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REVIEW

Anatomy of autophagy: from the beginning to the end

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Abstract Autophagy is a highly regulated process in eukaryotes to maintain homeostasis and manage stress responses. Understanding the regulatory mechanisms and key players involved in autophagy will provide critical insights into disease-related pathogenesis and potential clinical treatments. In this review, we describe the hallmark events involved in autophagy, from its initiation, to the final destruction of engulfed targets. Furthermore, based on structural and biochemical data, we evaluate the roles of key players in these processes and provide rationale as to how they control autophagic events in a highly ordered manner.

Keywords ATG · SNARE · Vps34 · Autophagosome · Lysosome

Introduction

Autophagy is a highly regulated self-clearance process in eukaryotes that delivers long-lived proteins, lipids, glycogens, and organelles from the cytosol to lysosomes for destruction [1–3]. It also functions as an adaptive cellular mechanism to deal with stress stimuli such as starvation, oxidation, and pathogen invasion [4, 5]. Malfunctions in autophagy are associated with a wide array of human diseases including cancer and neurodegeneration [6, 7]. Thus, studies of autophagy will provide the basis for the development of potential therapeutic strategies that target these diseases.

Autophagy is divided into roughly three types, according to the pathways that deliver cargo to the lysosomes: macroautophagy, microautophagy, and chaperone-mediated autophagy [4]. Macroautophagy, which is the focus of this review, and referred to as simply autophagy hereafter, is characterized by the formation of a double-membrane vesicular structure named autophagosome that sequesters bulk cytosol and fuses with lysosomes (or vacuole in yeasts and plants [8, 9].

As a hallmark event in autophagy, autophagosome formation is dynamically regulated by a group of structurally and functionally conserved proteins, which are encoded by autophagy-related genes (ATG) in yeast (Table 1) [10]. In response to autophagic signals, Atg proteins of the core autophagic machinery are mostly clustered in the vicinity of the vacuole/lysosome/and assembled into a structure named phagophore assembly site (or preautophagosomal structure, PAS) that also contains the forming vesicle (phagophore or isolation membrane) [4]. The phagophore undergoes an expansion process and eventually develops into a mature autophagosome in which a sealed double membrane enwraps the cargo. Then the outer membrane of the autophagosome quickly fuses with the lysosome membrane, exposing its inner membrane and enwrapped contents to degradation by lysosomal hydrolases. At last, the degradation products are transported back to the cytosol and used for protein synthesis

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Table 1 Structural and biochemical properties of Atg and related proteins during autophagosome formation

Autophagic events		Yeast	Mammals	Biochemical properties	Structural properties
Initiation of autophagy	The Atg1 complex	Atg1	ULK1/2	1. Phosphorylated by TORC1 2. Phosphorylates AMBRA1 and Beclin 1 3. Membrane targeting, membrane curvature sensing, and lipid vesicle tethering	1. Interaction with Atg13 via its C-terminal MIT domains (4P1N) 2. Kinase domain bound to inhibitors (4WNP, 4WNO, 5CI7)
		Atg13	Atg13	1. Phosphorylated by TORC1 2. Bridges Atg1 and Atg17–Atg31–Atg29 3. Recruits the Vps34 complex via Atg14 4. Binds to LC3 5. Interacts with Atg101	1. Interaction with Atg1 via its MIM domains (4P1N) 2. Interaction with Atg17–Atg31–Atg29 (4P1W, 5JHF) 3. The N-terminal HORMA domain (4J2G, 4YK8) 4. Interaction with LC3 via its LIR (3WAN, 3WAO, 3WAP)
		Atg17	–	1. Interacts with Atg13 and Atg9 2. Ternary complex with Atg31 and Atg29 3. Senses membrane curvature	1. Atg17–Atg31–Atg29 (4HPQ). 2. Atg17–Atg31–Atg29 in complex with Atg13 (fragment) (4P1W, 5JHF)
		Atg31	–	Ternary complex with Atg17 and Atg29	
		Atg29	–	Ternary complex with Atg17 and Atg31	
		–	Atg101	Interacts with Atg13	
		–	FIP200	Complexes with ULK1/2–Atg13–Atg101	
Biogenesis of phagophore	The Vps34 complex	Vps34	Class III PI3K	Lipid kinase to produce PI3P	1. Helical and catalytic domains alone or bound to inhibitors (2X6F, 2X6H, 2X6I, 2X6J, 2X6K) 2. Vps34 in complex with Vps15, Vps38, and Vps30 (5DFZ)
		Vps15	Vps15	1. Serine/threonine protein kinase 2. Membrane targeting via its N-terminal myristoylation	
		Atg6/Vps30	Beclin 1	1. Recruits Atg14 or Vps38 2. Interacts with Bcl-2 via its N-terminal BH3 motif 3. Lipid binding and membrane deformation	1. Beclin 1 BH3 in complex with Bcl-XL (2P1L) 2. The C-terminal BARA domain of Beclin 1 (4DDP, 3VP7, 5DFZ)

Table 1 (continued)

Autophagic events		Yeast	Mammals	Biochemical properties	Structural properties
		Atg14	Atg14(L)/Barkor	1. Interacts with Atg6 to assemble the autophagic-specific complex 2. Membrane targeting and membrane curvature sensing 3. Promote membrane fusion	
	Other proteins	–	VMP1	1. Transmembrane protein 2. Interacts with Atg6	
		–	DFCP1	Binds to PI3P via its FYVE motif	
		Atg18	WIPI1/2/3/4	1. Binds to PI3P via its FRRG motif 2. Required for the retrograde transport of Atg9 3. Complexes with Atg2	PROPPIN structure (4EXV)
		Atg2	Atg2A/B	Complexes with Atg18	
		Atg9	Atg9L1/2	1. Transmembrane protein 2. Shuttles between PAS and peripheral organelles to deliver lipids/factors 3. Self-interaction	
Expansion of phagophore	Atg12 conjugating system	Atg12	Atg12	1. Ubiquitin-like module, which is conjugated to Atg5 2. Forms an E3 complex with Atg5 and Atg16 3. Interacts with Atg3	Atg12–Atg5–Atg16N in complex with Atg3 (fragment) (4NAW)
		Atg5	Atg5	1. Conjugated by Atg12 2. Interacts with Atg16 3. Forms an E3 complex with Atg12 and Atg16 4. In vitro membrane association	
		Atg7	Atg7	1. E1 enzyme interacting with E2 enzyme Atg10 or Atg3 2. Homodimerization 3. Forms a thioester bond with Atg8	Atg7–Atg10 (4GSK) Atg7–Atg3 (4GSL) Atg7–Atg8 (3RUI)
		Atg10	Atg10	E2 enzyme	

Table 1 (continued)

Autophagic events		Yeast	Mammals	Biochemical properties	Structural properties
	Atg8 conjugating system	Atg8	LC3A/B/C, GABARAP, GABARAPL1/2/3	1. Ubiquitin-like module, which is conjugated to PE 2. Recognizes the cargo-specific adaptors via their LXXW motif/LIR 3. In vitro membrane tethering	
		Atg7	Atg7	E1 enzyme	
		Atg3	Atg3	E2 enzyme	
		Atg5	Atg5	Part of the E3 enzyme complex	
		Atg12	Atg12	Part of the E3 enzyme complex	
		Atg16	Atg16L1/2	1. Homodimerization 2. Part of the E3 enzyme complex	Atg16 dimer (3A7O)
		Atg4	Atg4A/B/C/D	1. Cysteine proteinease to process Atg8 by removing its last amino acid 2. Deconjugate Atg8–PE	Atg4B–LC3 (2Z0D, 2Z0E)

