cytosol to damaged mitochondria leading to polyubiquitination [42], which are recognized and clustered through polymerization by the p62 [42, 64]. Following mitochondrial translocation, Parkin induces two types of

polyubiquitin chain models. Firstly, Parkin-mediated K⁴⁸ chains on specific OMM proteins stimulate proteasome-dependent degradation of Mitofusin1/2 and mitochondrial protein import receptor, TOM20 [16]. Secondly, Parkin also triggers K⁶³ and K²⁷ ubiquitin chains that initiate p62-mediated a signaling cascade to activate mitophagy [81]. The ion channel VDAC1 (voltage-dependent anion-selective channel 1) in the OMM has also been reported to be a target for Parkin-mediated K⁶³ and K²⁷ polyubiquitination and promotes mitophagy [12, 42]. Furthermore, three integral receptor proteins in the OMM such as two homologous BH3-only proteins, BNIP3 (BCI-2/adenovirus E1B 19-kDa interacting protein 3), and NIX (also known as BNIP3L) as well as FUNDC1 that all have a LIR motif in their cytosolic N-terminal domain can directly link the damaged mitochondria to AP membranes in Ub-independent mitophagy pathways [68] (Fig. 5).

Transport of autophagosomes and position of lysosomes

Autophagosomes (APs) are supposed to occur haphazardly throughout the cytoplasm and transport bidirectionally in their maturing process. During AP maturation, interconnected MT network often serves as tracks for intracellular movement. MTs powered by specific motor proteins such as kinesin or dynein families utilize the energy generated by ATP hydrolysis [40]. In general, kinesins move cargo to peripheral that is plus-end of MTs, whereas dyneins involve in AP movement towards the minus-end in perinuclear center. Simple schematic molecular assemblies of both MT-mediated movements of APs are illustrated in Fig. 6 a and b.

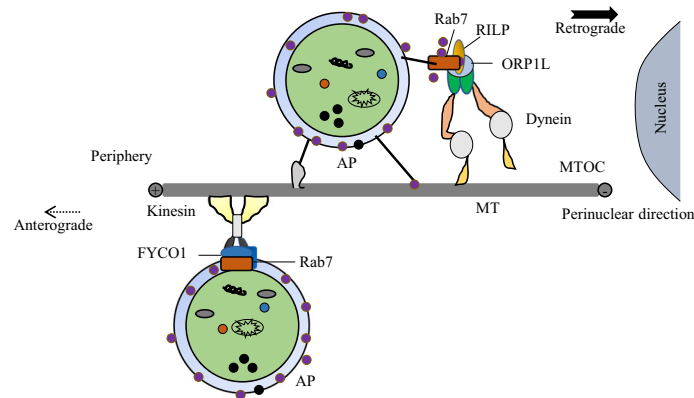
The Rab (Ras-related protein in brain) family small GTPases particularly Rab7 are the important coordinators of membrane trafficking in eukaryotic cells. Rab7 interlinks MT motors with the position of lysosomes and late APs through several downstream effector proteins such as RILP (Rab7-interacting lysosomal protein), ORP1L (oxysterol-binding protein (OSBP)-related protein 1 L), a cholesterol sensor, and FYCO1. Rab7 involves in both the minus-end and the plus-end direction transport on MTs based on the downstream interacting effector protein(s) and motor proteins such as RILP/dynein or FYCO1/kinesin [69] respectively. FYCO1 localizes on perinuclear cytosolic vesicles but upon starvation, it moves to the cell periphery in a MT-dependent manner [69]. In the plus-end directional movement along the MT, the C-terminal FYVE and Golgi dynamic (GOLD) domains of FYCO1 interacts with Rab7, LC3 and PI3P on AP membrane and its N-terminal coiled coil domain with the kinesin motor protein [69]. In the minus-end directional movement, Rab7 interacts with RILP, ORP1L, and dynein simultaneously in order to convey transport of APs to the perinuclear region [93] (Fig. 6a).

