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Review

Membrane-binding domains in autophagy

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ABSTRACT

Autophagy is an intracellular degradation system conserved among eukaryotes that mediates the degradation of various biomolecules and organelles. During autophagy, a double membrane-bound organelle termed an autophagosome is synthesized *de novo* and delivers targets from the cytoplasm to the lysosomes for degradation. Autophagosome formation involves complex and dynamic membrane rearrangements, which are regulated by dozens of autophagy-related (Atg) proteins. In this review, we summarize our current knowledge of membrane-binding domains and motifs in Atg proteins and discuss their roles in autophagy.

1. Overview of autophagy and autophagy-related (Atg) proteins

Autophagy is an intracellular degradation system conserved among eukaryotes and mediates the degradation of macromolecules (proteins, nucleic acids, and lipids), organelles, and invasive microbes (Mizushima and Komatsu, 2011). This versatile degradation system is made possible through a unique mechanism involving the sequestration of degradation targets inside the lumen of *de novo*-generated double-membrane organelles (termed autophagosomes) and subsequent delivery to the lytic compartment (lysosomes in mammals and vacuoles in yeasts and plants). On fusing an autophagosome with a lysosome, the sequestered materials, together with the inner membrane of the autophagosome, are degraded by lysosomal hydrolases. Autophagy indiscriminately degrades all materials sequestered within the autophagosome, and determining the targets of autophagy occurs at the step of autophagosome formation. Using yeast as a model organism, more than 40 autophagy-related (Atg) proteins have been identified (Mizushima et al., 2011; Tsukada and Ohsumi, 1993). These Atg proteins play various roles in autophagy, and in budding yeasts, 18 Atg proteins are essential for autophagosome formation. These “core Atg” proteins are classified into six functional groups: (i) the Atg1 complex, also known as the autophagy-initiation complex; (ii) Atg9; (iii) the Atg2–Atg18 complex; (iv) the autophagy-specific phosphatidylinositol (PI) 3-kinase complex; (v) the Atg12–Atg5 conjugation system; and (vi) the Atg8–-phosphatidylethanolamine (PE) conjugation system (Mizushima et al., 2011; Noda and Inagaki, 2015). When autophagy is induced in response to intracellular stresses, such as nutrient depletion, each functional group of the Atg proteins is hierarchically recruited to a peri-vacuolar site, forming the pre-autophagosomal structure (PAS) from which autophagosomes are believed to be generated (Suzuki et al., 2001). Most non-core Atg proteins are involved in selective-type autophagy, during which various organelles and biomolecules are selectively degraded (Wen and Klionsky, 2016).

Since autophagy involves unique membrane dynamics, Atg proteins interact in various ways with the membranes during autophagy. Among the Atg proteins identified to date (Atg1–42 at the time of writing), nine (Atg9, 15, 22, 27, 32, 33, 37, 39, and 40) encode transmembrane helices. Intriguingly, these transmembrane proteins are involved mainly in selective autophagy, with only one (Atg9) belonging to the core Atg proteins that regulate autophagosome formation. Therefore, other core Atg proteins must use membrane-binding domains or motifs to interact with the membrane. In this review, we summarize our knowledge of the membrane-binding domains and motifs of Atg proteins, focusing mainly on the core Atg proteins. We discuss the roles of these proteins in autophagosome formation (membrane-binding domains and motifs discussed throughout the article are summarized in Fig. 1 and Table 1, the functions of each protein complex in autophagy are summarized in Table 2, and the abbreviations used are summarized in Table 3).

2. The Atg1 complex

The Atg1 complex, which consists of five core Atg proteins (Atg1, 13, 17, 29, and 31) in the budding yeast, functions as the most upstream core Atg factor and mediates autophagy initiation (Noda and Fujioka, 2015). Under nutrient-rich conditions, Atg13 is hyperphosphorylated by the Tor kinase complex 1 (TORC1), which impairs the formation of the Atg1 complex. When TORC1 is inhibited during nutrient starvation, Atg13 is dephosphorylated, leading to the formation of the hetero-pentameric Atg1 complex (Fujioka et al., 2014; Kamada et al., 2000). Atg13 has another role in linking Atg1 complexes to one another,