The PDB codes are shown in the parentheses. Abbreviations:ULK1/2, Unc51-like kinase 1/2; TORC1, target of rapamycin complex 1; AMBRA1, activating molecule in Beclin 1 regulated autophagy; MIT, microtubule interacting and transport; MIM, MIT-interacting motif; HORMA domain, named after the Hop1p, Rev1p, and Mad2 proteins; LIR, LC3-interacting region; FIP200, focal adhesion kinase family interacting protein of 200KDa; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; BH3, Bcl-2 homology domain 3;BARA domain, beta-alpha repeated, autophagy-specific domain; Barkor, Beclin 1-associated autophagy-related key regulator; VMP1, vacuole membrane protein 1; DFCP1, double FYVE-containing protein 1; WIPI, WD-repeat protein interacting with phosphoinositides; PE, phosphatidylethanolamine; PROPPIN, β-propeller(s) that bind phosphoinositides; PAS, phagophore assembly site; LC3, microtubule-associated protein 1 light chain 3; GABARAP, gamma-aminobutyric acid receptor-associated protein

and other cellular functions [4, 8, 9, 11]. Figure 1 illustrates a simplified autophagic process that is directed by green arrowheads.

In this review, we dissect the autophagic process into several steps for ease of discussion, which includes (1) initiation of autophagy; (2) biogenesis of the phagophore; (3) expansion of the phagophore; (4) formation of the autophagosome; (5) fusion with the lysosome, and (6) reformation of the lysosome (also refer to Fig. 1). As we describe these steps in detail, we also summarize the biochemical and structural properties of key Atg proteins that participate in each respective step (Table 1). We use the yeast nomenclature Atg1–31 to represent autophagic proteins and the names of their mammalian homologues are shown in parentheses in Table 1. Not only we focus on the yeast Atg proteins for structural and functional analysis, but discuss important autophagic functions or events relating to their mammalian homologues. Of note, most yeast Atg proteins have obvious mammalian homologues (based on amino acid sequence), with the exception of a few specific proteins. For example, Atg17, Atg31, and Atg29 are found in yeast, but have no mammalian homologues, suggesting in these cases, homology may be based on function rather than sequence. In addition, the mammalian protein Atg101 does not have an apparent yeast counterpart [12, 13], thus demonstrating that yeast and mammalian cells may use different regulatory mechanisms in certain autophagic scenarios [5].

Initiation of autophagy: the Atg1 complex

Autophagy is induced in response to several stress signals, such as amino acid deprivation, DNA damage, low energy, and hypoxia. These signals trigger the activation of distinct pathways that primarily converge on the nutrient sensor, Tor kinase complex 1 (TORC1) (Fig. 2a). Lack of amino acids inhibits the translocation of TORC1 to lysosomal membranes, where TORC1 is typically activated by RAS-homologue enriched in brain (RHEB). This translocation process is mediated by RAG GTPases and their interacting protein complex, Ragulator [14]. Low oxygen levels induce the hypoxia-inducible gene (REDD1), which

