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Structural biology of the core autophagy machinery

Hironori Suzuki, Takuo Osawa, Yuko Fujioka and Nobuo N Noda

In autophagy, which is an intracellular degradation system that is conserved among eukaryotes, degradation targets are sequestered through the *de novo* synthesis of a double-membrane organelle, the autophagosome, which delivers them to the lysosomes for degradation. The core autophagy machinery comprising 18 autophagy-related (Atg) proteins in yeast plays an essential role in autophagosome formation; however, the molecular role of each Atg factor and the mechanism of autophagosome formation remain elusive. Recent years have seen remarkable progress in structural biological studies on the core autophagy machinery, opening new avenues for autophagy research. This review summarizes recent advances in structural biological and mechanistic studies on the core autophagy machinery and discusses the molecular mechanisms of autophagosome formation.

Address
Institute of Microbial Chemistry, Microbial Chemistry Research Foundation, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

Corresponding author: Noda, Nobuo N (nn@bikaken.or.jp)

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Introduction

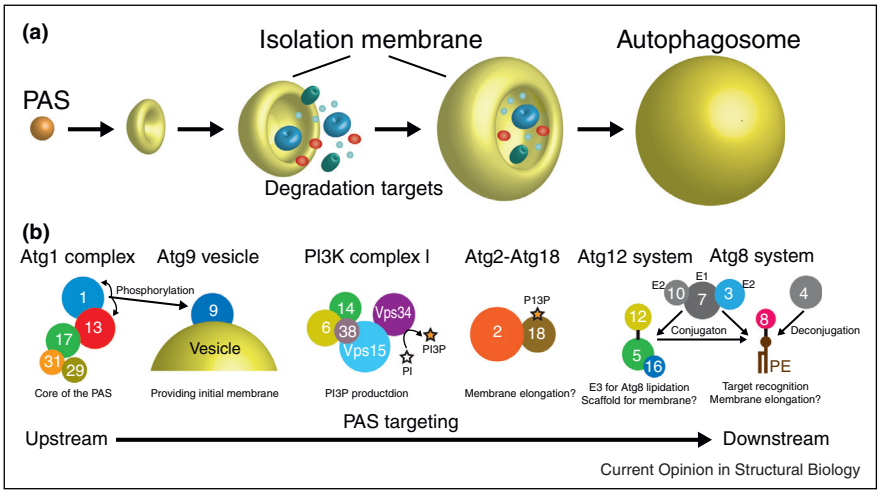
Autophagy is an intracellular degradation system conserved among eukaryotes ranging from yeast to mammals. In comparison with other degradation systems, the most distinguishing feature of autophagy is its ability to degrade almost everything in cells, including not only biomolecules (such as proteins) but also various organelles and invasive microbes [1]. The ability to degrade targets of various sizes is conferred by a unique mechanism of autophagy: the *de novo* formation of the autophagosome [2]. Autophagy induction is accompanied by the sudden appearance of an isolation membrane in the cytoplasm; this membrane expands and seals itself into an autophagosome while sequestering various materials inside it (Figure 1a). The autophagosome fuses with a lysosome, and all the contents of the autophagosome are degraded by lysosomal hydrolases. Thus, autophagosome formation determines the degradation of targets by autophagy.

Despite the crucial role of autophagosome formation in autophagy, its molecular mechanism remains elusive. Eighteen autophagy-related (Atg) proteins are required for starvation-induced autophagosome formation in budding yeast [2]. These proteins are classified into six functional groups: the Atg1 complex, also known as the autophagy initiation complex; Atg9; the Atg2–Atg18 complex; the autophagy-specific phosphatidylinositol (PI) 3-kinase (PI3K) complex; the Atg12–Atg5 conjugation system; and the Atg8–PE conjugation system [2]. These six groups localize to a perivacuolar site in a hierarchical manner to form the pre-autophagosomal structure (PAS), from which autophagosomes are generated [3] (Figure 1b). These six functional groups are evolutionarily conserved among eukaryotes, and thus are often called the 'core autophagy machinery.' To elucidate the molecular mechanism of autophagosome formation, understanding the function and structure of the core autophagy machinery is critical. Almost all previous structural biological studies focused on two conjugation systems [4,5]; however, recent studies have greatly increased our structural knowledge of the autophagy initiation and autophagy-specific PI3K complexes [6,7,8•]. We focused on findings regarding the structure and molecular function of the core autophagy machinery reported within the past few years and discussed the molecular mechanism of autophagosome formation.