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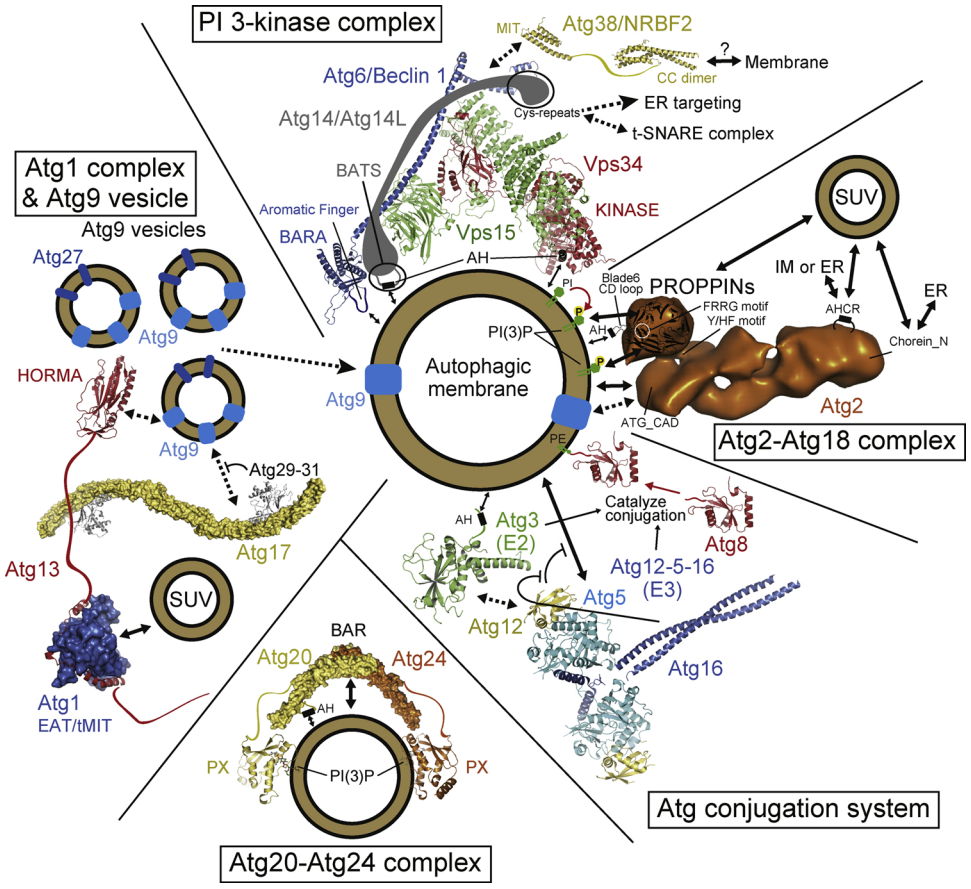

Fig. 1. Summary of the membrane-binding domains and motifs in autophagy-related proteins.

Atg1 complex and Atg9 vesicle: Atg1 complex consists of Atg1, Atg13, and Atg17-Atg29-Atg31 complex, where intrinsically disordered protein Atg13 links Atg1 and Atg17-Atg29-Atg31. Atg1 EAT/tMIT domain binds SUVs in vitro. The concave surface of Atg17 may bind Atg9 vesicles, which is inhibited by Atg29-Atg31. Atg13 HORMA domain recruits Atg9 vesicles via interaction with Atg9. Atg9 vesicles contain Atg9 and Atg27 and may function as initial membrane source for autophagosome formation.

PI 3-kinase complex: Autophagy-specific PI 3-kinase complex consists of Atg6/Beclin-1, Atg14/ATG14L, Atg38/NRBF2, Vps15 and Vps34. Vps34 KINASE, Atg14/ATG14L BATS, and Atg6/Beclin 1 BARA bind membranes using AH (KINASE and BATS) and an aromatic finger (BARA). Cys-repeats in ATG14L mediate ER targeting and t-SNARE complex formation. Atg38/NRBF2 CC may bind membranes. Vps34 KINASE phosphorylates PI to produce PI(3)P.

Atg2-Atg18 complex: The Atg2-Atg18 complex tethers IM to ER. Atg2 has at least three membrane-binding regions: Chorein_N, AHCR, and ATG2_CAD. Chorein_N and ATG2_CAD bind ER and IM, respectively, whereas it is not clear whether AHCR binds ER or IM. Atg2 binds PROPPINs using the Y/HF motif. PROPPINs bind two PI(3)P molecules using the FRRG motif. In addition, AH in the CD loop binds membranes.

Atg conjugation system: Atg8 is conjugated with PE through reactions similar to ubiquitination. Atg3, the E2 enzyme for Atg8, binds SUVs using AH. Atg3 also binds Atg12-Atg5-Atg16 complex, the E3 enzyme for Atg8. Atg5 binds membranes, which is inhibited by conjugation of Atg12 to Atg5 and this inhibition is canceled by Atg16. Atg3 and Atg12-Atg5-Atg16 complex cooperatively mediate conjugation between Atg8 and PE.