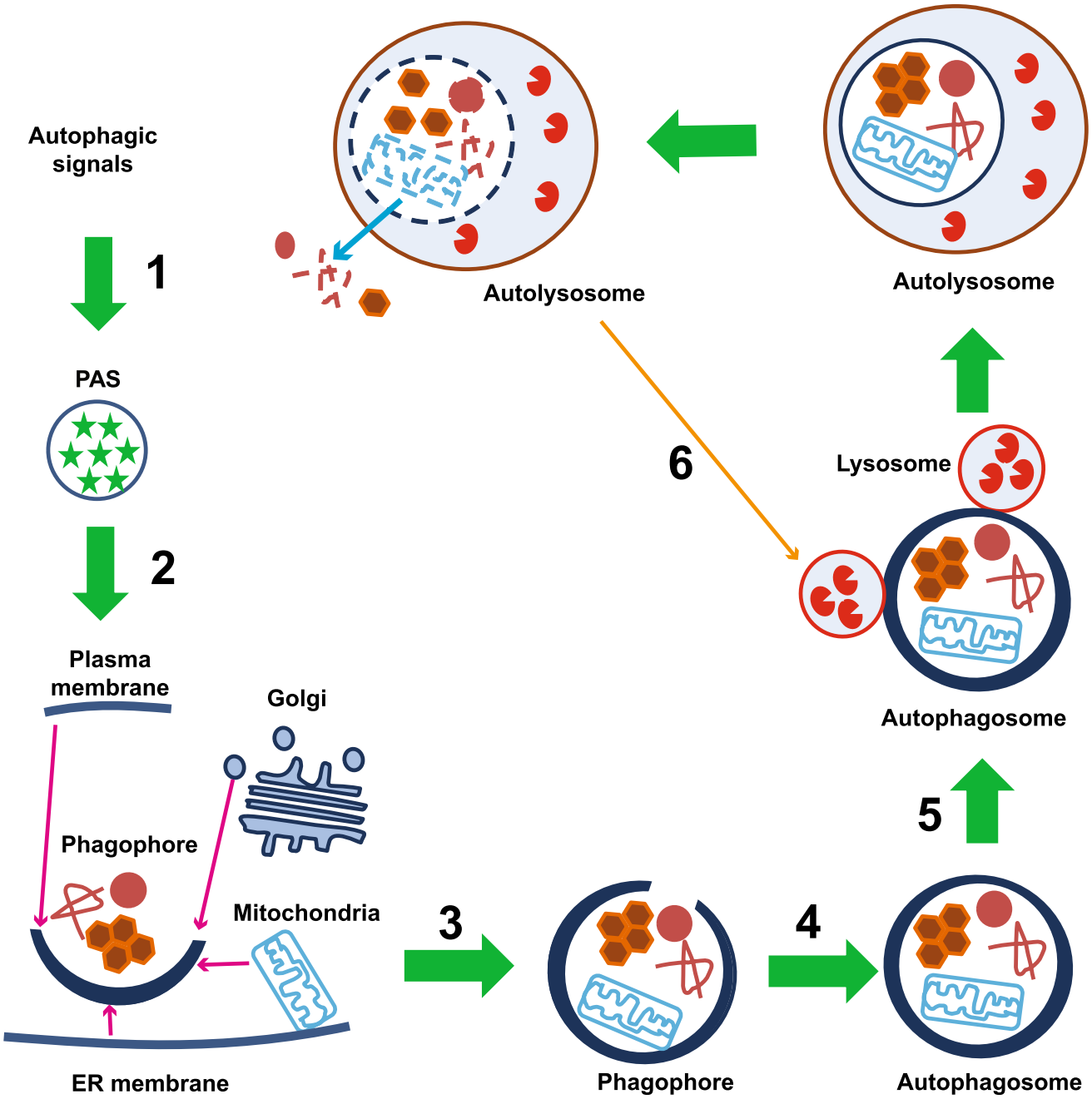
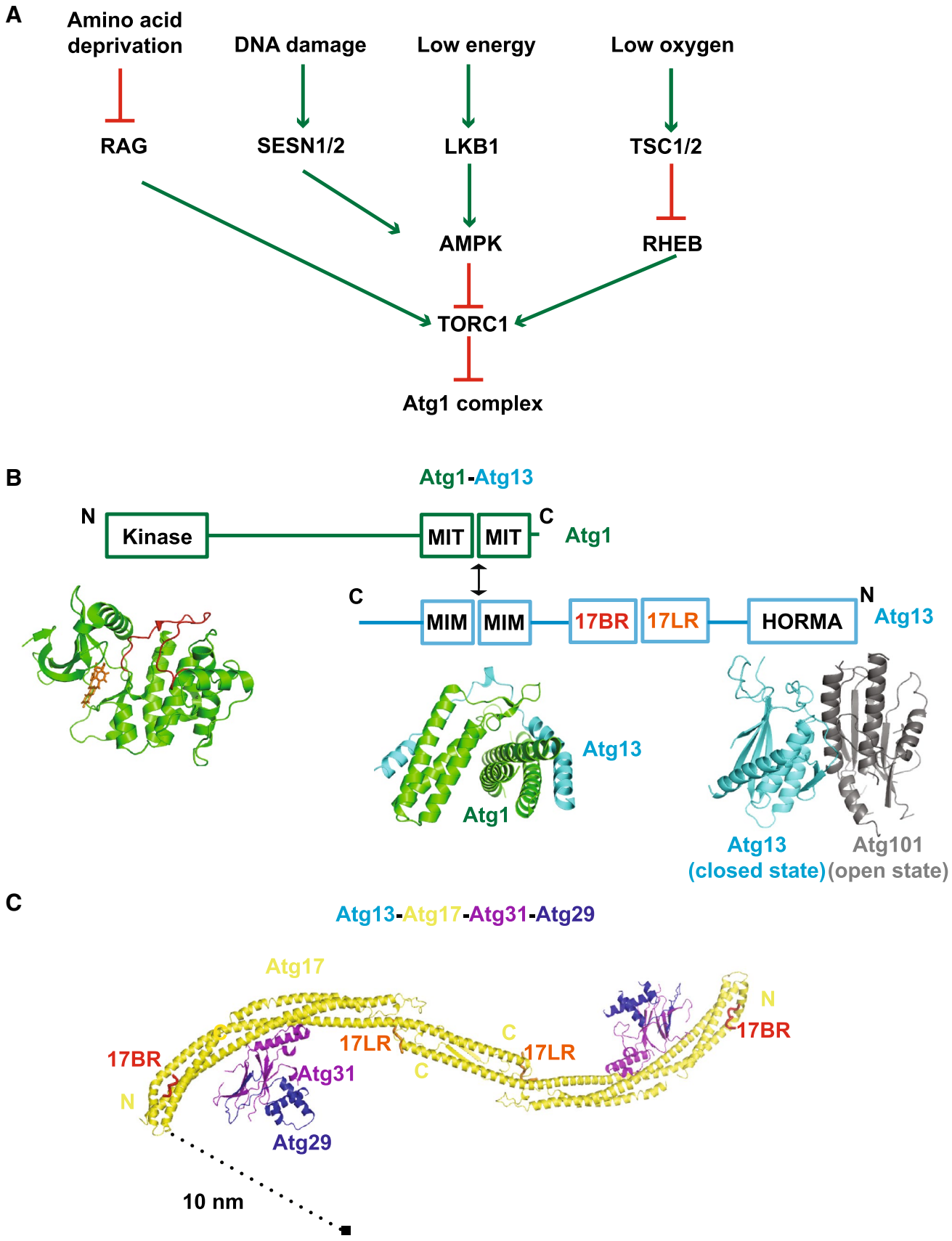


Fig. 1 Schematic depiction of the autophagic process. The autophagic process is directed by green arrowheads. The whole process can be roughly divided into six steps: (1) initiation of autophagy; (2) biogenesis of phagophore; (3) expansion of phagophore; (4) formation of autophagosome; (5) fusion with lysosome, and (6) reformation of lysosome. The red arrows indicate the potential membrane sources for autophagosomes, including cytoplasm, ER (endoplasmic reticulum), mitochondria, and Golgi. The blue arrow indicates that degraded materials are transported back to the cytosol for reuse via lysosomal transporters. *PAS* phagophore assembly site

in turn promotes the activity of the TSC1–TSC2 complex, a GTPase-activating protein for RHEB [15, 16]. As a result, GDP-loaded RHEB fails to activate TORC1. Low energy levels are sensed by a high cellular AMP-to-ATP ratio and this signal promotes phosphorylation and activation of AMPK by LKB1. Activated AMPK can regulate TORC1 activity positively or negatively through several signaling pathways. In one scenario, AMPK can inhibit TORC1 activation by phosphorylation of regulator-associated protein of TOR (RAPTOR) [17]. Alternatively, AMPK can also be activated by the sestrin family members (SESN1 or SESN2),


A
Amino acid deprivation
DNA damage
Low energy
Low oxygen
RAG
SESN1/2
LKB1
TSC1/2
AMPK
RHEB
TORC1
Atg1 complex
B
Atg1-Atg13
N
Kinase
MIT
MIT
C
Atg1
C
MIM
MIM
17BR
17LR
HORMA
N
Atg13
Atg13
Atg1
Atg13
(closed state)
Atg101
(open state)
C
Atg13-Atg17-Atg31-Atg29
Atg17
17BR
N
17LR
C
Atg31
C
17LR
N
17BR
Atg29
10 nm

◂**Fig. 2** Structural analysis of the Atg1 complex. **a** Signaling pathways that transmit stress signals to regulate TORC1 and autophagy. **b** The Atg1/Atg13 complex structure. The domain organization schemes of Atg1 and Atg13 are shown above. Below left is the kinase domain structure of Atg1 in complex with its inhibitor (yellow) (4WNP), where the activation loop is highlighted in red. Below middle is the domain complex structure of Atg1 MIT (green)/Atg13 MIM (cyan) (4P1N). Below right is the HORMA domain complex structure of Atg13 (cyan)/Atg101 (grey) (4YK8), where Atg13 is folded in the closed state, compared to Atg101's open state conformation. **c** The Atg13–Atg17–Atg31–Atg29 complex structure. Atg17 (yellow) forms a dimer with a curvature radius close to 10 nm and appears as a letter "S". Atg29 (blue) and Atg31 (magenta) are located at the concave face of Atg17. Atg13 interacts with the N-terminus and C-terminus via 17BR (red) and 17LR (orange), respectively. The ability of Atg13 to bind to different Atg17 molecules simultaneously allows the supramolecular molecular assembly. 5JHF is used as a reference here but 4HPQ and 4P1W should also be noted

which are induced by the p53 tumor suppressor protein in response to DNA damage [18].