The proper positioning of lysosomes and APs at perinuclear area is required for efficient fusion between these two organelles. Starvation causes an increase in the intracellular pH which induces lysosome relocation to perinuclear area and transports late APs to the same region of cell with the help of MTs [54]. Since lysosomes are predominantly found in the perinuclear regions, it is reasonable that the movement of mature APs along MT tracks towards the lysosomes is appeared to fuse [73]. Interestingly, mTORC1 signaling regulates the positioning of lysosomes [33, 54] and the intracellular pH (pHi) influences the docking of AP-lysosome in cells [59]. In response to amino acids, mTORC1 locates on peripheral lysosomes close to its upstream signaling elements, whereas in starvation associated higher pHi, the isolated mTORC1 and lysosomes are preferentially clustered in the perinuclear area. This elevated pHi condition also inhibits mTORC1 activity and favors the dissociation of ULK1 from mTORC1 complex and facilitates the docking of lysosomes with APs. More interestingly, pHi elevation also diminishes the recruitment of lysosomal attached KIF2B and KIF1B- β (kinesin family member 2B and 1B- β) as well as ARL8B [ADP ribosylation factor (ARF)-like GTPase 8B] to MTs [59]. Depletion of KIF2B and KIF1B- β disrupts centrifugal movement of lysosomes along MTs (Fig. 6b), and downregulation of ARL8B restricts lysosome transport to the cell periphery [59].

Fusion of autophagosome with lysosome

After acquiring docking machinery, APs fuse with endocytic vesicles and lysosomes. The double-membranous AP docks and fuses with lysosome to form single-membranous autolysosome (AL) [79]. It is also evident that prior to fusion with lysosome, APs merge with late endosomes to form amphisomes that deliver cargo in autophagic vesicle [59]. The machinery aspect of this fusion process is coordinated by three sets of protein families: Rab GTPases, HOPS (homotypic vacuole fusion and protein sorting) tethering complexes, and autophagosomal SNAREs (soluble N-ethylmaleimide-sensitive factor-attachment protein receptors), e.g., syntaxin17 (STX17). Late endosome- and lysosome-localized Rab7 is essential for membrane trafficking from late endosome to lysosome as well as AP-lysosome fusion [59]. Several Rab7 downstream effectors such as RILP, ORP1L, FYCO1, and PLEKHM1 (pleckstrin homology domain-containing family M member 1) have been characterized [69]. PLEKHM1 involves in AP-lysosome fusion through complex formation with Rab7, HOPS, and LC3 on late endosomes and lysosomes [62]. ORP1L binding to Rab7 in the presence of RILP negatively regulates fusion [93]. RUFY4 (RUN and PI3P-interacting FYVE domain-containing protein 4) promotes both autophagic flux and the tethering of APs with lysosomes [87]. Rubicon and UVRAG

a Transport of AP



b Positioning of AP and Lysosome

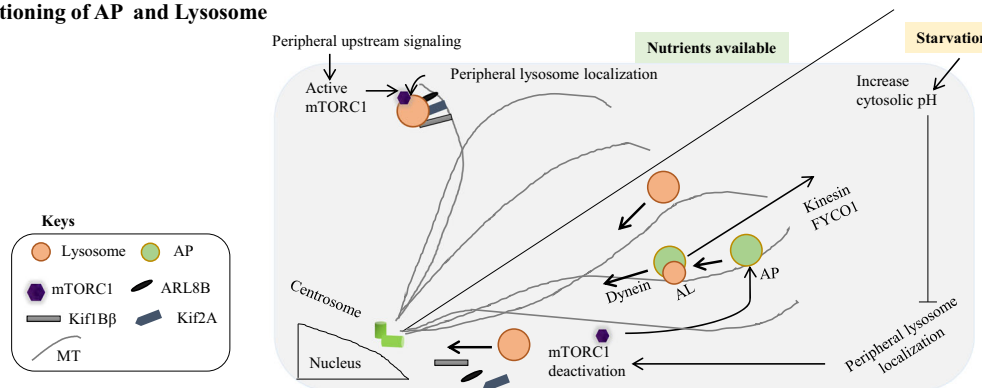


Fig. 6 Transport of autophagosome (AP) on microtubule; positioning of lysosome and AP. Microtubules (MTs) organize the substantial organelle positioning throughout the autophagy steps. The extremities of MT have two sides namely minus-end in perinuclear region and plus-end in cell periphery. The movement of APs along MTs depends on a precise balance between dynein, a minus-end-directed motor protein and kinesin, a plus-end-directed motor (Fig. 6a). Under sufficient nutrient conditions, lysosomal localized mTORC1 is transported towards plus end cell periphery where it is kept active. Under starvation intracellular pH increases, and recruitment of ARL8B and kinesin KIF2A to lysosomal membrane are inhibited; this favors the centripetal movement of lysosomes and