Autophagy initiation complex

The autophagy initiation complex, directly regulated by several kinases such as TOR kinase complex 1 and AMP-activated protein kinase, represents the core of the PAS and functions as the most upstream factor in the core autophagy machinery [9,10]. The budding yeast autophagy initiation complex comprises five components (Atg1, 13, 17, 29, and 31) (Figures 1a and 2a). Atg1 (897 aa) is the sole protein kinase in the core autophagy machinery and comprises an N-terminal kinase domain (KD); two C-terminal tandem microtubule-interacting and transport (MIT) domains (MIT1 and 2), which are responsible for Atg13 binding [11••]; and an intrinsically disordered region (IDR), which links the KD and MIT domains (Figure 2a). MIT2 is partially disordered when unbound to Atg13 [12]. Atg13 (738 aa) comprises an N-terminal Hop1, Rev7, and Mad2 (HORMA) domain [13] and an IDR comprising approximately 60% of Atg13 residues. The flexibility of the Atg13 IDR was revealed by high-speed atomic force microscopy [14•]. Unlike the flexible structural features of Atg1 and Atg13, Atg17 (417 aa) folds

Figure 1

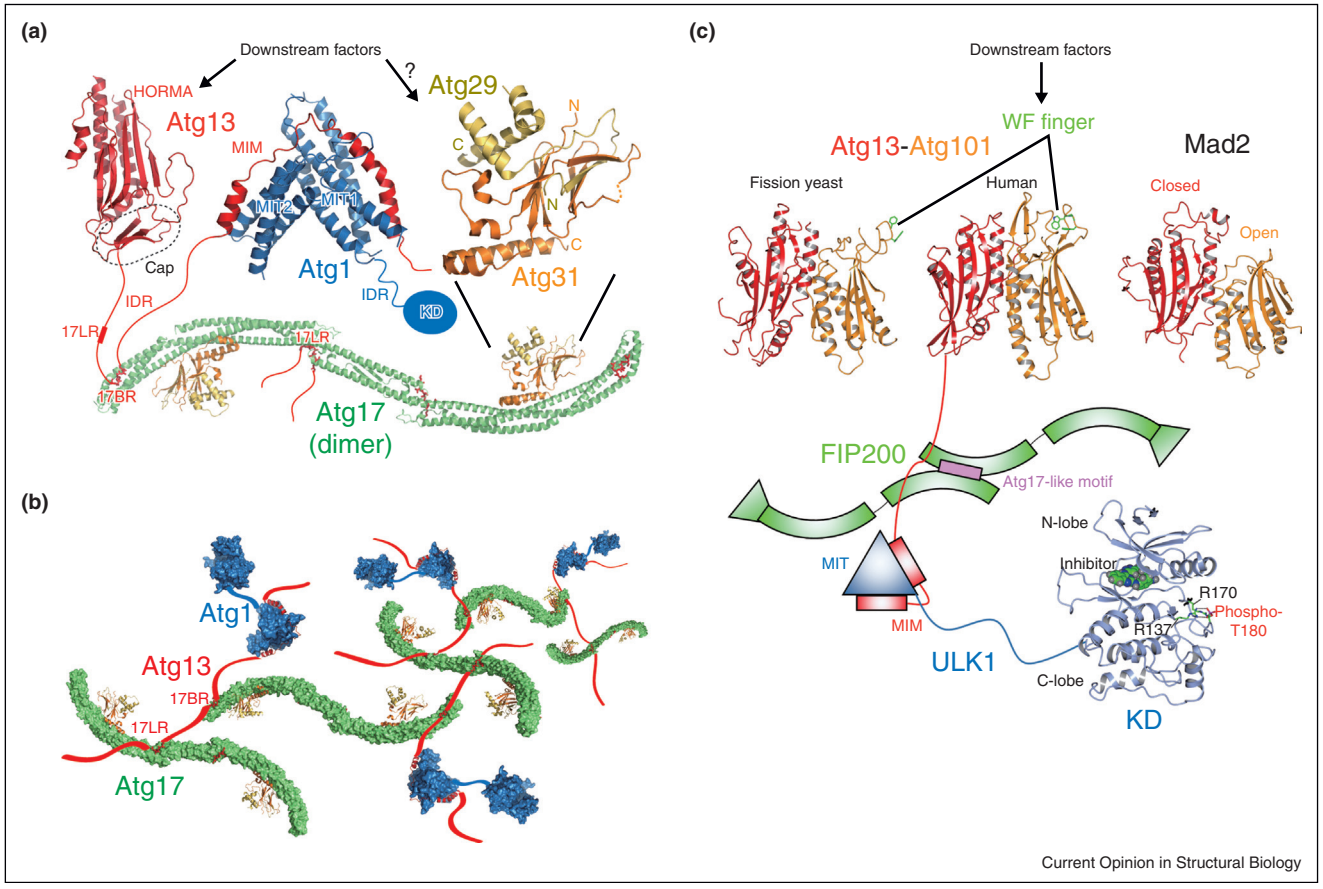


Principles of autophagosome formation. **(a)** Schematic drawing of autophagosome formation. **(b)** Core autophagy machineries. Six groups are targeted to the PAS in a hierarchical manner with the Atg1 complex and the Atg8 system as the most upstream and downstream factors, respectively. Atg proteins are indicated by spheres (roughly proportional to the protein size) with each number. Atg proteins that were not mentioned in this manuscript are colored gray. Some established functions of each group are also indicated.

into a single rigid architecture comprising four α-helices to form a unique S-shaped homodimer [15]. Atg29 (213 aa) and Atg31 (196 aa) form a stable heterodimer by sharing a single β-sheet and bind to the concave region of the S-shaped Atg17 [15]. The Atg17–Atg29–Atg31 complex is constitutively formed [16]; however, its interaction with Atg1 and Atg13 depends on nutrient conditions [11••,17]. The IDR of Atg13 possesses two functionally important Atg17-binding regions, 17BR (Atg17-binding region) and 17LR (Atg17-linking region), and Atg1-binding MIT-interacting motifs [11••,14•]. The formation of the pentameric complex is impaired under nutrient-rich conditions; this is attributed to the phosphorylation of serines in the binding regions [11••]. The 17BR and 17LR of Atg13 link Atg17 dimers to each other by binding to their binding pockets in different Atg17 dimers rather than in the same Atg17 dimer [14•] (Figure 2a,b). Direct weak interactions between Atg17 dimers were also revealed using small-angle X-ray scattering [18]. These interactions promote the supramolecular assembly of autophagy initiation complexes (Figure 2b), which function as the core of the PAS; dozens of each Atg protein were observed in the autophagy initiation complex *in vivo* [18,19].