Initiation of autophagy in yeast is characterized by assembly of the Atg1–Atg13–Atg17–Atg31–Atg29 complex (Atg1 complex) and its localization to the PAS. In contrast, a similar Atg1 complex (ULK1/2–Atg13–Atg101–FIP200, refer to abbreviations in the legend of Table 1) is constitutively formed in mammalian cells and translocated to the PAS in response to autophagic signals [5, 19–21]. Assembly of the Atg1 complex is determined by the phosphorylation status of Atg13, which serves as an adaptor protein bridging Atg1 and the Atg17 subcomplex (Atg17–Atg31–Atg29) [19, 22]. Under nutrient-rich conditions, TORC1 heavily phosphorylates the C-terminal domain of Atg13 [21, 23, 24], which in turn disrupts the interaction between Atg13 and Atg1 or Atg17, and the Atg1 complex fails to be formed [25]. In contrast, when TORC1 activity is inhibited (Fig. 2a), Atg13 is quickly dephosphorylated. Consequently, the interaction between Atg13 and Atg1 or Atg17 is increased, facilitating the formation of the Atg1 complex [21, 24]. Figure 2b indicates the interaction between Atg1 and Atg13, which is mediated by Atg1 microtubule interacting and transport (MIT) domains of Atg1 and MIT-interacting motif (MIM) domains of Atg13 [19]. When two MIT domains form two antiparallel three-helix bundles (like collars), two MIM domains fold into an extended helix–loop–helix, reminiscent of a short tie wrapping collars.

Atg13 contains a Hop1p, Rev1p, and Mad2 domain (HORMA) at its N-terminus (Fig. 2b), which corresponds to the "closed" state of Mad2 (the spindle checkpoint protein) and plays an essential role in recruiting the Vps34 complex to the PAS during autophagy via the interaction with Atg14 [26]. Additionally, it was reported that an LC3 interacting region (LIR) is present in human Atg13 and required for proper autophagosome formation [27]. As such, Atg13 is regarded as a key regulator and organizer during the early stage of autophagy.

Atg1 (ULK1/2) is a serine/threonine protein kinase whose activity is regulated by Atg13 binding and by TORC1 phosphorylation [21, 24]. The kinase activity of Atg1 is required for autophagy, whereas it is not required for localization of the Atg1 complex to the PAS [25, 28, 29]. Indeed, C-terminal early autophagy targeting/tethering (EAT) domain of Atg1 plays a rather important role in localization, due to its membrane functions including membrane targeting [29], membrane curvature sensing, and lipid vesicle tethering [30]. The structure of Atg1's kinase domain was solved most recently, which reveals a canonical bilobal kinase fold (Fig. 2b) [31]. The mechanistic roles of Atg1's kinase activity in autophagy have been intensely studied in recent years. A number of mammalian substrates have been identified including activating molecule in Beclin 1-regulated autophagy (AMBRA1) and Beclin 1 (Atg6 homolog) [32, 33]. Atg1 phosphorylates AMBRA1 and releases the core machinery from dynein [32]. In addition, Atg1 phosphorylates Beclin 1 and activates Vps34 lipid kinase activity [33]. These findings link Atg1 catalytic activity to earlier regulatory events in mammalian cells, including translocation of the Atg1 complex to the endoplasmic reticulum (ER) membrane and subsequent omegasome formation.

Atg17–Atg31–Atg29 is constitutively formed and serves as a platform for PAS assembly [30]. Crystallographic analysis of this subcomplex shows that Atg17 forms a dimer via its C-terminal helix (Fig. 2c) [30, 34]. Each Atg17 monomer appears in a crescent shape with a curvature radius close to 10 nm and two Atg17 molecules are arranged like a letter "S". Dimerization of Atg17 is required for PAS assembly. Intriguingly, the curvature radius of Atg17 is similar to that of Atg9 containing vesicles, which earliest cluster at the PAS. It should be noted that Atg29 and Atg31 sterically hinder lipid vesicle binding at the concave face of the Atg17 crescent in the crystal structure (Fig. 2c). Furthermore, the ability of the Atg1 EAT domain to tether vesicles is lost in the presence of Atg13–Atg17–Atg31–Atg29 [30]. Considering the structural hindrance and the dynamic properties of Atg1, other regulatory factors are likely involved in PAS construction. Most importantly, Atg13 interacts with both the N-terminus and C-terminus of Atg17 via its 17BR and 17LR domains, thus promoting the supramolecular assembly of the Atg1 complex (Fig. 2c) [34].

Biogenesis of phagophore: the Vps34 complex

Phagophore biogenesis requires the lipid kinase activity of the Vps34 complex, in which Vps34 is the core enzyme [35–37]. As the only phosphatidylinositol 3-kinase (PI3K) in yeast (and the only class III PI3K in mammalian cells), Vps34 phosphorylates the membrane lipid

phosphatidylinositol (PI) to produce phosphatidylinositol 3-phosphate (PI3P). Blockage of this reaction with PI3K inhibitors, such as wortmannin and LY294002, inhibits PI3P production and autophagy [37]. The structures of Vps34 alone or in complex with inhibitors provided an important model for its enzymatic activation and inhibition [38]. Interestingly, Vps34 has an inherent auto-repressive mechanism (Fig. 3a). When in solution, the last C-terminal helix of Vps34 (shown in magenta) covers and blocks the ATP-binding site so as to avoid futile ATP hydrolysis. When Vps34 is recruited to the membrane during autophagy, there is a conformational switch of this helix (shown in cyan), which binds to the membrane and sways from the top of the ATP-binding site. As a result, the membranous substrate gains access to the catalytic center. Noteworthily, the ATP-binding site of Vps34 is unique in the PI3K class members, because it is so constrained that only small adenine analogues (inhibitors) can fit [38].

The activity of Vps34 is regulated by interactions with its partners including Vps15 (regulatory subunit) and Atg6 (Beclin 1, Vps30) [39–42]. Vps15 is a protein kinase whose enzymatic activity is required for activation of Vps34 [41, 42]. Additionally, its N-terminally myristoylation plays a role in Vps34 membrane targeting [41, 43]. In general, Atg6 functions as a protein platform to recruit additional factors into the Vps34 complex, such as Atg14 and Vps38 (UVRAG), to form the autophagy-specific complex (complex I) and the endosome sorting complex (complex II), respectively [40, 44]. This notion is supported by a most recent structure of the Vps38–Vps30–Vps15–Vps34 complex (Fig. 3b) [45]. This exciting work reveals a unique path for the complex assembly and molecular basis of regulation of Vps34 activity by Vps15 [45]. It also sheds light on the differential activity of complex II and complex I on their specific phosphorylation targets.

While the Atg14–Atg6–Vps15–Vps34 complex structure has yet to be determined, like Vps38, Atg14 uses its N-terminal coiled-coil to assemble with Vps34–Vps15–Atg6 and its C-terminal Barkor/Atg14(L) autophagosome targeting sequence (BATS) domain for membrane functions such as binding to curved membranes enriched in PI3P [46]. Atg14 also serves as a regulatory nexus of autophagy by interacting directly or indirectly with autophagy activators such as Ambra1, Bif-1 (Bax interacting factor, also known as endophilin B1), and vacuole membrane protein 1 (VMP1) [47–49] or autophagy inhibitors such as RUN domain and cysteine-rich domain containing, Beclin 1-interacting (Rubicon) [5, 50]. A long-standing, unanswered question in the field of autophagy is where and how phagophore biogenesis occurs. The formation of omegasomes near the ER is one model to explain phagophore biogenesis in mammalian cells, which is supported by experimental evidence [51, 52]. Omegasomes are "Ω" like and PI3P-rich membrane subdomains that dynamically connect to the ER membrane and serve as a platform for phagophore biogenesis and expansion [53, 54].

