Review

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Getting ready for building: signaling and autophagosome biogenesis

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Abstract

Autophagy is the main cellular catabolic process responsible for degrading organelles and large protein aggregates. It is initiated by the formation of a unique membrane structure, the phagophore, which engulfs part of the cytoplasm and forms a double-membrane vesicle termed the autophagosome. Fusion of the outer autophagosomal membrane with the lysosome and degradation of the inner membrane contents complete the process. The extent of autophagy must be tightly regulated to avoid destruction of proteins and organelles essential for cell survival. Autophagic activity is thus regulated by external and internal cues, which initiate the formation of well-defined autophagy-related protein complexes that mediate autophagosome formation and selective cargo recruitment into these organelles. Autophagosome formation and the signaling pathways that regulate it have recently attracted substantial attention. In this review, we analyze the different signaling pathways that regulate autophagy and discuss recent progress in our understanding of autophagosome biogenesis.

Keywords Atgs; autophagosome biogenesis; autophagy; mTOR; signaling
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See the Glossary for abbreviations used in this article.

Introduction

Autophagy is a catabolic process by which proteins and organelles are delivered to the lysosome for degradation. As a self-degrading process that is conserved from yeast to man, autophagy is well established as a survival mechanism that maintains cellular homeostasis under normal growth conditions and enables adaptation under stress [1]. It has also been implicated in disease, development, and cell differentiation [2–4]. Although beneficial under normal conditions, autophagy can be detrimental in diseases such as cancer and neurodegenerative disorders. Therefore, cells have developed control mechanisms that tightly regulate their autophagic activity. More than 30 proteins have been identified as related to autophagy (Atgs), most of them directly associated with autophagosome biogenesis [5,6]. According to the current view, autophagy is a progressive process initiated by the elongation of a membrane to form a cup-shaped phagophore into which autophagic cargo is sequestered, a process that initially seemed random but now is mostly regarded as selective. The autophagic membrane is further expanded to its final size and, once sealed, results in a mature double-membrane autophagosome, the outer membrane of which subsequently fuses with the lysosome to create an autolysosome. The autophagosomal content is then degraded and recycled.

This review covers recent progress made in understanding the early stages of autophagy. The external signals and environmental conditions that regulate autophagy—such as growth factors and nutrient deprivation—and the signal transduction pathways involved in such regulation will be discussed, as well as changes in intracellular homeostasis that trigger the autophagic process, such as oxidative stress and internal energy levels. Finally, we will analyze new mechanistic insights into autophagosome biogenesis.

Regulation of autophagy

Eukaryotes have developed signaling networks that control transcription, translation, and protein modification to adapt to changing environmental conditions. At times of shortage, cells need to save energy and nutrients by maintaining basal and essential activities. As part of the cellular response to such conditions, autophagy—a major cellular catabolic process—is subjected to tight regulation by a network of canonical and unique signaling cascades. It is therefore important to examine not only cues originating within the cells, but also signaling initiated in response to external changes (Figs 1 and 2). Autophagy is known to be mainly under the control of the key regulator of cell homeostasis, the Ser/Thr kinase TOR (target of rapamycin), in yeast, or mTOR in mammals [7]. TOR is found in two distinct protein complexes, TORC1 or TORC2 [8,9]. Although both TOR complexes regulate cell metabolism, only TORC1 is directly linked to the regulation of autophagy.

Extracellular cues

Amino acid starvation The most extensively characterized inducer of autophagy is amino acid deprivation (Fig 1A). The absence of specific amino acids such as leucine and glutamine strongly induces

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Glossary

ALFY	autophagy-linked FYVE protein
Ambra1	activating molecule in Beclin1-regulated autophagy
AMPK	5′ AMP-activated protein kinase
ATM	ataxia-telangiectasia mutated
BAR	Bin1/amphiphysin/Rvs167
Bcl2	B-cell lymphoma 2
DFCP1	Double FYVE-containing protein 1
EGFR	epidermal growth factor receptor
eIF2α	eukaryotic initiation factor 2α
ER	endoplasmic reticulum
ERGIC	ER Golgi intermediate compartment
FIP200	200-kDa focal adhesion kinase family-interacting protein
FoxO	forkhead-box transcription factor class O
FYCO1	FYVE and coiled-coil domain containing 1
FYVE	Fab1, YOTB, Vac 1, EEA1
GAB2	GRB2-associated binding protein 2
GABARAP	GABA receptor-associated
GAP	GTPase activating protein
GARP	Golgi-associated retrograde protein
GATE16	Golgi-associated ADPase enhancer of 16 kDa
GCN	general control non-derepressible
GLUD	glutamate dehydrogenase
GRB2	growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GUVs	giant unilamellar vesicles
HOPS	homotypic fusion and protein sorting complex
IKKβ	inhibitor of nuclear factor κB kinase
IL-1β	interleukin 1β
IFN-γ	interferon γ
IP3R	IP3 receptor
JNK	c-JUN NH2-terminal kinase
LC3	light chain 3
LKB1	liver kinase B1
LPS	liposaccharide
LRS	Leucyl-tRNA synthetase
MyD88	myeloid differentiation factor 88
NBR1	neighbor of BRCA1 gene
NO	nitric oxide
NOS	nitric oxide synthase
NOX4	NADPH oxidase 4
Nrf2	nuclear factor erythroid 2-related factor 2
PDK1	phosphoinositide-dependent kinase 1
PERK	protein kinase RNA-like endoplasmic reticulum kinase
pex19	peroxisomal membrane protein
PI3KC1/3	phosphoinositide 3-kinase complex 1/3
PIP$_2$	phosphatidylinositol 4,5-bisphosphate
PKB	protein kinase B
PKR	protein kinase R
PtdIns3P/PI3P	phosphatidylinositol 3-phosphate
PTEN	phosphatase and tensin homolog
Rag	Ras-related GTP-binding protein
RalA	Ras-like protein
ROS	reactive oxygen species
SH3BP4	SH3-domain binding protein 4
SNARE	soluble NSF attachment protein receptor protein
SNX18	sorting nexin 18
STAT3	signal transducer and activator of transcription 3
SUVs	small unilamellar vesicles
TECPR1	tectonin β-propeller repeat containing 1
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF6	tumor necrosis factor receptor-associated factor 6
TRAPPIII	transport protein particle
TRIF	toll/interleukin-1 receptor homology domain-containing adaptor inducing interferon-β
TSC1/2	tuberous sclerosis protein 1 and 2
Ulk1	UNC-51-like kinase 1
UVRAG	UV irradiation resistance-associated gene
VAMP7	vesicle-associated membrane
Vps34	vacuolar protein sorting 34
WASH	Wiskott–Aldrich syndrome protein WASP and SCAR homolog
WIPI1/2	WD repeat proteins interacting with phosphoinositides 1/2

autophagy, whereas others have a lesser effect. Several mechanisms have been proposed as part of the amino acid sensing system, all of which finally mediate autophagic activity through TOR. A decline in amino acid content is initially sensed at the plasma membrane, and, in yeast, amino acid transporters were suggested to sense extracellular amino acid concentration to regulate TOR activity [10]. However, most studies in yeast induce autophagy by nitrogen starvation, and it has been recently demonstrated that sensing of nitrogen differs from that of amino acids [11]. A reduction of extracellular amino acids levels was shown to induce autophagy in a GCN2-GCN4-dependent manner, whereas how the lack of external nitrogen is sensed remains unknown.