Atg20-Atg24 complex: The Atg20-Atg24 complex functions in the Cvt pathway (selective autophagy of vacuolar enzymes). Both Atg20 and Atg24 consist of PX and BAR domains, which binds PI(3)P and senses membrane curvature, respectively. Atg20 BAR contains AH, which also binds membranes.

generating higher-order assemblages of Atg1 complexes that function as the core of the PAS (Yamamoto et al., 2016). After the PAS core is formed, Golgi-derived vesicles containing the transmembrane protein Atg9 (Atg9 vesicles) localize to the PAS in an Atg13-dependent process, and are proposed to represent the initial membrane source for autophagosome formation (Suzuki et al., 2015; Yamamoto et al., 2012). Therefore, recruitment of the Atg9 vesicles to the PAS is a critical event in autophagosome formation. The Atg13 HORMA domain was reported to mediate the recruitment of Atg9 to the PAS (Suzuki et al., 2015). Since Atg13 lacks membrane-binding activity, it probably recruits Atg9 vesicles through protein–protein interactions. All five components of the Atg1 complex are soluble and have no canonical membrane-binding domains or motifs. However, the C-terminal domain of Atg1, which consists of two tandem MIT domains (tMIT) that mediate Atg13 binding (Fujioka et al., 2014), has been reported to interact *in vitro* with small unilamellar vesicles (SUVs) ranging in size from 20 to 30 nm (Ragusa et al., 2012). The tMIT domain was shown to mediate the tethering of SUVs, and thus was named the “early autophagy targeting/tethering” (EAT) domain (Ragusa et al., 2012). The EAT/tMIT domain did not interact with large unilamellar vesicles (LUVs), suggesting a preference for highly curved membranes. Although the MIT domain is well known for mediating protein–protein interactions (Hurley and Yang, 2008), its membrane-binding activity has rarely been studied and the mechanisms underlying SUV recognition by Atg1 EAT/tMIT domains remain to be elucidated. Moreover, the role of this domain’s membrane-binding activity in autophagy also remains to be established.

Atg17 has an elongated coiled-coil (CC) architecture forming an “S” shape (Ragusa et al., 2012). Its two concave surfaces are suitable for recognizing the convex surfaces of SUVs with sizes of ~20 nm; this process is similar to BAR domains that use their concave surfaces to recognize membrane curvature (Frost et al., 2007). However, Atg17 does not interact with SUVs *in vitro* and it is unclear whether Atg17 actually uses its concave surface to recognize curved membranes (Ragusa et al., 2012). Recently, Atg9-containing proteoliposomes were shown to bind Atg17 via an Atg9–Atg17 interaction, which was negatively regulated by the Atg29–Atg31 subcomplex. Moreover, the Atg1–Atg13 interaction was shown to override negative regulation by Atg29–Atg31, thereby enabling the Atg1 complex to bind Atg9-containing proteoliposomes (Rao et al., 2016).

3. The autophagy-specific PI 3-kinase complex producing PI 3-phosphate

PI 3-phosphate (PI(3)P) is an essential phospholipid for autophagy because it enables the recruitment of various Atg factors equipped with PI(3)P-recognition domains. In budding yeast, phosphorylation of PI to yield PI(3)P is governed by the sole class III PI 3-kinase, Vps34, which forms two distinct complexes (I and II) with Vps15, Vps30/Atg6, and either Atg14 or Vps38, respectively (Kihara et al., 2001). Complex I functions specifically in autophagy. By contrast, mammals have multiple PI 3-kinases (classes I–III). In collaboration with a class II PI 3-kinase, the heterotetrameric complex, consisting of Vps34, Vps15, Beclin 1 (Vps30/Atg6 homolog), and ATG14L/Barkor (Atg14 homolog), is involved in PI(3)P synthesis for autophagy (Devereaux et al., 2013;

Table 1
Summary of the membrane-binding domains and motifs in autophagy-related proteins.