At the very beginning, autophagic signals activate the Atg1 complex and stimulate its localization to a subdomain of the ER membrane. Immediately, ER exit sites (ERESs) play an essential role in the hierarchical assembly of the autophagy machinery [55]. ERESs have been shown to be core autophagosomal biogenesis components, because autophagosomes are spatially, physically, and functionally linked to them [55]. ERESs are specialized regions of ER membranes that generate COPII transport vesicles. Interestingly, COPII-coated vesicles are also required for autophagy [56], suggesting ERESs might contribute one membrane source to both phagophore biogenesis and expansion via COPII transport vesicles. Consistently, COPII transport vesicles deliver the ER membranes containing the soluble *N*-ethylamaleimide-sensitive factor attachment protein receptors (SNARE) protein Ufe1 to sites of autophagosome formation [57].

In subsequent steps, the Atg1 complex recruits the Vps34 complex, likely via the interaction between Atg13 and Atg14 [26]. Vps34 is activated to produce PI3P. As a result, the local membrane concentration of PI3P is elevated, which in turn increases the membrane curvature. Considering that Atg14 has a high binding affinity towards curved membranes, a feed-forward loop is thus formed, leading to the facilitated recruitment of the Vps34 complex to the targeted region. Additionally, mammalian studies show that activation of Vps34 also induces the recruitment of COPII to the ER–Golgi intermediate compartment (ERGIC) to generate small vesicle active in Atg8/LC3 lipidation, which suggests ERGIC as another membrane source for autophagosome formation [58]. Finally, omegasomes are formed, from which the phagophore is generated as an extended membrane.

Besides increasing the membrane curvature, PI3P also functions as membrane anchors to recruit PI3P-dependent membrane-associated proteins that might assist or direct omegasome formation. These proteins include double FYVE-containing protein 1 (DFCP1) and Atg18 (WIPI1/2/3/4). DFCP1 binds to PI3P via its FYVE motif and clusters on the PAS in response to autophagic signals [54]. Of note, FYVE/PX/PH are the typical PI3P-binding motifs [59]. DFCP1 serves as a marker of omegasomes, but it is not required for autophagy [54]. Atg18 binds to PI3P via the FRRG motif. Noteworthily, Atg18's lipid binding activity is not required for the formation of the Atg18–Atg2 complex, whereas it is required for the recruitment of this complex to the PAS [60]. The precise location of DFCP1 and Atg18 is different in the PAS [61, 62]. DFCP1 is mainly localized to the omegasome, while Atg18 is concentrated on the phagophore protruding from omegasomes, suggesting that Atg18 might be mainly responsible for phagophore expansion. This

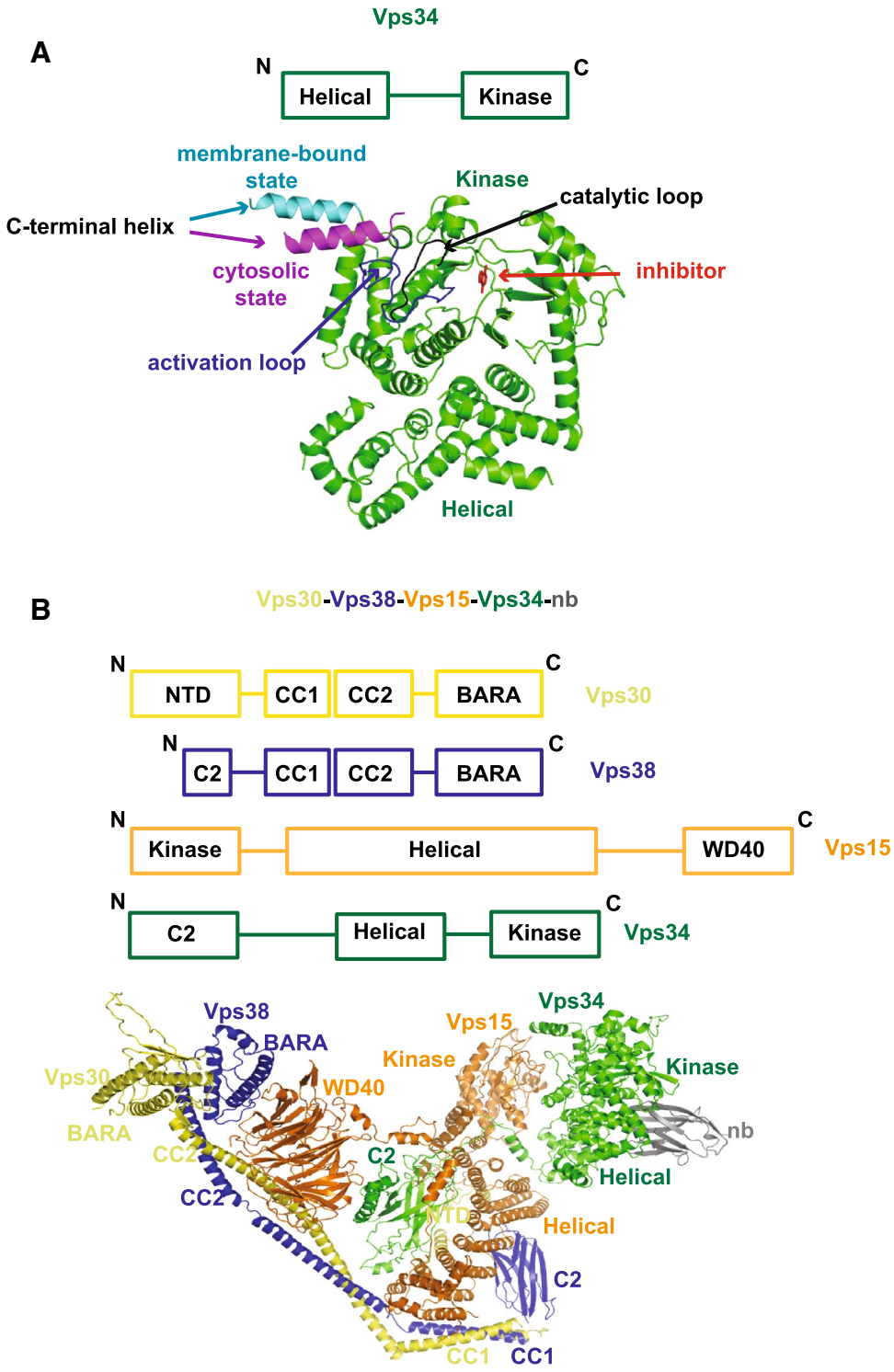
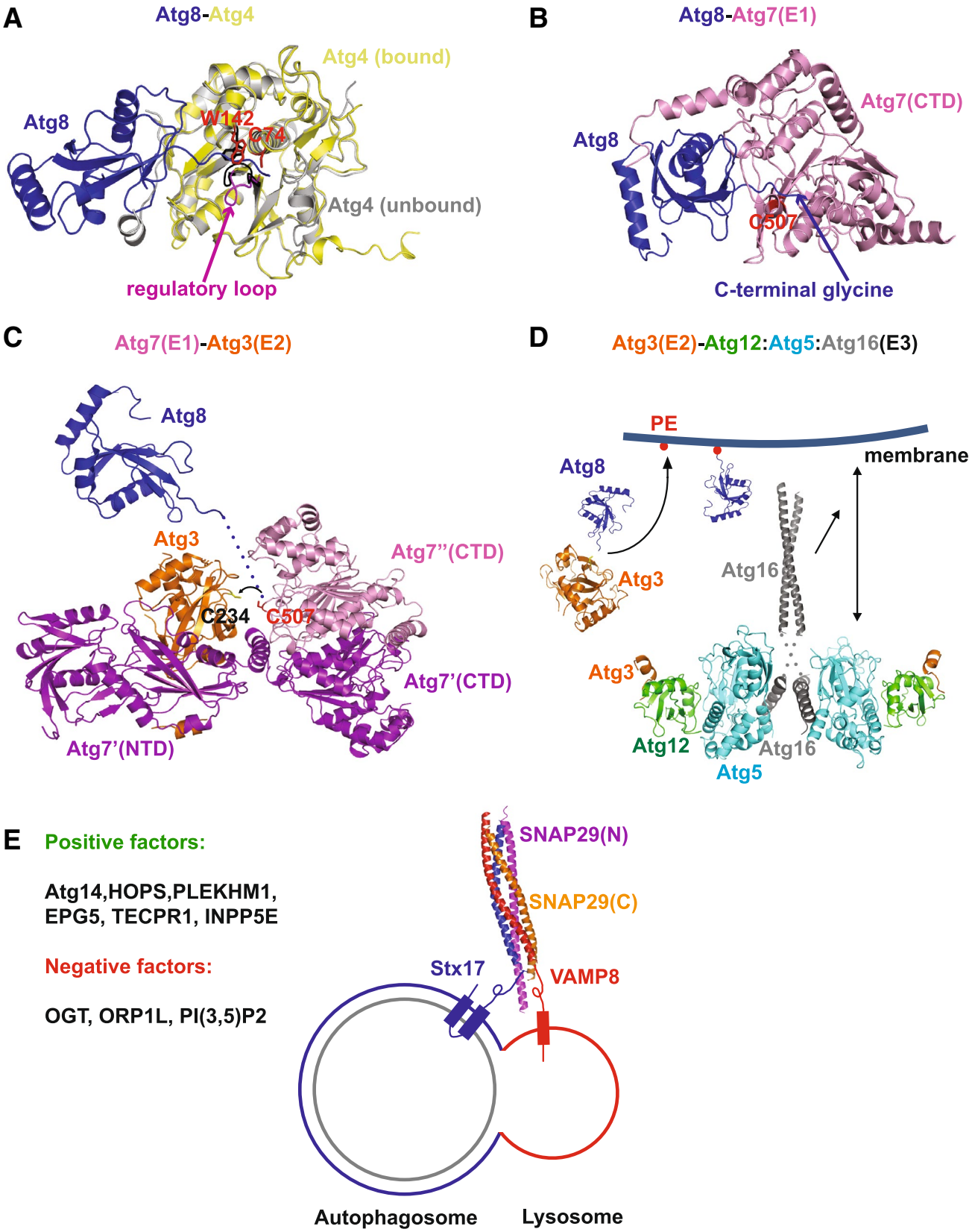