transport of APs in a perinuclear region close to the MT-organizing centre (MTOC). At the same time, mTORC1 is inhibited by ULK1, favoring the formation of new APs. Newly formed APs move along MTs in perinuclear directions as a result of opposing activities of dynein and kinesin/FYCO1. Thereafter, the APs cluster in a perinuclear region close to centrosome, where they fuse with lysosomes. Rab7 links APs to a MT motor through FYCO1 to facilitate kinesin driven movement towards the cell periphery. FYCO1 binds to LC3 and PI3P, a component of AP membrane as well as links to kinesin. Rab7 also binds to RILP and ORP1L in order to mediate dynein-driven movement towards the perinuclear region under normal cholesterol conditions

involve in endocytic transport and AP-lysosomal fusion through Rab7 [99] although they have opposite effects. OATL1 (ornithine aminotransferase-like 1, also called TBC1D25) is recruited onto APs through direct interaction with LC3, and OATL1 overexpression has been shown to inhibit AP-lysosome fusion. The generation of phosphatidylinositol 4-phosphate (PI4P) by PI3P phosphatases on APs is crucial for AP-lysosome fusion in mammals [89]. PI4KII α (phosphatidylinositol 4 kinase type 2- α) normally localizes to both perinuclear region and TGN. Upon starvation, PI4KII α exits from TGN, disperses into cytoplasm and some PI4KII α localize to APs in a palmitoylation dependent manner for AP-lysosomal fusion [89]. In addition, INPP5E (inositol polyphosphate-5 phosphatase E) [31] also promotes the fusion step in autophagy (Fig. 7). The lysosomal membrane-localized INPP5E mediates the conversion of phosphatidylinositol (3,5)-bisphosphate [PI(3,5)P₂] to PI3P

which is required for fusion. Lysosomal INPP5E activity has shown to be ultimately required for cortactin (CTTN) phosphorylation which leads to polymerize actin followed by AP-lysosome fusion [31]. Since the cytoskeleton plays a role in AL formation, several evidences suggest that actin involves in AP-lysosome fusion. For example, HDAC6 recruits the CTTN-dependent actin remodeling machinery, which in turn assembles the actin-network that stimulates the fusion step. PIKFYVE (1-phosphatidylinositol 3-phosphate 5- kinase) resided on lysosomes reverses the action of INPP5E and generates PI(3,5)P₂ from PI3P.

The AP-anchored SNARE proteins such as syntaxin17 (STX17), SNAP-29 (synaptosome associated protein 29), and VAMP8 (vesicle associated membrane protein 8) can interact with each other to form a highly energetically favorable complex. Moreover, several tethering factors such as HOPS complex, PI3P-binding protein, Atg14L, TECPR1