All the components of the core autophagy machinery are highly evolutionarily conserved, except for those that compose the autophagy initiation complex. In mammals, the autophagy initiation complex comprises four components: Unc-51-like kinase (ULK)1/2 (Atg1 homologs), FIP200 also known as RB1CC1, Atg13, and Atg101 (Figure 2c). FIP200 (1594 aa) may be a functional counterpart of Atg17, despite markedly different size and little sequence homology, and Atg13 interacts with ULK1/2 and FIP200 *via* its C-terminal IDR in a manner similar to budding yeast Atg13 [20]. Atg101 (218 aa), not conserved in budding yeast, directly interacts with the HORMA domain of Atg13 [21]. This is unlike Atg29 and Atg31, which bind Atg17 but not Atg13 [15,22], indicating that Atg101 is not the counterpart of Atg29 or Atg31. The crystal structure of $ULK1^{KD}$ shows a typical bilobal kinase fold with phosphorylated Thr180 in the activation loop, which forms salt bridges with Arg137 and Arg170, thereby fixing the activation loop in the active conformation [23]. These residues are highly conserved among Atg1/ULK family kinases, indicating that autophosphorylation at Thr180 is a common mechanism for activating Atg1/ULK family kinases. Structural study has never been reported for FIP200 and thus its structure remains elusive. However, FIP200 possesses predicted coiled coils and a conserved sequence responsible for Atg17 dimerization [24], suggesting that FIP200 has at least partial structural similarity to Atg17. Structural studies on Atg101 alone [25] or complexed with Atg13 [26•,27] revealed that Atg101 is a HORMA protein. Structural studies on a representative HORMA protein, Mad2, established that the HORMA fold may assume two distinct conformations, open and closed. The HORMA domains of Atg101 and Atg13 correspond to the open and closed conformations, respectively, and the Atg101–Atg13 heterodimer is structurally quite similar to the dimer comprising the open and closed conformations of Mad2, wherein closed Mad2 is stabilized by open Mad2. These observations suggest that Atg13 is similarly stabilized by Atg101. In budding yeast, which does not have a conserved Atg101, $Atg13^{HORMA}$ has an additional β-sheet