In mammalian cells, the G protein-coupled receptors (GPCRs) T1R1/T1R3 have been implicated in the extracellular sensing of amino acid availability, which leads to autophagy induction mediated by a decrease in mTOR activity [12]. Leucyl-tRNA synthetase (LRS) was recently shown to sense increases in intracellular leucine levels and to mediate TOR activation by slightly different mechanisms in yeast and mammalian cells [13,14]. Moreover, leucine also regulates mTOR activity through glutamate dehydrogenase (GLUD) [15]. In addition to its leucine-mediated regulation, mTOR is regulated by α-ketoglutarate, which is produced during glutamine metabolism [16]. Notably, glutamine plays a key role in the internalization of essential amino acids by the bidirectional transporter SLC7A5-SLC3A5 [17].

Upon starvation, the low amino acid concentration is sensed by Rag GTPases on the lysosomal surface [18,19], which then form inactive heterodimers of RagA/RagB bound to RagC/RagD [19]. Under amino acid-rich conditions, the Rag complex, together with a multi-protein signaling complex known as the Ragulator and the vacuolar protein pump v-ATPase, targets mTOR to the lysosome for its activation [18,20]. It is tempting to speculate that the localization of active mTOR on the lysosomal membrane allows it to be directly regulated by free amino acids produced by protein degradation within the lysosome. Indeed, the v-ATPase was suggested to serve as a link between mTOR and the amino acids generated by the lysosome [20]. Active mTORC1 is localized mainly on the lysosomal membrane [21,22]; under amino acid deprivation, inactivation of the Rag complex causes its detachment from raptor (part of mTORC1) and separation of mTOR from the lysosome and its activator Rheb, resulting in autophagic stimulation [23]. An important connection between the Rag complex and tumorigenesis was recently suggested, as this complex is negatively regulated by the tumor suppressor SH3BP4 [24]. Sabatini and co-workers also identified a protein complex termed GATOR, which is comprised of the subunits GATOR-1 and GATOR-2. GATOR-1 is a GAP for RagA/RagB, mediating

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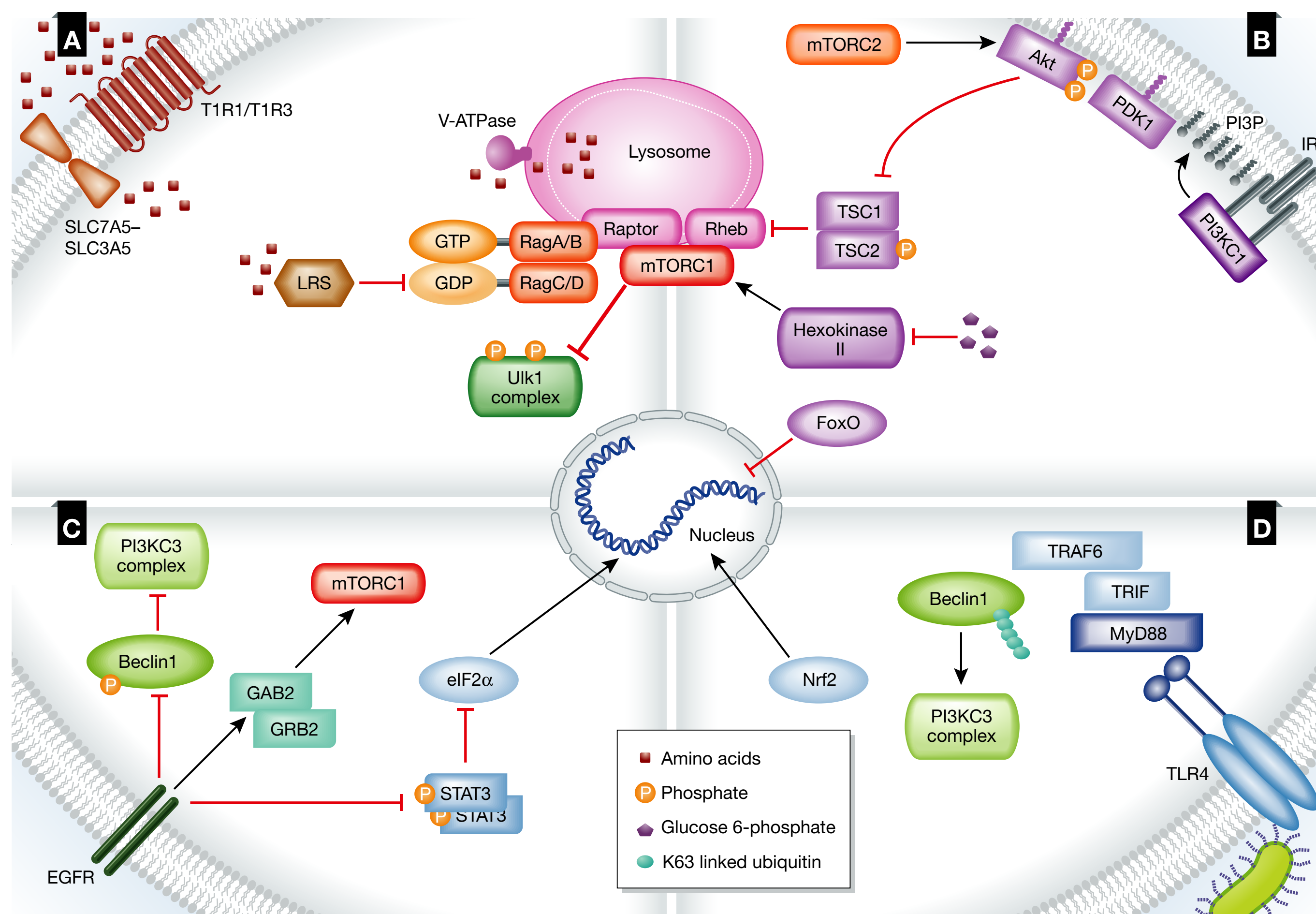

Figure 1. Regulation of autophagy by extracellular cues.
(A) Amino acids are key regulators of autophagy. When they are in excess, mTORC1 is targeted to the lysosomal membrane, where it is activated by Rheb and inhibits autophagy through phosphorylation of Ulk1 complex subunits. (B) Binding of insulin to its receptor (IR) activates mTOR via the PI3KC1/Akt/TSC pathway, inhibiting autophagy. The expression of autophagy-related proteins is inhibited after the inhibition of FoxO transcription factors by Akt. Glucose 6-phosphate inhibits the activity of hexokinase-II, an mTOR activator, inhibiting autophagy. (C) Activation of EGFR by its ligand inhibits autophagy directly by the phosphorylation of Beclin1 or indirectly via GRB2 and GAB2, as well as via the phosphorylation of STAT3, which releases eIF2α to induce the expression of autophagy-related proteins. (D) TLR4 is activated upon binding of LPS, leading to the recruitment of adaptor proteins to the plasma membrane. As a consequence, TRAF6 is recruited, resulting in the Lys63-linked ubiquitination of Beclin1, allowing it to bind PI3KC3 and induce autophagy. Nrf2 is activated, up-regulating the expression of p62.

See Glossary for definitions and the text for details.

mTORC1 sensitivity to amino acids [25]. Importantly, mutations in the GAP activity of GATOR-1 are associated with human cancer.