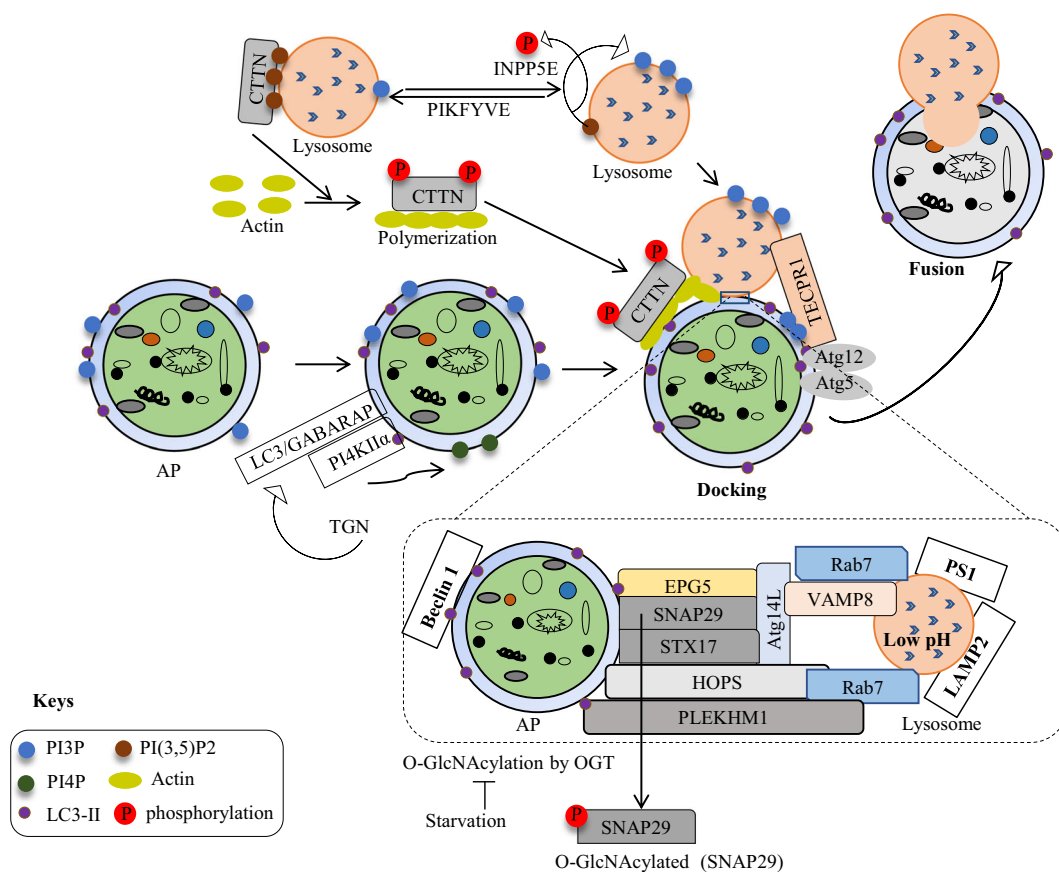


Fig. 7 Docking and fusion of autophagosome with lysosome. Cortactin (CTTN) binds with lysosomal localized PI(3,5)P₂. During fusion step, cellular INPP5E are partially localized on lysosomes where it converts PI(3,5)P₂ to PI3P. As a result, INPP5E decreases the amount of PI(3,5)P₂ level and releases CTTN from trapped lysosomal membrane. This released CTTN in turn is phosphorylated by other kinases and the activated CTTN acts as a promoter for stabilization of actin polymerized filaments on lysosome, an event required for fusion step. On the other hand, PIKfyve resides on lysosomes reverses the action of INPP5E and generates PI(3,5)P₂ from PI3P. TECPR1 on lysosome interacts with Atg12-Atg5 complex and binds to PI3P on the outer membrane of AP, thus functioning as a tethering factor for AP-lysosome docking. LC3/GABARAP recruits palmitoylated PI4KII α from TGN to APs, and this PI4KII α produces PI4P, an essential phospholipid for APs-lysosome

fusion. In machinery aspect of AP-lysosome fusion process, three sets of protein families involve: Rab GTPases, HOPS tethering complexes and autophagosomal SNAREs such as STX17, SNAP-29, and VAMP8. The recruitment of EPG5 on late endosomes/lysosomes together with Rab7 and VAMP8 causes a tethering action by binding to LC3-II and STX17/SNAP29. The adaptor protein PLEKHM1 interacts with Rab7, HOPS-SNARE complexes and LC3 and/or GABARAP proteins to facilitate AP-lysosome fusion. Under normal conditions, SNAP-29 is O-GlcNAcylated which is generated by O-linked β -N-acetylglucosamine (O-GlcNAc) transferase (OGT). O-GlcNAcylated SNAP-29 has a decrease binding affinity for its partner SNAREs. This modification is suppressed by starvation and a reduced level of O-GlcNAcylated SNAP-29 acts as a signal for the assembly of SNAP-29-containing transSNARE complexes, thus stimulating autophagy