Figure 2



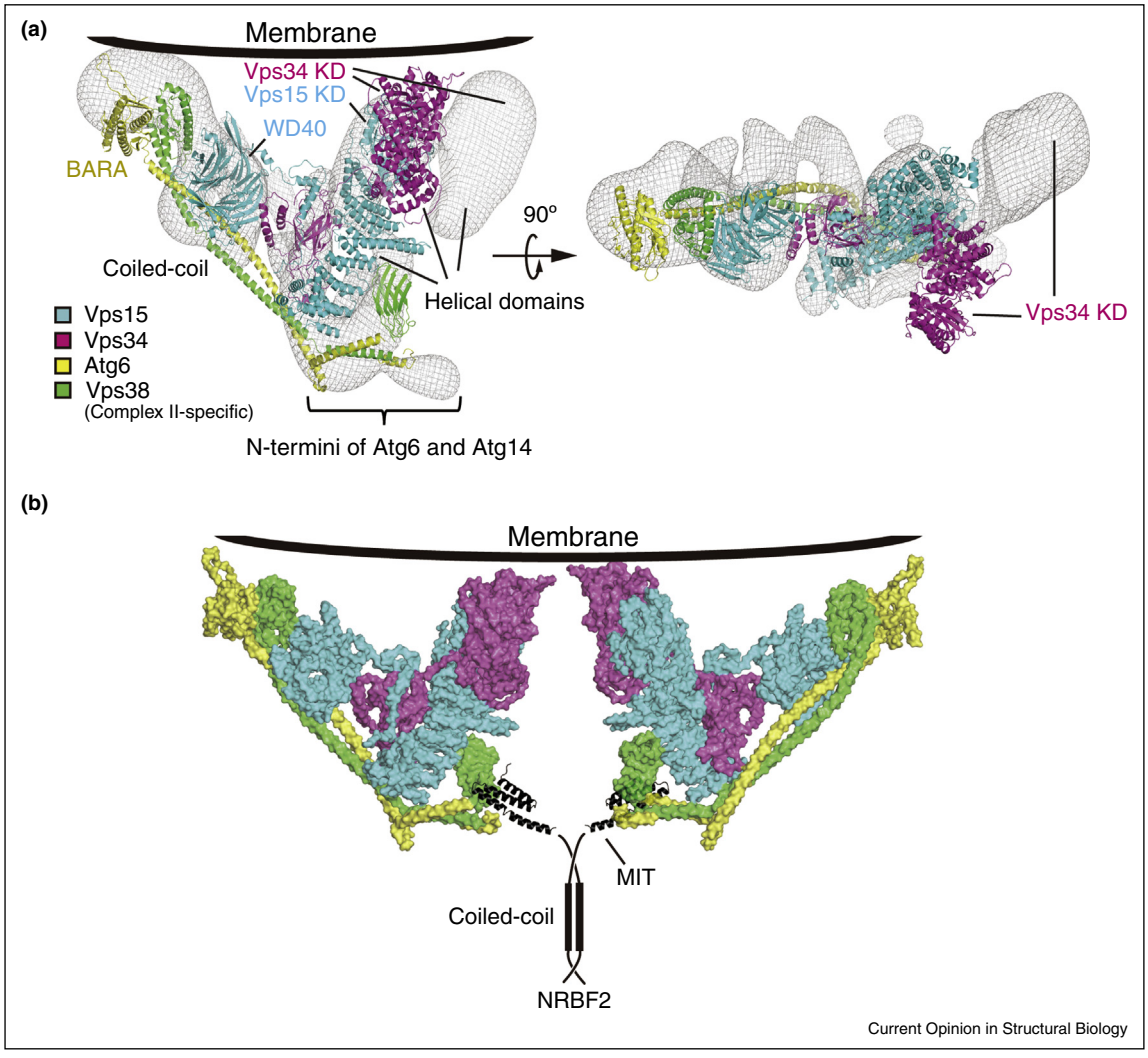
Structural basis of the autophagy initiation complex. **(a)** Structure of the components of the yeast autophagy initiation complex. **(b)** String-like Atg13 (red) binds multiple Atg17 dimers (green) using 17BR and 17LR, thereby promotes the supramolecular assembly of the autophagy initiation complexes. **(c)** Structure of the components of the mammalian autophagy initiation complex. For comparison, the Mad2 dimer structure is also shown. For model production, crystal structures of the Atg13–Atg17–Atg29–Atg31 complex (PDB 5JHF), the $Atg1^{MIT}$–$Atg13^{MIM}$ complex (PDB 4P1N), $Atg13^{HORMA}$ (PDB 4J2G), $ULK1^{KD}$ (PDB 4WNO), human and fission yeast Atg101–Atg13 complex (PDB 5C50 and 4YK8, respectively), and the Mad2 dimer (PDB 2V64) were used. Structural models in this manuscript were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) or CCP4MG [62].