A more direct mechanistic connection of TOR to autophagy is illustrated by its ability to phosphorylate the Atg1 complex (ULK1 complex in mammals) [5]. In yeast, TOR binds and phosphorylates Atg13, detaching it from the Atg1 complex, whereas in mammals, it regulates the constantly assembled Ulk1 complex—Atg13, ULK1, and FIP200—through direct binding and phosphorylation of Atg13 and Ulk1 [26–29]. Under unfavorable conditions, the Atg1 complex in yeast is activated by the release of Atg13 from inactive TOR, whereas in mammals, the whole Ulk1 complex is activated by its detachment from mTOR [30]. Ulk1 can be then auto-phosphorylated and phosphorylate Atg13 and FIP200, triggering complex activity in the initial steps of autophagosome biogenesis [29].

An important link exists between mTORC1, the Rag GTPase complex, and the scaffold protein p62, which is also an autophagic cargo receptor [31]. p62 recruits TRAF6, an E3 ubiquitin ligase that is essential for activation of mTOR through Lys63 ubiquitination [32]. It would therefore be interesting to determine whether p62 serves as a molecular switch modulating mTOR activity during changes in growth conditions. If this is indeed the case, it could explain why p62 mediates mTOR activity and autophagic inhibition under normal conditions, yet sequesters cargo for lysosomal degradation upon autophagic induction. Phosphorylation of Bcl2 by JNK-1 was recently shown to induce its dissociation from Beclin1, enabling it to form the PI3KC3 essential for autophagosome formation [33,34], thereby providing another important insight into the mechanism by which starvation induces autophagy.

Insulin and glucose starvation At high glucose concentrations, autophagy is down-regulated through insulin receptor signaling [35]. Binding of insulin to its receptor activates PI3KC1 to generate

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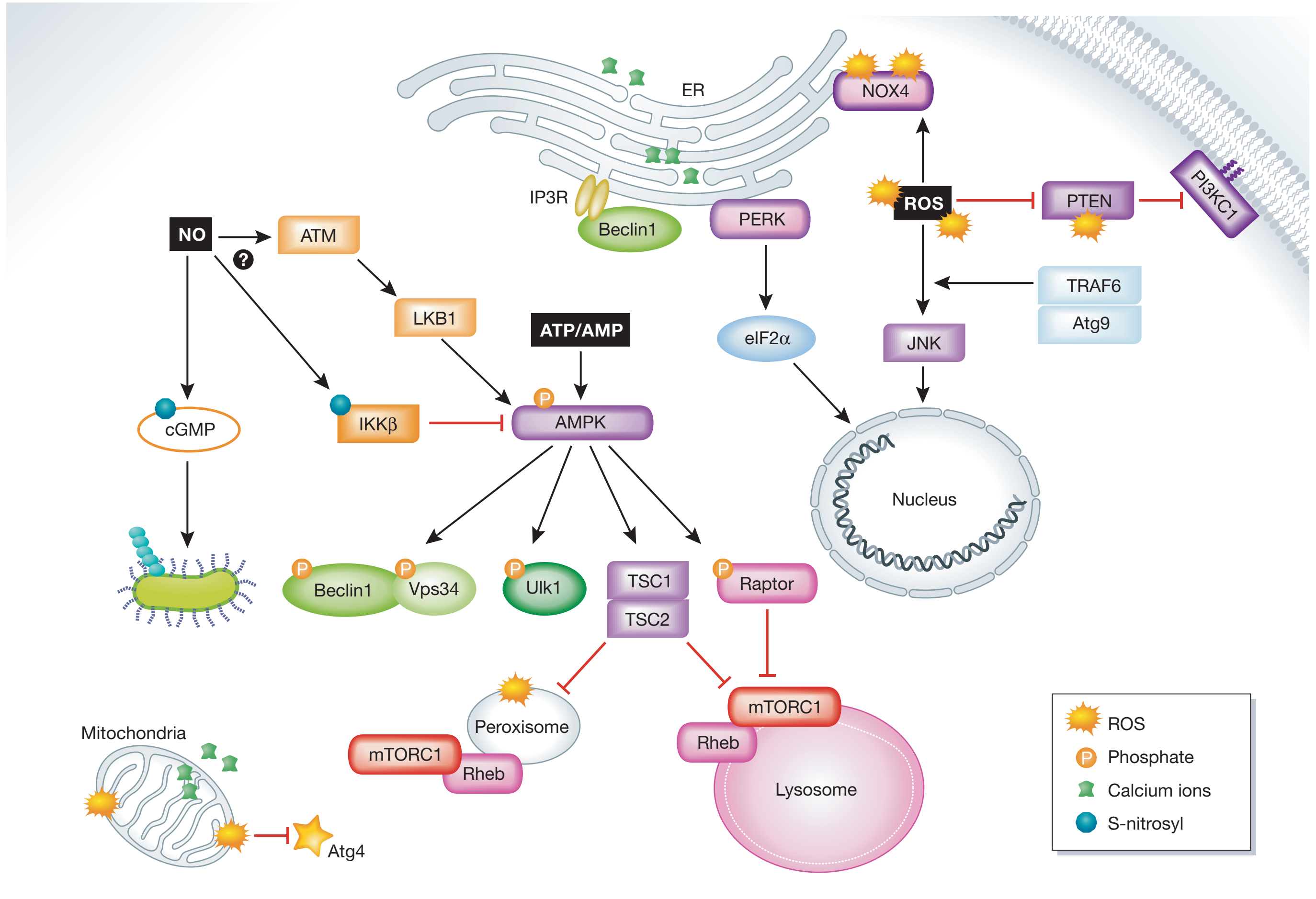

Figure 2. Regulation of autophagy by intracellular cues.

Internal cues regulate autophagy on different levels from many intracellular locations. The activity of mTORC1 is regulated at the lysosome and the peroxisome through AMPK. Active AMPK indirectly inhibits autophagy by activating the TSC1/2 complex and via inhibition of raptor by phosphorylation, both of which lead to the inhibition of mTORC1. Autophagy is inhibited directly by Ulk1, Vps34, and Beclin1 phosphorylation. ROS molecules activate autophagy at the plasma membrane, the ER, and mitochondria, as well as by up-regulating the expression of autophagy-related proteins. Ca^{2+} signaling is mediated from its intracellular storages in the mitochondria and the ER. The regulation of autophagy through AMPK induced by NO remains poorly understood. NO regulates mitophagy through cGMP.

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PtdIns3P at the plasma membrane [36], thereby recruiting and activating both PDK1 and Akt/PKB (Fig 1B). Upon insulin signaling, Akt is activated by two pathways, PDK1 and mTORC2, thus mediating an indirect regulation of the non-autophagic mTOR complex on autophagy [37,38]. Akt activation leads to the inhibition of TSC1/2, an mTOR inhibitor, resulting in the inhibition of autophagy [8]. Interestingly, loss of the TSC1/2 complex induces constitutive mTOR activity at the plasma membrane due to the enhanced activity of RalA/RalB through the exocyst complex [39]. The long-term regulation of autophagy by Akt includes down-regulation of the expression of many autophagic proteins through a process mediated by the FoxO transcription factor family [40–42].

Autophagy was recently directly linked to glucose deprivation in cardiomyocytes [43]. In low glucose conditions, hexokinase-II—the first enzyme in the glycolysis pathway—was postulated to directly bind and thereby inhibit mTOR. This binding would be inhibited by glucose 6-phosphate, the substrate of hexokinase-II (Fig 1B). Furthermore, glucose deprivation in neonatal mice was shown to induce the lysosomal targeting of mTOR through the Rag GTPase pathway, previously implicated only in amino acid starvation [44]. This issue has not yet been fully resolved, however, as another study in mice indicated that long periods of glucose deprivation do not inhibit autophagy [45].