(tectonin β -propeller-repeat-containing protein 1), and EPG5 (Ectopic P-granules autophagy protein 5) help SNARE proteins for physically driving the AP-lysosomal fusion (Fig. 7). As a tethering factor, Atg14L acts by directly binding to a binary STX17-SNAP29 complex on APs and facilitates its interaction with VAMP8 for promoting STX17-SNAP29-VAMP8-mediated fusion. Absence of TECPR1 in cells results in impaired autophagic flux and accumulated APs which apparently unable fuse efficiently with lysosomes. TECPR1 also interacts with the subunits of lipidated LC3 (LC3-II) complex in an Atg12-Atg5-conjugation dependent manner. EPG5 identified as a Rab7-VAMP7/8 interactor on endosomes/lysosomes determines

the specificity of fusion between APs with endosomes/lysosomes [71, 90]. Interestingly, EPG5 also binds to LC3 through its LIR motif and assembles the STX17-SNAP29 complex on APs [45, 59]. Upon starvation, the recruitment of STX17 from the cytosol to matured APs mediates fusion by binding partners HOPS complex, SNAP29, and lysosomal R-SNARE VAMP8 [37]. Furthermore, modification of O-GlcNAc (O-linked N-acetylglucosamine) in SNAP29 negatively regulates SNARE-dependent fusion [30]. Consequently, knockdown of O-GlcNAc transferase or mutating SNAP29-O-GlcNAc sites promotes SNAP29-containing SNARE complex formation and enhances the fusion step.

Autophagic lysosome reformation

After fusion with matured lysosome, the intraAP constituents get degraded and released the degraded end products such as amino acids and fatty acids into the cytosol, thereby, causing a local rise in nutrient availability for energy production. These phenomena lead to reactivation of mTORC1 signaling pathway and regeneration of mature lysosomes from ALs in a dynamic remodeling process known as autophagic lysosome regeneration (ALR) [9, 10]. During ALR, tubular structures are extruded from AP membrane and several small vesicles are generated from these tubules called protolysosomes. Initially, the protolysosomes are in neutral pH and absent in lysosomal luminal proteins. Subsequently, the protolysosomes convert into nascent lysosomes by acquiring acidity and lysosomal luminal proteins through ALR, and lysosomal homeostasis is maintained [9, 10]. Although mTORC1 is inhibited and autophagy is induced in short-term starvation, prolonged starvation, and/or nutrient availability by degrading autophagic cargo, reactivation of mTORC1 signaling acts as a negative feedback mechanism to avoid excess autophagy. As part of mTORC1 mechanism, the protolysosomal expansions dynamically emerge from ALs and eventually detaches from the AL as renewed lysosome pool by converting into functional mature lysosomes. Based on proteomic analysis of purified ALs, three key molecular regulatory pathways have been identified to regulate ALR and lysosome homeostasis such as (i) clathrin and phosphatidylinositol (4,5)-biphosphate, [PI(4,5)P₂] mediating budding of tubules, (ii) motor protein KIF5B (kinesin family member 5B) driving tubule structure elongation, and (iii) large GTPase Dynamin 2 (Dyn2) mediating scission of protolysosomes (Fig. 8).

Phosphatidylinositol (PI) such as PI(4,5)P₂ and PI4P play essential roles in controlling lysosomal tubule initiation. Clathrin and its associated adapter protein complexes AP2 and AP4 mediate the formation of PI(4,5)P₂-enriched microdomains on ALs. Here, one clathrin molecule recruits two AP2 proteins and one AP2 molecule recruits two PI(4,5)P₂ molecules. Thus, AP2 links clathrin to PI(4,5)P₂ in order to form PI(4,5)P₂-enriched microdomains in the clathrin lattices on AL membrane. Intriguingly, PI(4,5)P₂ appears on the main body of ALs and on reformation tubules by two different PI4P kinases such as PI4P 5-kinase type 1 beta (PIP5K1 β) and PIP5K1 α . Here, PIP5K1 β initiates AL tubulation by generating membrane PI(4,5)P₂-enriched microdomains from PI4P at the AL surface, and induces membrane budding by recruiting clathrin and its associated proteins AP2 and AP4. Another related kinase, PIP5K1 α localized in the leading edges of tubules produce PI(4,5)P₂ in protolysosomes aided for tubule scission. Besides PI(4,5)P₂, PI4P, and PI3P also involve in different