named cap that stabilizes the HORMA fold [13,26•], allowing the protein to be stable without Atg101 (Figure 2a). Besides stabilizing Atg13, Atg101 plays another important role in autophagy: recruiting downstream factors to the autophagosome formation site. This process is mediated by the WF finger, a loop containing conserved Trp and Phe residues [26•], which seems to be mediated by $Atg13^{HORMA}$ [28] and Atg29–Atg31 in the case of budding yeast that lacks Atg101. It remains to be established how the WF finger exerts its role in recruiting downstream factors.

Autophagy-specific PI3K complex

The autophagy-specific PI3K complex, also known as PI3K complex I in yeast and class III PI3K complex I in mammals, comprises Vps34 (875 aa), Vps15 (1454 aa), Atg6/Vps30 (557 aa) (Beclin 1 in mammals), Atg14 (344 aa), and the recently identified Atg38 (226 aa) (NRBF2 in mammals) [2,29] (Figure 1b). PI3K complex I localizes to the PAS, dependent on the autophagy initiation complex and Atg9, and recruits downstream factors by producing PI 3-phosphate (PI3P) at the PAS [2]. The tetrameric human complex I (Vps34–Vps15–Beclin 1–Atg14) structure was determined by negative-stain electron microscopy at 28 Å resolution, which revealed a unique V-shaped architecture [30••] (Figure 3a, meshes). One arm of the V comprises a parallel coiled-coil heterodimer of Atg6 and Atg14 and the WD40 repeat domain of Vps15, whereas the other arm comprises two KDs and helical domains from Vps34 and Vps15. The edge of one arm corresponds to the C-terminal region of Atg6 and Atg14, which contains the beta-alpha repeated, autophagy-specific domain responsible for PAS targeting [31] and the Barkor/Atg14 autophagosome targeting sequence domain responsible for binding the curved PI3P-enriched membrane [32]. The edge of another