Fig. 3 Structural analysis of the Vps34 complex. **a** The helical and kinase domain structure of Vps34. The domain organization scheme of Vps34 is shown above. Below is the structure of Vps34 helical and catalytic domains (2X6H). The different colors of C-terminal helix indicate a potential conformational switch from its cytosolic state (magenta) to its membrane-associated state (cyan). The activation loop is highlighted in blue and the catalytic loop in black, when the inhibitor is shown in red. **b** The complex structure of Vps30–Vps38–Vps15–Vps34-nb. The domain organization schemes of individual subunits are shown above. Below is the complex structure (5DFZ) that appears as a letter "Y". One arm of "Y" is formed by intertwined Vps15 (orange) and Vps34 (green), where Vps15 contacts the activation loop of Vps34 to regulate its activity. The other arm is formed by intertwined Vps30 (yellow) and Vps38 (blue), which embraces the WD40 domain of Vps15 and the C2 domain of Vps34. nb (nanobody) is used as a crystallization chaperone

notion is supported by the role of Atg18 role in regulating two key events in phagophore expansion: Atg9 shuttling and Atg8 (LC3) lipidation [63, 64]. Reportedly, the Atg18–Atg2 complex, together with the Atg1 complex, regulates the retrograde transport of Atg9 from PAS to peripheral organelles [65, 66]. Additionally, Atg18, together with its family member Atg21, facilitates the localization of Atg8 and Atg16 to the PAS and protects Atg8–PE from cleavage by Atg4 before the phagophore expansion is completed [67].

Transmembrane proteins VMP1 and Atg9 also play a critical role during omegasome formation. VMP1 is localized to the PAS during autophagy and promotes Vps34 complex activity via its interaction with Atg6 [49, 62, 68]. Atg9 contributes to omegasome formation and phagophore


A
Atg8-Atg4
Atg4 (bound)
Atg8
W142
G74
Atg4 (unbound)
regulatory loop
B
Atg8-Atg7(E1)
Atg7(CTD)
Atg8
C507
C-terminal glycine
C
Atg7(E1)-Atg3(E2)
Atg8
Atg3
Atg7''(CTD)
C234
C507
Atg7'(CTD)
Atg7'(NTD)
D
Atg3(E2)-Atg12:Atg5:Atg16(E3)
PE
membrane
Atg8
Atg3
Atg16
Atg3
Atg12
Atg16
Atg5
E
Positive factors:
Atg14,HOPS,PLEKHM1,
EPG5, TECPR1, INPP5E
Negative factors:
OGT, ORP1L, PI(3,5)P2
SNAP29(N)
SNAP29(C)
Stx17
VAMP8
Autophagosome
Lysosome

◂**Fig. 4** Structural analysis of the Atg8 conjugating system. **a** The complex structure of Atg8–Atg4. The tail of Atg8 (blue) is inserted into the catalytic center of Atg4 (yellow) (2ZOD). C74 (red) stands for the catalytic residue of Atg4. W142 (red) and the regulatory loop (G257-A263, magenta) undergo a large conformational change, compared to Atg4's apo structure (grey, 2CY7). **b** The complex structure of Atg8–Atg7. The tail of Atg8 (blue) is inserted into the catalytic center of Atg7 (pink) (3RUI). C507 (red) stands for the catalytic residue of Atg7, which is located in the proximity of the last amino acid glycine of Atg8. **c** The complex structure of Atg7–Atg3. Two Atg7 molecules (Atg7′ and Atg7″ labeled here) form a dimer via their CTDs and coordinate in Atg3 binding and catalysis (4GSL). Atg7' NTD (purple) (Atg7″ NTD is not shown) pushes the backside of Atg3 (orange) to force its front side (containing the catalytic residue C234 in black) facing the catalytic center of Atg7″ CTD (pink) (containing the catalytic residue C507 in red). The juxtaposition of active sites of Atg7″ and Atg3 facilitates the transfer of Atg8 (modeled here) between two enzymes, as indicated by the arrow. **d** The complex structure of Atg3–Atg12–Atg5–Atg16. The Atg12–Atg5–Atg16 complex is present as a tetramer due to Atg16 dimerization (3A7O), and anchored to the target membrane via the association of Atg16 and Atg5 with the membrane, as indicated by the arrows. From the interaction between Atg3 (orange) and Atg12 (green) (4NAW), it is speculated that Atg8-conjugated Atg3 is recruited to the target membrane by Atg12. Then, Atg8 is transferred from Atg3 to the membrane substrate PE, as indicated by the arrow. **e** The factors involved in the autophagosomal fusion. Stx17, SNAP29, and VAMP8 form a SNARE complex that mediates the membrane tethering and fusion (4WY4). The regulatory factors are listed, which play either a positive or negative role in this process