Epidermal growth factor Autophagy plays a crucial metabolic role, especially when supplies are limited, implying that it is tightly linked to growth factors. Recent studies have indeed demonstrated that the EGFR system inhibits autophagy, either indirectly through GRB2 and GAB2 [46], or directly by phosphorylating Beclin1, prompting its dimerization to prevent its activity [47] (Fig 1C). Alternatively, autophagy can be induced through EGF-dependent phosphorylation and dimerization of STAT3, releasing its binding of PKR, the catalytic domain of eIF2α [48,49]. This up-regulates the transcription and translation of the core autophagic proteins LC3 and Atg5 [50]. Interestingly, expression of the constitutively active EGFR mutant EGFRvIII results in extensive autophagic activity, promoting cell survival in tumor cells especially under stressful conditions, such as uncontrolled Ras signaling and oxidative stress

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[51]. It would thus be interesting to further elucidate the molecular switches dictating the regulation of autophagy by the EGFR family upon changes in growth conditions. Accordingly, serum starvation was recently reported to induce autophagy through GSK3, which phosphorylates and activates the acetyltransferase TIP60, leading to acetylation and activation of Ulk1 [52].

Toll-like receptors Autophagy has been widely implicated in immunity through its support of immune-cell activity. The toll-like receptor (TLR) family, an essential part of the innate immune system, has been implicated in the regulation of autophagy, and the mechanism governing this process was recently elucidated [53] (Fig 1D). Two adaptor proteins, MyD88 and TRIF [54], recruit the E3 ligase TRAF6 to the autophagic regulator Beclin1 via its two TRAF6-binding domains [55]. Beclin1 is polyubiquitinated with a Lys63-linked ubiquitin chain on Lys117, which is located within its BH3 domain. This induces Beclin1 detachment from Bcl2 to induce autophagy in a process regulated by the deubiquitinating enzyme A20 [55]. Beclin1 is then free to form, together with Atg14, Vps34, and Vps15, the PI3KC3 complex essential in the initial stages of autophagosome biogenesis [5]. Moreover, the up-regulation of p62 expression by the TLR4 pathway, which is mediated by the transcription factor Nrf2, further extends the induction of autophagy in a MyD88- and p38-dependent manner [56]. Activation of TLR4 thus leads to lysosomal elimination of invading bacteria, a process termed xenophagy, which is beyond the scope of the present review [57].

Cytokines, a large group of immune signaling molecules that are secreted to promote differentiation, recruitment, and activation of immune cells, have also been implicated in the regulation of autophagy. The proinflammatory cytokines IL-1β, IFN-γ, and TNF were found to induce autophagy to protect macrophages from bacterial infection [58]. In contrast, the cytokines IL-4, IL-13, IL-10, and IL-6 signal for autophagic inhibition, each via a different signaling pathway. IL-10 inhibits autophagy through the Akt signaling pathway, whereas IL-4 and IL-13 are inhibitory only when autophagy is induced by starvation. IL-6 inhibits autophagy by down-regulating the expression of autophagic proteins mediated by STAT3 regulation [58].

Intracellular cues

Autophagy is the main intracellular process responsible for the clearance of defective organelles and protein aggregates caused by aging, cellular malfunction, or both. This is particularly important in long-lived cells such as neurons. The internal state of the cell is monitored to maintain homeostasis under different growth conditions (Fig 2).

Energy level The energy status of the cell is typically sensed by the ATP/AMP ratio and regulated by AMPK binding [59]. When AMP is in excess, indicating low energy levels, it binds AMPK, leading to phosphorylation and activation by LKB1 [60]. This consequently activates autophagy via two main signaling pathways: mTOR inhibition through the TSC1/2 complex [61,62] or the phosphorylation of raptor and binding to 14-3-3 proteins, which also inhibits mTOR [63]. An alternative mechanism was recently described whereby upon glucose deprivation, AMPK directly phosphorylates Ulk1 [64], Vps34, and Beclin1 (members of the PI3KC3 complex), leading to PI3KC3 stabilization and activation of autophagy, which ensures cell survival [65].

Oxidative cues Intracellular pathways lead to the production of ROS, which serve as signaling molecules at low concentrations yet are highly hazardous and must be eliminated. The main ROS molecules that participate in autophagic signaling are H_2O_2 and O_2^-. ROS regulate autophagy at various intracellular locations. At the plasma membrane, H_2O_2 directly modifies and inactivates PTEN, which inhibits PI3KC1 activity, thus eventually activating mTOR [66]. ROS have also been implicated in the activation of JNK, in a process regulated through the interaction of Atg9 with TRAF6 [67] that induces autophagy by up-regulating the expression of different Atg proteins [68–70]. In the ER of cardiomyocytes, ROS levels are intentionally up-regulated by NOX4 upon glucose deprivation to induce autophagy through the PERK and eIF2α pathway, thus preventing cell death [71]. TSC1 and TSC2 are targeted to the peroxisomal membrane by pex19 and pex5, respectively, where they inhibit mTOR activity by hydrolyzing GTP-Rheb upon exposure to ROS [72], suggesting that peroxisomes may induce autophagy in response to oxidative stress.

In mammalian systems, ROS production at the mitochondria is elevated upon starvation and was found to directly regulate the activity of Atg4, the priming and delipidating enzyme of Atg8 [73]. Upon oxidation, Atg4 is transiently and locally inactivated to stabilize Atg8s in their lipidated active form. Moreover, ROS production by mitochondria could serve as a signal for their elimination by mitophagy [74]. In this regard, GLUD activity at the mitochondria inhibits autophagy, probably through the generation of NADPH, which prevents ROS accumulation [15].

Nitric oxide NO is produced in cells by NOS and acts as a signaling molecule in different immune response pathways and the cardiovascular system, among other contexts [75]. NO was initially shown to inhibit autophagosome biogenesis in HeLa cells by inducing mTOR activation via the AMPK-TSC pathway, through S-nitrosylation of IKKβ [76]. In MCF-7 breast cancer cells, however, NO was reported to lead to the induction of autophagy in an ATM- and mTOR-dependent manner [77]. The difference observed between the two cell lines may be explained by the lack of LKB1 in HeLa cells, which is essential in the autophagic regulation of the IKKβ signaling pathway by NO.

NO regulates immune responses and was recently shown to be implicated in xenophagy [78]. NO was shown to nitrosylate cGMP following cell stimulation by LPS and IFN-γ, forming 8-nitro-cGMP, which can subsequently modify cysteine residues in target proteins. Proteins on the bacterial surface of group A Streptococcus (GAS) were modified by *S*-guanylation after cellular invasion, which marked bacteria for K63-linked polyubiquitination, inducing their engulfment by autophagosomes and lysosomal targeting. It would be interesting to investigate whether *S*-guanylation by 8-nitro-cGMP serves as a broad modification marking substrates in additional forms of selective autophagy.

Ca^{2+} ions Ca^{2+} is a well-established signaling molecule implicated in numerous cellular processes, and its cytosolic concentration is tightly regulated. The ER and the mitochondria serve as the primary Ca^{2+} storage organelles. ER stress, for example, leads to the release of Ca^{2+} from internal ER pools into the cytosol to regulate different stages along the autophagy pathway, yet this process remains poorly understood at a mechanistic level [79–81].