Figure 3



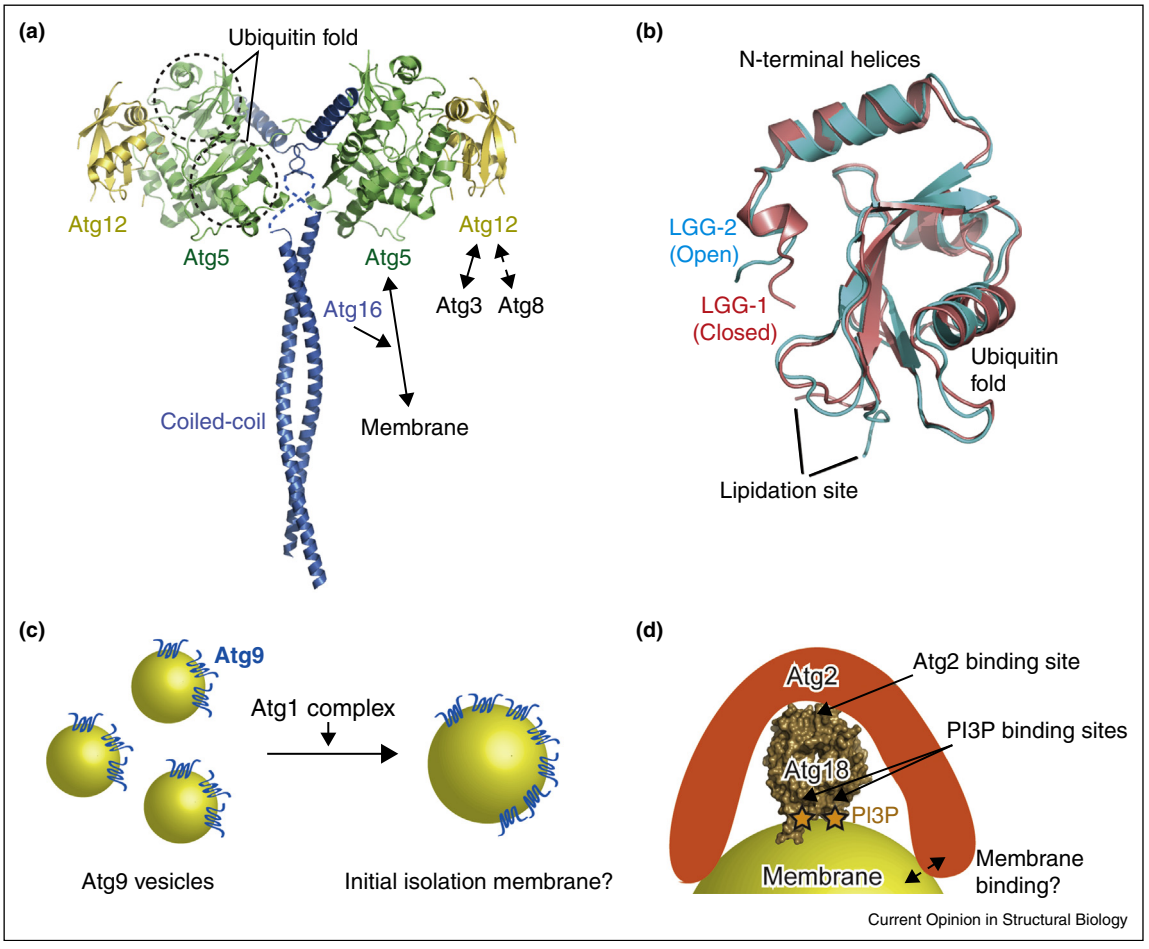
Architecture of the autophagy-specific PI3K complex. **(a)** 3D reconstruction of human PI3K complex I (EMDB 2846) on which the crystal structure of yeast PI3K complex II (PDB 5DFZ) was superimposed. **(b)** NRBF2 (Atg38 in yeast) binds to the bottom of the V-shape using MIT and dimerizes using the coiled-coil, thereby dimerizing the V-shaped PI3K complex I.