Protein	Domains/motifs	Binding target
Atg1 complex		
Atg1	EAT/tMIT	Highly curved membrane
Atg17	CC	Curvature sensing (no binding)?
PI3-kinase complex		
Beclin 1	Aromatic finger in BARA	Membrane (cardiolipin preference)
ATG14	BATS (containing AH)	Membrane with packing defects
Vps34	C-terminal helix (AH)	Membrane
Atg38	CC	Membrane or Membrane protein?
PI3P effectors		
Atg2	Chorein_N	ER, Highly curved membrane
	ATG2_CAD	Curvature sensing, IM binding?
	AHCR (containing AH)	IM or ER
Atg18 (PROPPINs)	β-propeller	PI(3)P, PI(3,5)P2
	AH	Membrane with packing defects
Atg20	PX	PI(3)P
	BAR (containing AH)	Curvature sensing (binding)
Atg24	PX	PI(3)P
	BAR	Curvature sensing (no binding?)
Atg conjugation system		
Atg3	N-terminal helix (AH)	Membrane with packing defects
Atg5	Unknown	Binding to GUVs
Atg8	Ubiquitin fold	PE (via conjugation reaction)
Atg16	Unknown	Supporting Atg5 to bind GUVs
Transmembrane proteins		
Atg9	6 transmembrane helices	Atg9 vesicle, IM
Atg15	Single transmembrane helix	Vacuolar membrane
Atg22	10-12 transmembrane helices	Vacuolar membrane
Atg27	Single transmembrane helix	Atg9 vesicle
Atg32	Single transmembrane helix	Mitochondrial outer membrane
Atg33	4 transmembrane helices	Mitochondrial outer membrane
Atg37	Single transmembrane helix	Peroxisome membrane
Atg39	Single transmembrane helix	Nuclear membrane
Atg40	2 transmembrane helices	ER membrane

Table 2
Proposed functions of the membrane-localized proteins in autophagy.

Protein complexes	Proposed functions in autophagy
Atg1 complex	Construction of autophagosome formation sites on the vacuolar membrane (in yeast) or on the ER (in mammals). Recruitment of Atg9 vesicles. Phosphorylation of Atg proteins.
PI3-kinase complex	Production of PI(3)P at autophagic membranes.
PI3P effectors	
Atg2-Atg18 complex	Tethering of ER to IM. Lipid transport from ER to IM?
Atg20-Atg24 complex	Promotion of the Cvt pathway.
Atg conjation system	
Atg8-PE	Selective target recognition. IM expansion and closure. Degradation of autophagic bodies.
Atg3	Conjugation of Atg8 to PE.
Atg12-Atg5-Atg16	Conjugation of Atg8 to PE.
Transmembrane proteins	
Atg9, Atg27	Construction of Atg9 vesicles. Supply of initial membranes for autophagosome formation.
Atg32, Atg33	Promotion of mitophagy.
Atg37	Promotion of pexophagy.
Atg39	Promotion of nucleophagy.
Atg40	Promotion of ERphagy.
Atg15	Degradation of autophagic bodies.
Atg22	Efflux of amino acids from the vacuole to the cytoplasm.

Table 3
Abbreviations used in this manuscript.

AH	amphipathic α-helix
AHCR	AH-containing region
Atg	autophagy-related
BARA	beta-alpha-repeated, autophagy specific
BATS	Barkor/Atg14(L) autophagosome-targeting sequence
CC	coiled-coil
Cvt	cytoplasm-to-vacuole targeting
EAT	early autophagy targeting/tethering
ER	endoplasmic reticulum
ERES	ER exit site
GUV	giant unilamellar vesicle
IM	isolation membrane
LUV	large unilamellar vesicle
PAS	pre-autophagosomal structure
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI(3)P	PI 3-phosphate
PROPPIN	polyphosphoinositides
ERphagy	selective autophagy of ER
nucleophagy	selective autophagy of nucleus
mitophagy	selective autophagy of mitochondria
pexophagy	selective autophagy of peroxisome
SUV	small unilamellar vesicle
tMIT	tandem MIT domains
TORC1	Tor kinase complex 1

Itakura et al., 2008; Volinia et al., 1995). Structural studies revealed that both complexes have V-shaped architectures (Baskaran et al., 2014; Ma et al., 2017; Rostislavleva et al., 2015). The Vps34 and Vps15 kinase domains are located at the tip of the right arm of the complex, and the tip of the left arm consists of the beta-alpha-repeated, autophagy specific (BARA) domain of Atg6/Beclin 1 and the Barkor/Atg14(L) autophagosome-targeting sequence (BATS) domain of ATG14L. The membrane-binding ability of complex I depends on the narrow tips of the arms, formed from an amphipathic α-helix (AH) at the C-terminus of the Vps34 kinase domain, the BATS domain of ATG14L, and an aromatic finger of the Beclin 1 BARA domain (Fan et al., 2011; Huang et al., 2012). The C-terminal α-helix of the Vps34 kinase domain is involved in membrane binding, which cancels its autoinhibition function and increases the kinase activity of Vps34 (Miller et al., 2010).

Deformation of the complex's V-shape is also necessary to activate the Vps34 kinase through cancelling the negative regulation by Vps15 (Baskaran et al., 2014; Stjepanovic et al., 2017).