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The inositol 1,4,5-trisphosphate receptor, IP3R, is a Ca^{2+} channel activated by IP3 binding [82]. It is located on various membranes and regulates Ca^{2+} levels in organelles, and consequently in the cytosol. The N-terminal region of the receptor interacts with Beclin1 through a non-Bcl2-interacting region to regulate autophagy and was shown to sensitize the receptor to IP3 binding during autophagy [83]. Interestingly, the IP3R also regulates low Ca^{2+} levels in the mitochondria, impairing ATP production and increasing the AMP/ATP ratio, thereby inducing autophagy in an AMPK-dependent manner [84].

Autophagosome biogenesis

According to the current view, autophagosomes originate from a membrane that elongates until it is finally sealed as a mature double-membrane autophagosome, which subsequently fuses with the lysosome, where its content is degraded. The search for the membrane origin of the phagophore has been an enticing quest for many years. The introduction of autophagy-specific molecular tools and sophisticated imaging techniques led the way to the identification of multiple cellular membranes as possible sources of the isolation membrane. The first report in this regard utilized a GFP-tagged FYVE zinc finger domain of DFCP1, an ER resident protein that does not participate in autophagy but has high affinity for PtdIns3P on membranes [85]. The induction of autophagy apparently leads to the recruitment of this artificial reporter protein into an ER subdomain that contains autophagic factors such as Atg14 and WIPI and to the formation of a cup-shaped membrane termed omegasome [85–87]. However, additional membrane sources for phagophore formation have been suggested, such as plasma membrane [88,89], mitochondria [90], Golgi [91], ERGIC [92], ER–mitochondria contact sites [93], ER exit sites [94], and recycling endosomes [95]. This issue has been extensively reviewed and is therefore not discussed here [5,6,96].

Nucleation

The initial step in membrane nucleation for phagophore formation is the recruitment of autophagic proteins to a membrane in the cell designated by the presence of PtdIns3P. Yeast phagophores are initiated at one location termed the pre-autophagosomal structure (PAS), whereas in mammals, they are synthesized throughout the cell. Microscopic analysis of the recruitment order of the autophagy-related proteins in both yeast and mammalian systems suggested well-defined hierarchies for the order of incorporation of complexes into the site of autophagosome formation [97,98]. Regulation of the stability of PI3KC3, which is composed of Vps34, Vps15, Atg14, and Beclin1, and that of the Ulk1 complex, is essential for the nucleation process in mammalian cells and is regulated by post-translational modifications (Fig 3). Vps34 is a class III PI3K that phosphorylates phosphatidylinositol at the designated membrane, generating PtdIns3P [99]. EM analysis utilizing quick-freezing and freeze-fracture replica labeling revealed differences in the dispersion of PtdIns3P in yeast and mammalian autophagosomes [100]. In yeast, PtdIns3P was found mostly in the inner membrane leaflets facing the luminal barrier within the double membrane, whereas in mammals, this lipid was mostly localized to the outer autophagosomal membrane leaflets, suggesting differences in the autophagosome formation process in the different organisms. The site of PtdIns3P formation dictates the location of phagophore formation, as it leads to the recruitment of early autophagosome biogenesis factors, such as the WD-40-repeat-domain containing proteins WIPI1 and WIPI2 [101,102]. In addition, the FYVE-domain containing protein ALFY is recruited [103] and was recently defined as an adaptor protein able to concentrate several factors that are essential

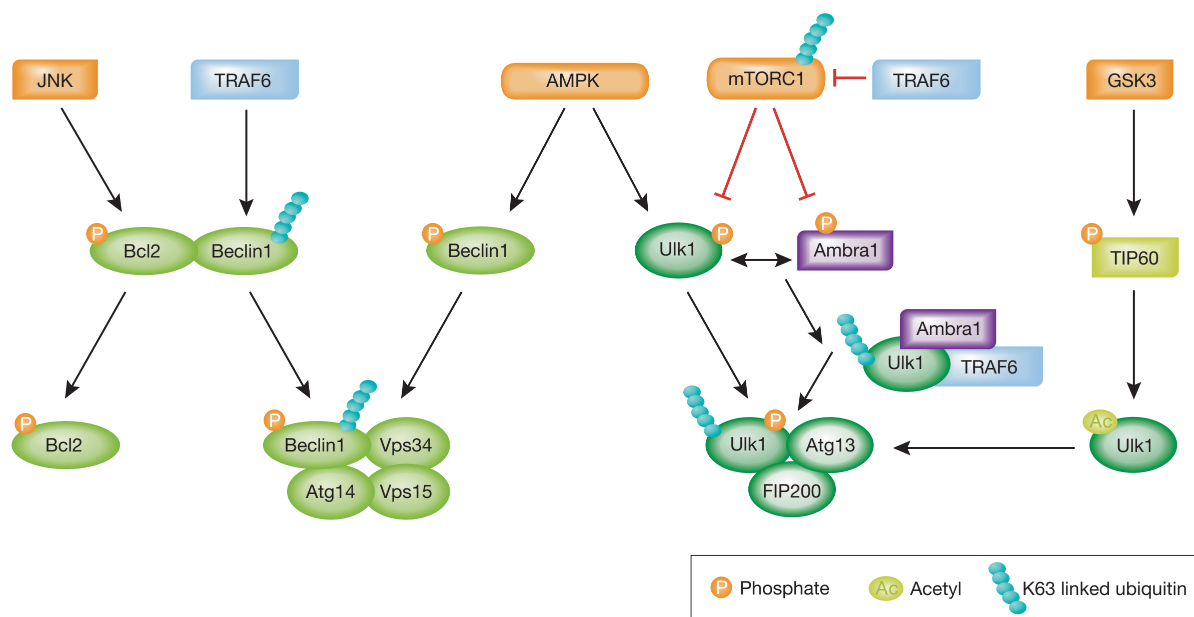

Figure 3. Post-translational modifications regulate PI3KC3 and the Ulk1 complex.
Ubiquitination, phosphorylation, and acetylation of Ulk1 and Beclin1 regulate autophagy by promoting or preventing the formation of the Ulk1 complex or PI3KC3.

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in the different stages of autophagosome biogenesis to the phagophore [104]. E3 ubiquitin ligases, such as TRAF6, ubiquitinated protein aggregates, the core autophagic protein Atg5, and the cargo recruiters p62 and NBR1, have all been shown to bind ALFY [105,106]. Following protein aggregation in cells, ALFY is exported from the nucleus and targets the protein aggregates to the phagophore via p62. The implication of ALFY in neurodegenerative diseases is consistent with its importance in aggregate clearance [107]. However, the signaling pathways that dictate the cellular location of ALFY and its targeting to the membrane remain unknown. Importantly, starvation leads to a decrease in ALFY level, suggesting that this protein is important for the clearance of protein aggregates yet may be toxic under stressful conditions [108]. It would be interesting to determine whether ALFY is implicated in additional forms of selective autophagy and whether the budding of the phagophore occurs in parallel to ALFY recruitment or sequentially. The order by which proteins are recruited by ALFY and the time point of membrane binding is likely to shed new light on the early stages of autophagosome biogenesis.