arm contains the lipid kinase domain of Vps34. These observations suggest that the PI3K complex faces the membrane using these two edges and catalyzes the phosphorylation of PI while exposing the bottom of the V shape to the cytoplasm. This orientation would enable the N-terminal region of Atg6 and Atg14 (located at the bottom of the V) to mediate interactions with various proteins and undergo various modifications [33]. The crystal structure of yeast PI3K complex II, which possesses Vps38 instead of Atg14 and is responsible for the multivesicular body pathway rather than autophagy, revealed a similar V-shaped or Y-shaped overall architecture with more precise and detailed structural information [34••] (Figure 3a, ribbon model). A large positional difference at $Vps34^{KD}$ was observed between the PI3K complex I and II structures. This finding, with studies of PI3K complex I showing high mobility of $Vps34^{KD}$ [30••], suggests that $Vps34^{KD}$ is highly mobile and changes its relative arrangement in the complex in solution. Negative-stain electron microscopic analysis of the pentameric human PI3K complex I showed that NRBF2 binds to the bottom of the V shape [8•]. Atg38/NRBF2 comprises an N-terminal MIT domain, responsible for interactions with other components of PI3K complex I, and a C-terminal coiled-coil domain, responsible for dimerization [29]. These observations indicate a model of two V-shaped complexes connected to each other at the bottom (Figure 3b). Although the molecular roles of the V shape and its dimerization remain elusive, such structural information would help accelerate the elucidation of the functions and regulation of the autophagy-specific PI3K complex.

Ubiquitin-like proteins Atg8 and Atg12

Among the 18 Atg proteins constituting the core autophagy machinery, as many as eight are involved in the Atg8 and Atg12 ubiquitin-like conjugation systems. Extensive structural studies were conducted on these systems and are summarized in several reviews [4–7]. Here we focus on the molecular roles of the two conjugation products in autophagosome formation. The Atg12–Atg5 conjugate (Atg12: 186 aa; Atg5: 294 aa) contains three ubiquitin folds, one from Atg12 and two from Atg5, which interact to create a globular architecture [35,36] (Figure 4a). The Atg12–Atg5 conjugate functions as an E3-like enzyme in the Atg8 system [37] and rearranges the catalytic site of Atg3 (310 aa), the E2 enzyme for Atg8, to allow a conjugation reaction [38]. Atg12 mediates the E2–E3 interaction by directly recognizing the flexible region of Atg3 [39]. The molecular mechanism underlying the conformational change in Atg3 induced by the Atg12–Atg5 conjugate remains elusive owing to a lack of clarity regarding the overall E2–E3 complex structure. The Atg12–Atg5 conjugate plays another important role as an E3 *in vivo*: it targets the E2 to the membrane, thereby providing the conjugation target PE. The latter role absolutely requires Atg16 (150 aa), a dimeric coiled-coil protein that binds Atg5 [40,41], enhancing the membrane-binding activity of the Atg12–Atg5 conjugate [42]. Besides its role as an E3 enzyme, the Atg12–Atg5 conjugate (in complex with Atg16) interacts with Atg8–PE *via* an Atg12–Atg8 interaction to form a membrane scaffold crucial for autophagosome formation, according to an *in vitro* study using giant liposomes [43]. More detailed characterization, including structural studies on the Atg12–Atg8 complex, would provide further support for this scaffolding model.

Figure 4



Structural insights into two Atg conjugates, Atg9 vesicle, and the Atg2–Atg18 complex. **(a)** Architecture of the Atg12–Atg5 conjugate bound to Atg16. Crystal structures of the Atg12–Atg5 conjugate bound to the N-terminal domain of Atg16 (PDB 3W1S) and the coiled-coil of Atg16 (PDB 3A7P) were used for model production. Critical interacting partners are indicated. **(b)** Open and closed conformations of Atg8 homologs. Crystal structures of LGG-1 (PDB 5AZF) and LGG-2 (PDB 5E6O) are shown as a representative of the closed and open conformations, respectively. **(c)** Model of initial isolation membrane formation from Atg9 vesicles. **(d)** Model of the Atg2–Atg18 complex on the curved membrane. Atg18 model is generated using the crystal structure of an Atg18 paralog, Hsv2 (PDB 3VU4).