stages of ALR. PI4K variant PI4KIII β produced PI4P on lysosomes also regulate lysosomal membrane tubule production. In ALR, the generated PI(4,5)P₂ from PI4P by PIP5Ks acts in both restricted clathrin recruitment at lysosomal membrane and vesicular scission at the tips of tubules. MT-based motor protein, KIF5B, plays essential roles in transport of lysosome and tubulation of AL [19]. Lysosomal PI3P produced by PI3KC3 complex plays an important role in the scission of protolysosomes in ALR [58]. HSP autosomal recessive gene products, SPG15 (spastic gait 15 protein, also called spastizin) and SPG11 protein (also called spatacsin) also act as PI3P-binding proteins that may pivotally regulate lysosomal tabulation in ALR [7]. Moreover, spastizin and spatacsin are also essential components for the initiation of lysosomal tubulation [9]. However, it remains unclear the detailed mechanisms of how these phospholipids and their kinases regulate ALR. Dyn2 involves in scission of protolysosome through binding to PI(4,5)P₂ and forming microtubule bundles. Genetic depletion or treatment of cells with dynasore, a chemical inhibitor of dynamins, Dyn2 prevent lysosomal tubular scission resulting in decreased lysosomal pools. Mechanistically, a set of elegant studies demonstrate that mTOR regulates generation of lysosomal PI3P produced by PI3KC3 complex, and this PI3P in turn regulates the scission of ALR tubules [9, 10, 58]. When mTOR activity is inhibited, the omegasomal PI3P pool increases, and autophagy is initiated [33]. However, when mTOR is reactivated at the termination of autophagy, the lysosomal pool of PI3P increases due to activation of PI3KC3 complex and results in scission of ALR tubules [74]. Thus, PI3KC3 with different complexes in different localizations acts as a regulatory node for coordinating initiation and termination of autophagy (Fig. 8). However, lysosome homeostasis and autophagic function are also regulated by transcription factor EB (TFEB), a protein belonging to the MiTF/TFE family of transcription factors. In addition to inhibiting autophagy, mTORC1 regulates lysosomal biogenesis through the TFEB-dependent pathway [56]. In normal feeding conditions, TFEB is associated with Rag GTPases at the lysosomal surface and undergoes phosphorylation (at residues Ser211 and Ser142) by mTORC1. mTORC1 also promotes the secretion of phosphorylated TFEB to the cytosol and the interaction with chaperones, 14-3-3 proteins [56], thus stabilizing TFEB in the cytosol and preventing its nuclear translocation [56]. Conversely in starvation, mTORC1 and Rag GTPase are inhibited, mTORC1 dissociates from its lysosomal surface, and TFEB no longer undergoes mTORC1-associated phosphorylation. As a result, phosphorylated TFEB-14-3-3 complex is no longer available, TFEB diffuses from the surface of lysosomes to undergo nuclear translocation and TFEB induces lysosomal-autophagy gene expressions [56].