The formation of the PI3KC3 is supported by UVRAG [109] and by Ambra1, a Beclin1-interacting protein [110]. Ambra1 was recently shown to be a target of mTOR and to be inhibited by its phosphorylation at Ser52 under normal growth conditions [111]. Upon autophagic induction, Ambra1 is phosphorylated by Ulk1, which detaches it from dynein on microtubules and targets it to the ER [112]. Ambra1 then binds Ulk1 and TRAF6, promoting the Ulk1 Lys63-linked polyubiquitination that is essential for creation of the Ulk1 complex [111]. Interestingly, WASH—an endosome-associated protein—was shown to compete with Ulk1 ubiquitination and with Beclin1 binding by Ambra1 [113]. Ambra1 therefore appears to act as a novel link between PI3KC3 and the Ulk1 complex, both of which are essential in the initial steps of autophagosome biogenesis.

Notably, although its activity is crucial, only a limited number of Ulk1 effectors have been identified. A recent study in yeast shows that Atg9 is a direct substrate of Atg1 [114], the yeast homolog of Ulk1. As active mTORC1 resides on the lysosomal membrane, the inhibited Ulk1 complex could share the same location. To become active, Ulk1 needs to be shuttled from the lysosomal membrane by a mechanism yet to be resolved. A key factor in this process might be Ambra1, owing to its location along microtubules.

Connexins, a family of multispan transmembrane proteins that form plasma membrane gap junctions, were recently suggested to negatively regulate autophagosome formation by direct interaction with the PI3KC3 at the plasma membrane. According to the proposed model, under starvation conditions, Atg14 is incorporated into the plasma membrane, where it releases connexin-induced inhibition by directing these proteins to lysosomal degradation [115].

Phagophore formation and elongation

Downstream of the recruitment of WIPI1/2 are two ubiquitin-like (UBL) systems specific to the autophagic process. The first is the conjugation of Atg12, a UBL protein, to Atg5 by the E1 enzyme Atg7 and the E2 enzyme Atg10 [116]. Atg5 binds the N-terminal region of Atg16 through a non-covalent bond, independently of its interaction with Atg12 [117]. Atg16 creates homodimers, each capable of binding an Atg12–Atg5 conjugate resulting in a heterohexamer [118,119]. The Atg12–Atg5–Atg16 complex is known to dictate the site of autophagosome formation by acting as an E3 ligase in the second UBL conjugation system, that of Atg8 to phosphatidylethanolamine (PE) [117]. The conjugation process is mediated by Atg7 as the E1-conjugating enzyme, and Atg3 as the E2-conjugating enzyme. The mammalian Atg8 is a UBL protein family consisting of eight family members grouped into the LC3 and GABARAP subfamilies [120]. WIPI2 was recently reported to recruit the Atg12–Atg5–Atg16 complex to the site of autophagosome formation by directly interacting with Atg16 [121].

The exact mechanism whereby the Atg12–Atg5–Atg16 complex induces phagophore elongation is still unclear. Using GUVs and purified recombinant proteins, it was initially suggested that the complex participates in vesicle tethering [122]. Atg12 has also been shown to bind Atg3 and carry it to the membrane, promoting Atg8 lipidation, which supports its role as an E3 in Atg8 conjugation. Interestingly, conjugation of Atg8 to PE is promoted by the Atg12–Atg5–Atg16 complex on SUVs but not on GUVs, indicating that membrane curvature is a significant factor in the activity of this complex. In agreement with this hypothesis, Atg3 was shown to be targeted to highly curved membranes, where it promotes Atg8–PE conjugation [123]. A more recent study in GUVs suggested a slightly different scenario, in which Atg12–5–16 initially catalyzes the lipidation of Atg8 to the membrane, which in turn acts to stabilize the association of the Atg12–5–16 complex on the membrane [124]. Thus, Atg8 is suggested to play a structural role and Atg12–5–16 to function as a coat. Both of these *in vitro* studies need further clarification. The observed differences might be associated with alterations in membrane curvature (Fig 4). During the initial stages of autophagosome biogenesis, when the phagophore is still relatively small and highly curved, the Atg12–Atg5–Atg16 complex might promote lipidation. As the phagophore continues to grow, its elongation points exhibit high curvature, whereas the sites already built are less curved and need to be stabilized. At these locations, the Atg12–Atg5–Atg16 complex might be essential in maintaining membrane structure and stability.

Further support for the notion that curvature is important for the recruitment and activity of the biogenesis machinery comes from the association of SNX18 with autophagic induction [125]. SNX18 contains a PX domain that targets it to PIP_2 on membranes, as well as SH3 and a BAR domains known to sense and endorse membrane curvature. It was shown to target Atg16 to perinuclear recycling endosomes and interact directly with LC3 [125], suggesting that Atg16 mediates LC3 lipidation on highly curved membranes. SNX18 was further suggested to induce the tubulation of recycling endosomes that supply membranes for the elongating phagophore. Interestingly, endosomal tubulation driven by the overexpression of TBC1D14, a Rab11 binding protein, was found to be inhibitory for autophagosome formation, suggesting an antagonist role for SNX18 [126]. Another pathway thought to target Atg16 to the phagophore is through interaction with FIP200, a subunit of the Ulk1 complex, as it relieves Atg16 auto-inhibition [127]. The discovery of multiple Atg16 targeting pathways to membranes reflects the vital role of Atg16 in autophagosome biogenesis and strengthens the hypothesis that it plays multiple roles in this process.

The phagophore membrane is subsequently elongated through a process that is not fully characterized. A given membrane source could continue to elongate until the autophagosome is completed or, alternatively, small vesicles could fuse with the phagophore to expand its membrane [128]. In mammals, the VAMP7 SNARE

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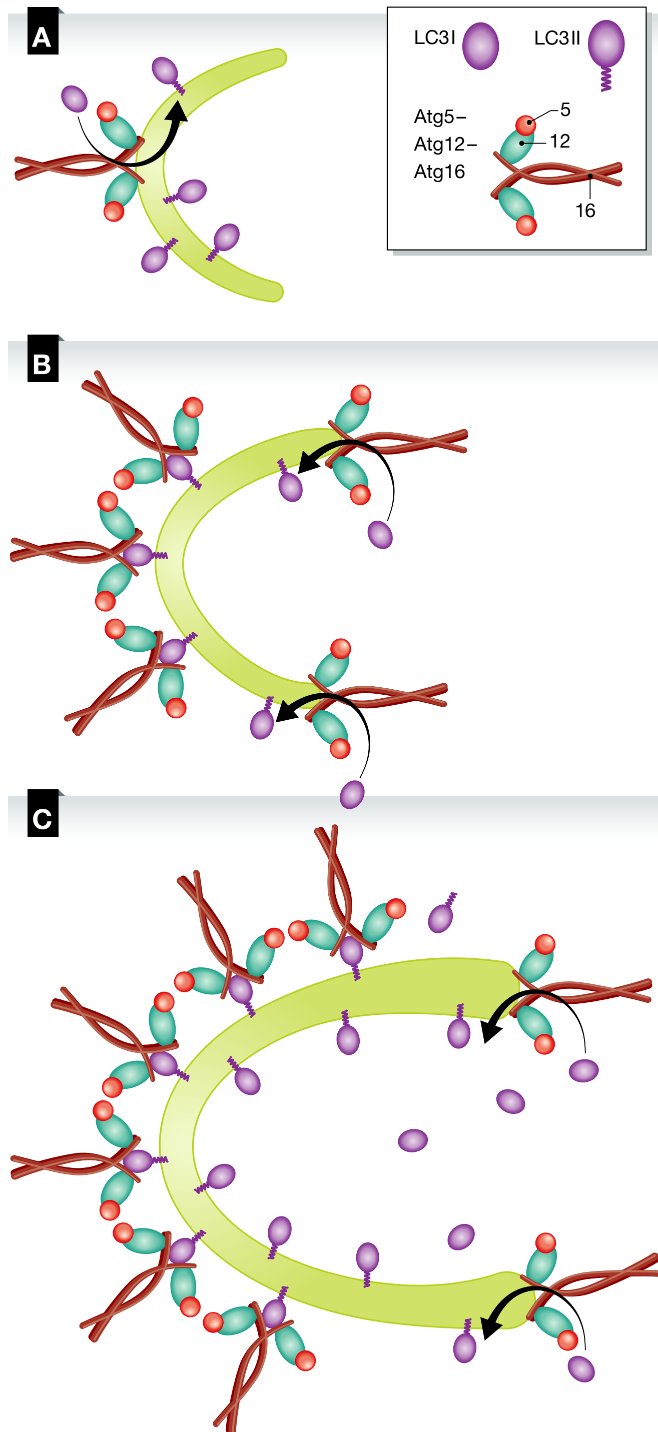