Atg8 (117 aa) comprises a ubiquitin-like fold and two unique α-helices at the N-terminus [44]. One established function of the Atg8–PE conjugate is as a receptor for recognizing cargos during selective autophagy, usually with the help of adaptors/cargo receptors [44,45]. Besides cargo recognition, the Atg8–PE conjugate appears to have a critical role in autophagosome formation. In mammals, Atg8 lipidation-active vesicles are generated from the ER–Golgi intermediate compartment. These vesicles may be a membrane source of the autophagosomes [46,47]. In yeast, Atg3 was observed at the isolation membrane, suggesting that Atg8 lipidation occurs there [48,49]. The molecular role of the Atg8–PE conjugate in membrane dynamics is unclear. *In vitro* experiments showed that yeast Atg8–PE can tether and hemifuse liposomes [50], whereas mammalian Atg8 homologs can tether and fully fuse liposomes [51]. The worm Atg8 homologs LGG-1 and LGG-2 were shown to mediate the tethering and full fusion (LGG-1) or tethering alone (LGG-2) of liposomes [52]. All these studies indicate the importance of the unique N-terminal region of Atg8-family proteins. Structural comparisons suggested that the N-terminal conformation of Atg8 homologs can be characterized as open or closed [52]; this may determine the different activities of these proteins (Figure 4b). It is imperative to establish the molecular role of these membrane-related activities of the Atg8–PE conjugate in the context of autophagosome formation.

Atg9 and the Atg2–Atg18 complex: unexplored but absolutely critical factors

Despite advanced structural studies on other members of the core autophagy machinery, the structures of Atg9 and the Atg2–Atg18 complex remain unexplored. This omission is merely owing to technical difficulties and does not reflect a dearth of interest in these two members.

Atg9 (997 aa), the sole transmembrane protein in the core autophagy machinery, is predicted to possess six transmembrane helices. In cells, Atg9 is mainly incorporated into the Atg9 vesicle, a single-membrane vesicle that is generated from the Golgi apparatus and moves around in the cytoplasm [53]. When autophagy is induced, no more than three Atg9 vesicles localize to the PAS through interactions with the HORMA domain of Atg13 [28,53] and receive phosphorylation by Atg1 [54], both of which require the supramolecular assembly of the autophagy initiation complex [14•]. Furthermore, *in vitro* experiments showed that the autophagy initiation complex tethers Atg9 vesicles to each other [55]. These data suggest that the autophagy initiation complex recruits several Atg9 vesicles, which then fuse together to form an initial membrane source of the autophagosomes (Figure 4c).

Atg2 is a large, soluble protein (1592 aa) with no known domains/motifs. In mammals, Atg2 was reported to localize to lipid droplets without Atg18, suggesting that Atg2 itself is able to interact with lipids [56]. Unlike Atg2, the structure of Atg18 (500 aa) is predicted to be a seven-bladed β-propeller with two binding pockets for PI3P based on the crystal structure of an Atg18 paralog, Hsv2 [57–59]. Atg2 and PI3P bind to the opposite sides of Atg18 [57,60]. However, as a massive protein, Atg2 could simultaneously interact directly with the membrane and membrane-bound Atg18 (Figure 4d). The Atg2–Atg18 complex localizes to the expanding edge of the isolation membrane [61], suggesting a preference for highly curved membranes. This localization also indicates that the Atg2–Atg18 complex plays a key role in the elongation and/or closure of the isolation membrane. Structural studies on the Atg2–Atg18 complex are expected to provide valuable insights into these substantive questions.

Conclusions

Recently, structural studies on the core autophagy machinery have substantially progressed. However, the molecular mechanisms of autophagy, especially that of autophagosome formation, remain masked in mystery owing to the remarkable complexity of this phenomenon. Sporadic structural studies cannot answer essential questions. Consecutive, comprehensive structural studies on the core autophagy machinery in tight collaboration with biochemical and cell biological studies are indispensable for fully elucidating the molecular mechanisms of autophagosome formation.

Note added in proof

In a recent study on Atg38 and NRBF2 [63], crystal structure of the C-terminal dimerization domain of Atg38 was reported. Furthermore, it was shown that one Atg38 homodimer engages a single PI3K complex I, whereas human NRBF2 homodimer can bridge two PI3K complex I assemblies.

Conflict of interest statement

The authors declare no competing financial/personal interests.

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