biogenesis by transporting lipids and/or critical autophagic factors from peripheral organelles (e.g., the trans-Golgi network and endosomes) to the PAS [64].

The phagophore membrane is connected to the omegasome/ER membrane, which suggests that the ER membrane (e.g., ERES) is indeed the membrane source autophagosome. However, we cannot exclude the possibility that the omegasome only functions as a membrane anchor, while the phagophore forms most of its membrane through Atg9-mediated vesicle fusion in the expanding step [69–72]. Several membrane origins have been indicated for the autophagosome (Fig. 1). In the mitochondria model, the phagophore is derived from the outer membrane of mitochondria and developed with the lipid transfer from mitochondria [73]. Interestingly, this model requires the connections between ER and mitochondria and is unique to starvation-induced autophagy. In the cytoplasm membrane model, the phagophore comes from clathrin-mediated budding of the cytoplasmic membrane, which is dependent on the interaction between Atg16 (Atg16L1/2) and the heavy chain of clathrin [74]. With all the models, it is very likely that the autophagosome has multiple mechanisms to obtain membranes, all of which are dynamically regulated in response to different stress signals.

Expansion of phagophore: Atg9 and Atg8 lipidation

Atg9 shuttling and Atg8 lipidation are two key events in the phagophore expansion [63, 64]. Atg9 is the only transmembrane protein in the core Atg machinery and strictly required for autophagosome formation [75]. It is recruited to the PAS during autophagy in an Atg17-dependent manner, which is based on the Atg9–Atg17 interaction and requires the presence of Atg1 [76]. Atg9 shuttles between the PAS and peripheral organelles, and mediates material exchange between them [1, 4, 64, 66, 77]. In doing so, Atg9 containing vesicles are budded from peripheral organelles, anterograde transport lipids and/or critical autophagic factors to PAS, and then fused with the existing phagophore. During this process, Atg9 self-interaction plays a critical role [78, 79]. On the other hand, Atg9-containing vesicles are retrograde transported to peripheral organelles to recycle Atg9 and/or retrieve PAS-resident factors [65]. In this way, Atg9 shuttling regulates phagophore expansion. Importantly, SNARE proteins control Atg9-mediated vesicle fusion [80].

Atg8 lipidation is dependent on two ubiquitin-like conjugating systems: Atg12 and Atg8 conjugating systems. (1) The Atg12 conjugating system comprises Atg12 (ubiquitin-like module), Atg5 (substrate), Atg7 (E1 enzyme), and Atg10 (E2 enzyme) (Table 1). This system does not require an E3 enzyme and Atg12–Atg5 conjugate is constantly formed. In comparison, Atg12–Atg5 further complexes with Atg16 to form an E3 enzyme for the Atg8 conjugating system. (2) The Atg8 conjugating system consists of Atg8 (ubiquitin-like module), Atg4 (a cysteine protease to process Atg8), phosphatidylethanolamine (PE, substrate), Atg7 (E1 enzyme), Atg3 (E2 enzyme), and Atg5–Atg12–Atg16 (E3 enzyme). This system has been well characterized by structural biology approaches. First, Atg4 processes Atg8 into an active form by cleaving its last residue (R) and exposing the second last residue (G). It shows the structure of Atg4 (yellow) in complex with Atg8 (blue) in Fig. 4a. In the structure, the C-terminal tail of Atg8 is inserted into the catalytic core of Atg4 containing the catalytic residue C74 (marked in red), and induces a considerable conformational change of W142 and the regulatory loop (G257-A263) at the entrance of Atg4's active site, compared to Atg4's apo structure [81]. This structure also reveals the structural basis for Atg8–PE deconjugation by Atg4, which functions to recycle Atg8 or sensitize the autophagosome for its fusion with lysosomes [82]. Next, the structure in Fig. 4b shows how Atg8 (blue) is conjugated to Atg7 (pink). In the structure, the C-terminal tail of Atg8 is inserted into the catalytic core of Atg7's C-terminal domain (CTD) and positioned next to Atg7's catalytic residue, C507 (marked in red) [83].

While the structures of Atg3–Atg8 and Atg7–Atg3–Atg8 are yet known, the structure of Atg7–Atg3 sheds lights on

how Atg8 might be transferred from E1 to E2 (Fig. 4c). Two Atg7s form a dimer via their own CTDs and coordinate in Atg3 binding and catalysis. With this spatial arrangement, active sites of Atg7 (C507 in red) and Atg3 (C234 in black) are juxtaposed so as to facilitate the transfer of Atg8 (blue) between two enzymes (the curved arrowhead line). Also the structure of Atg8 in complex with Atg3–Atg12–Atg5–Atg16 has not been determined. Nevertheless, the structures of Atg3–Atg12–Atg5–Atg16 and Atg16–Atg16 provide helpful hints as to how Atg8 is finally conjugated to PE (Fig. 4d). The Atg12–Atg5–Atg16 complex is present as a tetramer due to Atg16 dimerization [84], and anchored to the target membrane via the association of Atg16 and Atg5 with the membrane [85]. Atg8-conjugated Atg3 is recruited to the target membrane by Atg12. Eventually, Atg8 is transferred from Atg3 to the membrane substrate PE, ending one round of Atg8 lipidation.

Maturation of autophagosome: Atg8 and Atg4

The expanding phagophore develops as a cup-shaped structure and surrounds the nearby cargo to sequester them from the cytosol. How cargoes are recruited to the expanding phagophores is a mystery. Recently, a number of cargo-specific receptors have been identified including Atg19, p62, neighbor of BRCA1 gene 1(NBR1) in degrading ubiquitinated protein aggregates, or Atg32, Nix (also called Bnip3L) in mitophagy, indicating that cargo recruitment is a selective process [86]. Interestingly, these receptors contain the LXXW motif/LIR that is specifically recognized by Atg8 [87], indicating Atg8 is involved in the selective cargo recruitment besides its role in phagophore expansion. It is intriguing to speculate that this Atg8-mediated interaction between expanding phagophores and encapsulating cargo is key to stabilization of the expanding membrane curvature [88]. In this regard, Atg8 serves as a sensor of membrane curvature during autophagosome formation. Consistent with this notion, the amount of Atg8 controls the size of autophagosomes [89]. Eventually, the expanding phagophore becomes a mature double-membrane vesicle with both ends sealed. While the exact underlying regulatory mechanisms of this process remain unknown, it is wellknown that Atg8–PE deconjugation by Atg4 is critical to the sealing step [90].