Figure 4. Model of the Atg12–Atg5–Atg16 complex function in autophagy.
(A) The Atg12–Atg5–Atg16 complex is recruited to the phagophore after its initial nucleation. At this stage, the membrane is curved and the complex promotes the lipidation of LC3 with PE. (B) Once the membrane elongates, the complex remains associated with the membrane through LC3 on membranes with low curvature for their stabilization and continues to promote LC3 lipidation at the highly curved edges of the phagophore. (C) As the elongation continues, the Atg12–Atg5–Atg16 complex, together with the lipid-conjugated LC3, forms a coat-like structure that stabilizes the structure of the phagophore.

complex—which includes VAMP7, syntaxin 7, syntaxin 8, and Vti1b—was the first reported to mediate autophagosome biogenesis [88]. In a parallel yeast study, in which trafficking of ectopically expressed Atg9 was studied, the t-SNARE Tlg2 and the v-SNAREs Sec22 and Ykt6 were shown to be essential for autophagosome biogenesis through mediation of Atg9 trafficking [89]. Notably, Atg9 overexpression results in the formation of tubular structures suggested to serve as Atg9 reservoirs and as a membrane source for the PAS [129], a finding recapitulated also in mammalian cells [130]. When Atg9 expression was regulated by its endogenous promoter, however, this phenomenon was observed only on small unilaminar vesicles synthesized *de novo* from the Golgi membrane [128]. These vesicles were shown to fuse with autophagosomes that contain Atg9 on the outer membrane, which is detached only after autophagosome maturation. Furthermore, relatively few Atg9-tagged vesicles were shown to fuse with the phagophore, and therefore, additional membranes sources are required. Atg9 trafficking is regulated by a complex machinery, as became recently apparent [131]. TRAPPIII, which is part of the general trafficking machinery, was implicated in Atg9 trafficking under normal growth conditions, but shown to be less necessary under starvation conditions, in which the GARP pathway is essential. In mammalian cells, Atg9 was initially found in the Golgi and is transported to endosomal compartments upon autophagic induction [132], yet it was recently detected on additional intracellular compartments [130]. It was also shown to be essential in the early stages of autophagosome formation by transiently interacting with the expanding phagophore [130]. In addition, Atg9 has been recently reported to localize to the plasma membrane, from which it is internalized and fused with Atg16-tagged vesicles, a finding consistent with the suggested involvement of both the plasma membrane and the ER–Golgi system in autophagosome formation [95].

Atg8 and its mammalian orthologs have been implicated in the elongation of the phagophore [5,133–135]. In yeast, phagophore elongation and autophagosome size are controlled by Atg8 [134,135]. Notably, an *in vitro* system using liposomes containing different concentrations of PE yielded contradictory results regarding the fusogenic activity of Atg8s [89]. LC3, a mammalian homolog of Atg8, is also involved in phagophore expansion [136]. Interestingly, the N-terminal region of LC3 and GATE-16 promotes vesicle tethering and fusion *in vitro*, suggesting their involvement in elongation of the phagophore membrane [137]. It would therefore be interesting to study the significance of Atg8 in the fusion of Atg9-containing vesicles to the phagophore.

Although typically described as a progressive process whereby the edges of a crescent-shaped phagophore elongate continuously to form autophagosomes, it may be also worth considering an alternative view whereby formation of the phagophore membrane is initiated at several sites, possibly by different membrane sources (Fig 5). Different pieces of membranes could then be tied together by membrane fusion events. This would require many autophagy biogenesis complexes acting on several sites at different stages of biogenesis, and attempting to reconstitute such a process in the test tube would be extremely challenging. The available cell-free systems aimed at reconstituting autophagosome formation can reflect only fragments of the complex process. Accordingly, GUVs could represent only events that take place on membranes with relatively low curvature, whereas small liposomes may mimic highly

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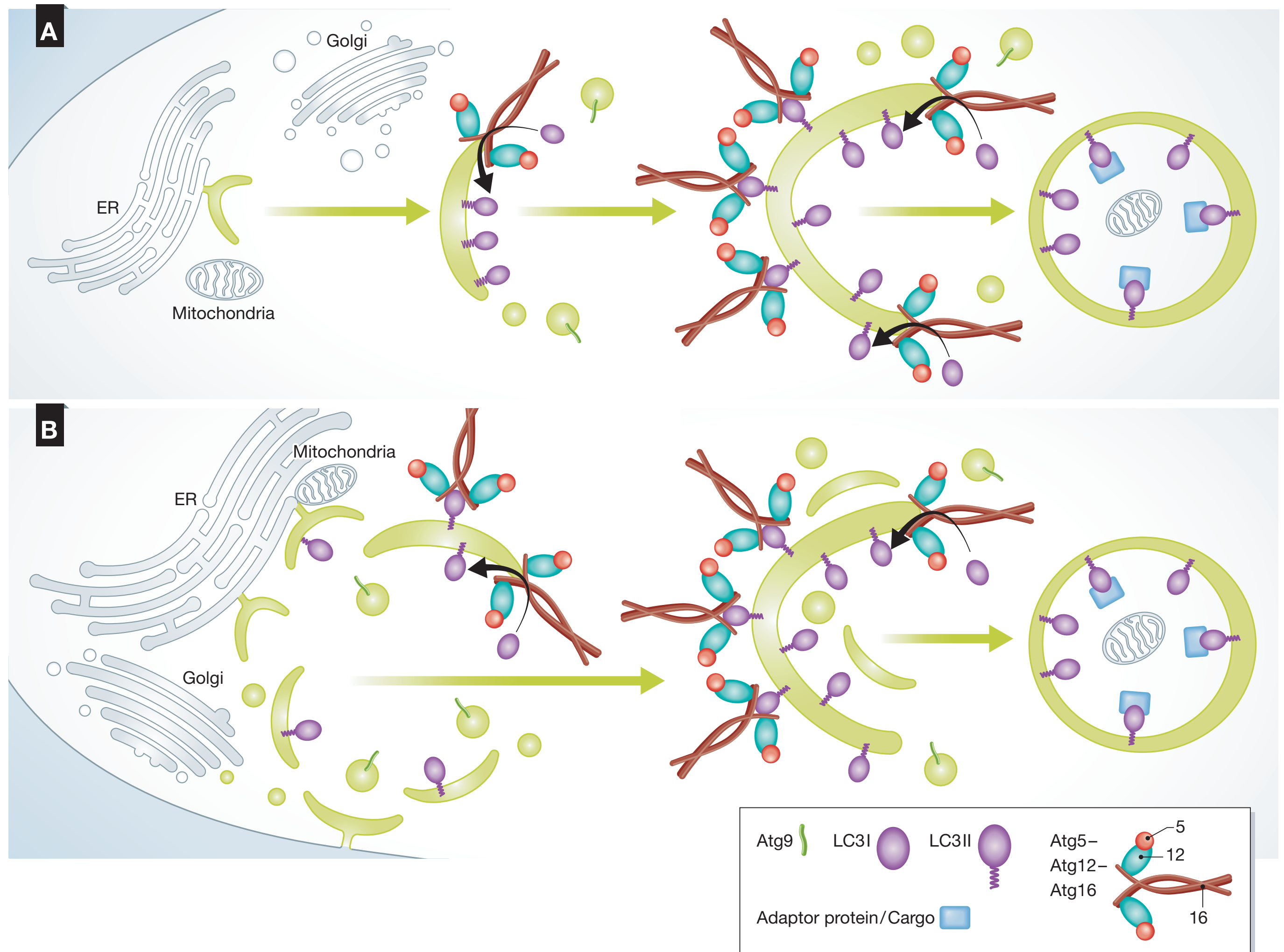