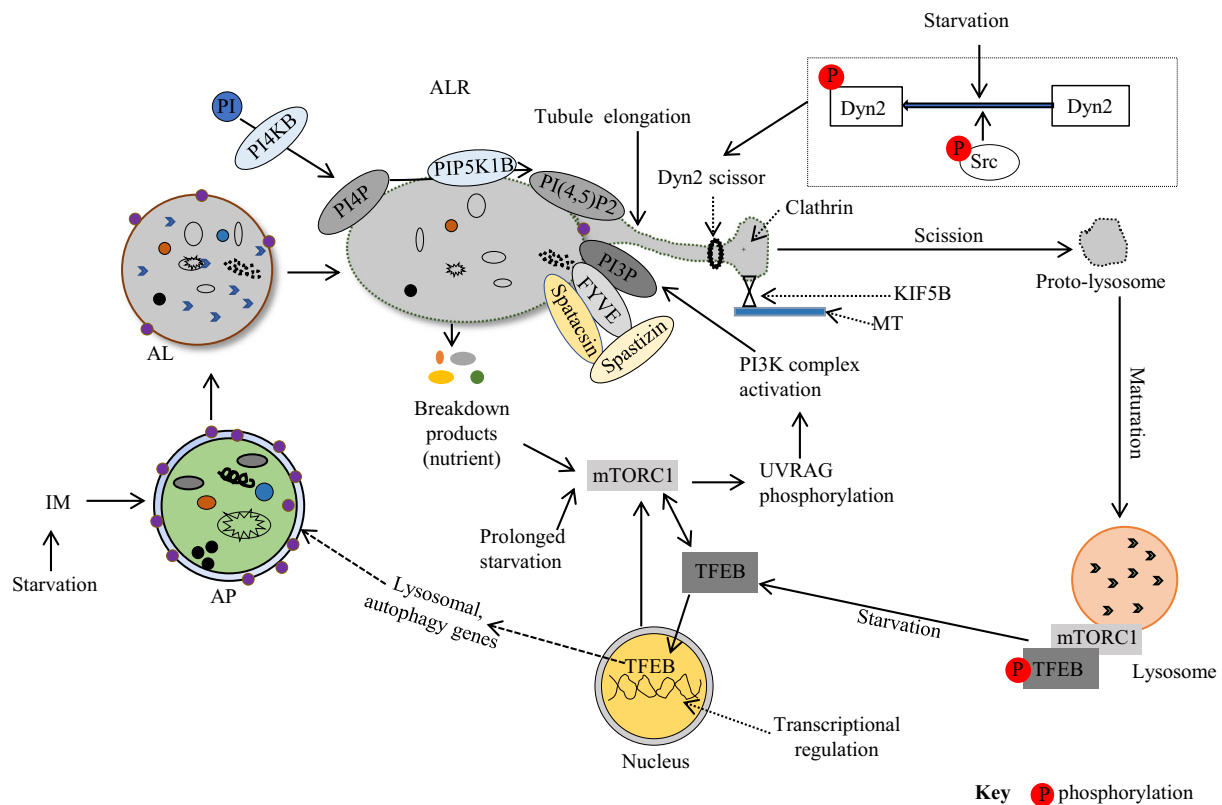


Fig. 8 Autophagic lysosome reformation cycle. At autolysosome (AL), PI4KB converts PI to PI4P, which suppresses uncontrolled lysosomal tubulation from ALs and facilitates lysosome budding organized by clathrin and the GTPase Dyn2. The localization of spatzin-spatacsin complex on lysosome/AL is governed by interaction of spatzin FYVE domain and PI3P, and this complex is an important component for initiation of lysosome budding. The conversion of PI(4,5)P2 from PI4P by PIP5K1B is an essential event for tubule elongation. It is also known that Dyn2 is phosphorylated by starvation as well as Src kinase, a nonreceptor protein tyrosine kinase and this activated Dyn2 acts as scissor for separation of proto-lysosome from ALR [50]. In prolonged starvation, energy

sources are rapidly generated by degradation of autophagy cargo in ALR pathway which in turn activate mTORC1 and reform lysosomes efficiently. The phosphorylated mTORC1 acts as a regulator of UVRAG phosphorylation and PI3K complex activation in order to increase lysosomal PI3P formation that appears to be critical for tubule scission, a process regulated by Dyn2. In starvation, mTORC1 is inhibited and dissociated from its lysosomal surface. As a result, TFEB is no longer mTORC1-associated phosphorylation, diffuse it from lysosomal surface to nuclear translocation and induces lysosome-autophagy gene expressions

Conclusions and future perspectives

In this review, it is summarized the findings regarding several sequential steps of autophagy process. Understanding about the variation of IM sources are in cell-, tissue specific, or in different contexts to environmental or pathological conditions. Thus, different conclusions reached by the different laboratories might be in part due to different experimental approaches and techniques used in the various laboratories. The tissue and cell specificity as well as time-dependent manner of the dissection of autophagy must be addressed in future studies. Answering these questions will provide the opportunity to develop new therapeutic strategies in order to manipulate autophagy for combating established diseases including cancer and neurodegeneration. Moreover, the precise involvement of individual steps of autophagy in human individual diseases remains partially understood. Thus, precise dissecting of autophagy process in specific disease contexts and determining

whether manipulation of these steps in autophagy are more beneficial future goals.

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Author's contributions AAB contributed in the writing, editing, and revised of whole manuscript.

Compliance with ethical standards

Conflict of interest The author declares that he has no competing interests.

Ethics approval and consent to participate The MS is a review paper only and does not involve human participants, human data, or human tissue. So, ethics approval and consent to participate are "Not applicable".

Consent for publication “Not applicable”.

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