Fusion with lysosome: the SNARE complex

Mature autophagosomes dissociate from the PAS and fuse with lysosomes to form autolysosomes, during which process, the outer membrane of the autophagosome quickly fuses with the lysosome membrane (see Fig. 1) [4, 8, 9, 11]. The fusion step is controlled by a number of factors that play a positive or negative role.

A SNARE complex is key to the fusion step [91]. Syntaxin 17 (Stx17) specifically recognizes and localizes to the outer membrane of mature autophagosomes but not of expanding phagophores, where it forms a complex with SNAP29 and the lysosomal SNARE protein VAMP8 to facilitate membrane tethering and fusion (Fig. 4e). Atg14 has been shown to bind to Stx17 and SNAP29, promoting Stx17-mediated fusion events [92]. Interestingly, Atg14 is recruited by Stx17 to the ER–mitochondria contact site to participate in autophagosome formation [93]. O-GlcNAc-modification of SNAP29 negatively regulates fusion, which is mediated by the glycan transferase OGT [94]. Furthermore, the Vici syndrome protein EPG5 interacts with molecules on both lysosomes (Rab7, VAMP7/8) and autophagosomes (LC3, assembled Stx17–SNAP29 SNARE complex). As a result, it promotes the assembly of Stx17–SNAP29–VAMP7/8 complex and facilitates fusion [95].

Stx17 also associates with the homotypic fusion and protein sorting (HOPS) complex proteins, which participate in the regulation of the autophagosomal fusion. Additionally, the HOPS complex interacts with pleckstrin homology domain containing protein family member 1 (PLEKHM1), which functions as an adaptor protein that connects endocytic and autophagy pathways to lysosomes [96]. PLEKHM1 facilitates the fusion step through its binding to the HOPS complex and LC3 on the autophagosome membranes. Interestingly, the cholesterol-sensing Rab7 effector, ORP1L regulates the formation of ER–autophagosome contact sites, which inhibits the localization of PLEKHM1 to Rab7 and consequently suppresses the autophagosome–lysosome fusion [97]. Moreover, tectonin beta-propeller repeat-containing protein 1 (TECPR1), also plays a role in the fusion step [98].

Lastly, emerging evidence indicates that certain lysosomal lipids regulate autophagosome–lysosome fusion. Phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) antagonizes the fusion step by counteracting actin filament stabilization on the lysosomal surface. The enzyme inositol polyphosphate-5-phosphatase E (INPP5E), which catalyzes PI(3,5)P2, was shown to be involved in autophagosome–lysosome fusion process. Mutations in INPP5E are responsible for autophagy-related diseases such as Joubert syndrome [99].

After fusion, the inner membrane and inside cargo are attacked by a series of lysosome-resident acidic hydrolases, such as cathepsins, lipases, and glycosidases that break down the cargo into basic building blocks of proteins, lipids, and sugars, respectively [100, 101]. Disruption of hydrolases always causes autophagy defect and lysosome dysfunction. Atg15, a lysosomal lipase which is delivered to lysosome from ER through MVB pathway, is required for autophagic bodies breakdown, the first step of autophagic protein

turnover in lysosome/vacuole [102, 103]. Since the role of Atg15 in membrane lytic events, depletion of Atg15 inhibits both lipophagy and pexophagy [104, 105].

Subsequently, the resultant monomeric units from autophagic degradation are transported back to the cytosol through lysosomal membrane transporters (permeases), and participate in the cellular maintenance of energy, metabolic, and organelle homeostasis under autophagic conditions [106]. Atg22, Avt3 and Avt4 serve as partially redundant vacuolar effluxes, which mediate the efflux of leucine and other amino acids resulting from autophagy [107–109]. As far, no fatty acid or sterol effluxer protein has been identified, though some evidence derived from electron microscopic investigation indicates Atg22 might be a candidate in this process [105].

Reformation of lysosome–clathrin

During autophagy, lysosomes are exhausted to form autolysosomes, which are hybrid vesicles with mixed membrane compositions and containing components. Lysosomes are regenerated through a cellular process named autophagic lysosomal reformation (ALR), which is characterized by the formation of tubular structures extended from the autolysosomes [110]. Clathrin plays a key role in this process by regulating budding off of the autolysosome membranes to form reformation tubules, and also by regulating pitching off of the reformation tubular membranes to generate proto-lysosomes (lysosomal vesicles containing no hydrolases) [111, 112]. Proto-lysosomes inherit the ancestral lysosomal membranes and membrane transporters via a sorting mechanism [113], and further mature into functional lysosomes by taking in newly synthesized hydrolases through the Golgi–endosome pathway [101] (Fig. 1). Noteworthily, ALR is strictly coupled to autophagy, because ALR is eliminated when activity of TORC1, lysosomal proteases, or permeases is inhibited [110, 114].

As a conserved degradation pathway, autophagy has been reported to relate to several critical human diseases, such as neuronal degeneration diseases and cancer [6, 7]. It is a highly dynamic and complex process coupled with many steps. Although tremendous progress was made for understanding of autophagy in the past 24 years, the mechanisms of autophagy remain far from clear.

So far, the structures of many Atg proteins (Atg8 homologues) and their complexes (ATG5–ATG12–ATG16, Class III PI3K and Atg17–Atg29–Atg31, et al.) have been solved which facilitates drug development. For example, the structure of ECD domain in Beclin 1 shed light on the autophagy induction mechanism of tat-Beclin 1 peptides. Most recently, the co-crystal structures of Vps34 with two high-specificity inhibitors, PIK-III and SAR405, provide better understanding of two novel action mechanisms for PI3K kinase inhibition. Since conjugation system and autophagic ClassIII PI3K complex were established, autophagosome–lysosome fusogenic system is also reconstituted in vitro recently. These in vitro biochemistry systems provide promising strategies which can be exploited to develop more autophagy-specific inhibitors or inducers. Most structural biology studies mainly focus on the early stage of autophagy. With the development of autophagy studies, the structural information of important autophagy complex is urgently needed, especially in the fusion and lysosome reformation steps.

In addition, research on several parts in autophagy is still missing, such as omegasome formation, the composition of autophagosome, the content between inner membrane and outer membrane. How is the curvature of the isolation membrane determined? How is isolation membrane stitched? How is autophagic SNARE recruited to complete autophagosome but not to isolation membrane?

Selective autophagy including mitophagy, xenophagy, lipophagy, pexophagy stands out due to their critical physiological and pathological function in vivo. A subset of special genes has been identified such as different receptors for different types of selective autophagy. However, whether there are non-overlapping genes with non-selective autophagy machinery remain largely unknown. And it is also not clear about the mechanical difference between non-selective autophagy and selective autophagy. Recently, noncanonical autophagy was discovered, which bypasses a subset of autophagy essential genes or complexes. Of note, it is still elusive that these processes are real autophagy or just autophagy genes-involved processes.

In the future, both structural biology and biochemistry studies on potential drug targets in the different autophagic pathways will provide more critical information which probably further facilitates drug design and ultimately benefit patients.

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