Figure 5. Models of autophagosome biogenesis.
(A) The current view of autophagosome biogenesis is a continuous process, where an initial membrane for autophagosome formation buds out from an existing organelle and is further elongated by the fusion of vesicles, some containing Atg9. The Atg12–Atg5–Atg16 complex promotes LC3 lipidation on the highly curved membranes while supporting the membrane's structure as it elongates. Once the autophagosome is sealed, it encapsulates cargo for degradation and the external biogenesis machinery is removed. (B) A new model for autophagosome biogenesis. Multiple nucleation membranes bud from several organelles to contribute to the formation of the initial membrane of the autophagosome. Each membrane elongates individually until all are fused to create an autophagosome that encapsulates cargo for lysosomal degradation.

curved membrane. If multiple events occur in parallel on different sites, it is possible that several biogenesis pathways occur simultaneously *in vivo*. Therefore, various membrane sources can contribute to biogenesis, since within the cell's third dimension, there are many organelles and membranes in close proximity to the phagophore.

Sealing and maturation

The final step of autophagosome biogenesis is the sealing of the phagophore to form a double-membrane vesicle. This rather understudied process dictates the final size of the autophagic organelle. Regulation of this process also serves as part of the regulation of fusion with the lysosomes. The mammalian homologs of Atg8 have been suggested to promote this stage of autophagosome biogenesis. Consistently, studies in *Caenorhabditis elegans* showed that the GABARAP homolog LGG-1 and the LC3 homolog LGG-2 act consecutively [138]. LGG-2 activity downstream of LGG-1 promotes autophagosome maturation through a pathway that also involves the HOPS subunit VPS39.

Atg4 plays a dual role by priming Atg8 both for its lipid conjugation and for its removal from membranes [139]. Interestingly, deconjugation of Atg8 by Atg4 was shown to be important in both early and late stages of biogenesis, allowing the fusion of autophagosomes with lysosomes. There are four members of the Atg4 mammalian family, each able to specifically modify different Atg8 family members. In erythrocytes, mammalian Atg4B participates in the regulation of autophagosome maturation that is necessary for cell differentiation [140]. The protein FYCO1, previously shown to bind LC3 and mediate autophagosome trafficking, was recently implicated in autophagosome maturation as well [141].

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Sidebar A: In need of answers

(i) How are amino acids sensed by TORC1?
(ii) How are various internal signals integrated to regulate autophagy?
(iii) How does the lysosomal location of mTORC1 affect its downstream signaling on the Ulk1 complex?
(iv) How do Atg9-containing vesicles contribute to autophagosome biogenesis?
(v) How are the SNARE molecules needed for fusion of the autophagosome with the lysosome incorporated into the outer autophagosomal membrane?
(vi) How are the inner and outer membranes of the phagophore sorted into two sub-domains?
(vii) How are the cup-shaped phagophores formed and stabilized?
(viii) What dictates the final sealing of the autophagosome?

The participation of trafficking factors in the sealing and maturation of autophagosomes is well established. Several Rab proteins are known to play a role in autophagosome maturation [142], a process in which SNARE molecules have also been implicated, as discussed above. In a recent study, immuno-electron microscopy was used to show that syntaxin 13 localizes with LC3 on immature autophagosomes [143]. Depletion of syntaxin 13 leads to the accumulation of immature autophagosomes, indicating that it participates in the maturation process. In addition, syntaxin 17 was suggested to promote autophagosomal sealing by trafficking through a still unknown mechanism to phagophores at late stages of biogenesis, enabling them to fuse with the lysosome [144]. Recent studies in *Drosophila* and mammalian cells suggested that syntaxin 17 binds the HOPS complex, thereby promoting the fusion between autophagosomal and lysosomal membranes [145,146]. Syntaxin 17 was also shown to induce, together with Atg14, budding of the source membrane at ER contact sites [93]. Additional studies are clearly needed to clarify the exact role of syntaxin 17 along the autophagic pathway.

Interestingly, the lysosome-located protein TECPR1 was implicated in fusion between the autophagosome and the lysosome by binding the Atg12–Atg5 conjugate [147]. Depletion of TECPR1 resulted in the accumulation of autophagosomes that do not fuse with lysosomes. As the Atg12–Atg5 conjugate is removed from autophagosomes prior to their final sealing, these results suggest a direct link between autophagosome sealing and removal of this complex. It is possible that autophagosomes only reach their final maturation, cued by the removal of all biogenesis machinery, immediately before their fusion with the lysosomes. Importantly, Atg5 binding by TECPR1 was also suggested to promote the initial stages of autophagosome formation and to target bacteria to the phagophore during xenophagy, but not upon starvation [148]. The exact role of TECPR1 in autophagy thus remains to be elucidated.

Concluding remarks

Autophagy lies at a fundamental junction in cell fate, determining death or survival. The integration between stress-induced pathways and autophagic proteins is rather complex, given that different cues are funneled to activate a relatively small pool of autophagic proteins. The continuing discovery of connections between well-established signaling pathways and autophagy-related proteins, as well as of new regulators of both processes, contributes substantially to our understanding of the regulation of autophagy in response to changes in the extracellular and intracellular environment.

The mechanism of autophagosome biogenesis is still elusive. Our knowledge concerning the order of incorporation of the different factors into sites of autophagosome formation, as well as on the functional complexes involved in this process, has increased substantially in recent years. However, the exact details concerning the way in which all of these factors act in concert are still missing. The reconstitution of such a step in the test tube will clearly be very challenging, but if successful, it will provide invaluable information on this process. There are several open questions remaining in this field (Sidebar A). One of them is how the membrane of the phagophore is sorted into two sub-domains, leading to the formation of distinct inner and outer membranes. The former membrane will be degraded in the lysosome, whereas the latter fuses with the lysosome-limiting membrane to become an integral part of it. Understanding this process in molecular terms is of broad interest, as it may shed light on the mechanism by which the lysosome-limiting membrane is protected from degradation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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