The ATG14L BATS domain serves as a PI(3)P-dependent sensor for membrane curvature (Fan et al., 2011). *In vitro*, the BATS domain preferentially binds smaller liposomes containing ~2% PI(3)P; liposomes containing a larger amount of PI(3)P negate the domain's preference for highly curved membranes. The BATS domain also bears an AH sequence at its C-terminus, which is essential for the targeting of mammalian complex I to early autophagic membranes with high curvatures. Although a sequence homologous to the BATS domain was undetectable in yeast Atg14, the C-terminal region of Atg14 appears to contain an AH motif according to computational predictions (Gautier et al., 2008) although its function has not yet been experimentally validated. By contrast, N-terminal regions of Atg14 orthologs are conserved across species. Characteristic cysteine repeats in the N-terminal region localize ATG14L to endoplasmic reticulum (ER) membranes, thus forming a platform for autophagosome formation by recruiting the remaining mammalian complex I subunits (Matsunaga et al., 2010). Furthermore, in the late stages of autophagy, the cysteine repeats mediate ATG14L oligomerization, which is essential for binding to a t-SNARE complex consisting of STX17 and SNAP29 (Diao et al., 2015). The t-SNARE complex, stabilized by oligomeric ATG14L, facilitates membrane fusion between autophagosomes and endolysosomes containing v-SNARE VAMP8. Thus, the complex I-specific subunit ATG14L participates in various ways in autophagic membrane binding through both its termini.

BARA domain is conserved at the C-terminus of Beclin 1 and Vps30/Atg6 (Huang et al., 2012; Noda et al., 2012). In budding yeast, the BARA domain is crucial for the targeting of complex I to the PAS (Noda et al., 2012). The membrane-binding region of the BARA domain has been identified in Beclin 1 (Huang et al., 2012). Partially conserved Phe–Phe–Trp loop-forming sequence, known as an aromatic finger, is required for membrane binding, with preference to cardiolipin, of the Beclin 1 BARA domain *in vitro* and for autophagic activity. The structural and biochemical properties of complex I suggest that two edges of the V-shaped complex I face membranes. A recent EM study of mammalian complex I on a lipid monolayer demonstrated that the BATS domain anchors the complex to a lipid monolayer, and the Vps34 kinase domain determines the orientation of the complex (Ma et al., 2017). The bottom of complex I interacts with Atg38 in yeast and its homolog NRBF2 in mammals (Araki et al., 2013). They comprise an N-terminal MIT domain that binds Atg6 and Atg14 and a C-terminal CC domain that mediates homodimerization (Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Ohashi et al., 2016). During starvation-induced autophagy, Atg38 promotes the localization of complex I to the PAS and enhances autophagic activity. Subcellular fractionation experiments suggested that the CC domain of Atg38 is required for the association with membranes or membrane proteins (Ohashi et al., 2016).

4. PI(3)P effectors

PI(3)P produced at the PAS by the autophagy-specific PI 3-kinase complex I recruits downstream Atg proteins either directly or indirectly. Direct PI(3)P effectors include the Atg2–Atg18 and the Atg20–Atg24 complexes. The Atg conjugation system is recruited to the PAS in a PI(3)P-dependent manner, but there has been no report of a direct interaction between the components of the conjugation system and PI(3)P. In mammals, DFCP1 is recruited to a specific ER compartment, termed an omegasome, through interactions of the FYVE domains of DFCP1 with PI(3)P (Axe et al., 2008). Although DFCP1 has been widely used as a marker for omegasomes, the role of DFCP1 in autophagy remains unknown because it is not conserved in yeasts and its deletion has no effect on the autophagic activity in mammals. Therefore, DFCP1 is not further discussed here.

4.1. The Atg2–Atg18 complex

The Atg2–Atg18 complex localizes to the PAS in a PI(3)P-dependent manner. During expansion of the isolation membrane (IM; autophagosome intermediate) for target sequestration, the Atg2–Atg18 complex localizes to the edge of the IM where it makes contact with ER exit sites (ERESs), where COPII-coated vesicles are generated (Graef et al., 2013; Suzuki et al., 2013). Considering that ER membranes are candidate membrane sources in autophagosome formation (Tooze and Yoshimori, 2010), the Atg2–Atg18 complex, which bridges the putative membrane donor and the IM, may play a central role in IM expansion. Hence, elucidating the molecular function of the Atg2–Atg18 complex is critical to understand the molecular mechanisms of autophagosome formation; however, much less is known about the function of this complex in comparison with other proteins and complexes.

Atg18 belongs to the β-propellers that bind polyphosphoinositides (PROPPIN) family and possesses binding activity with both PI(3)P and phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2). The PI(3)P-binding ability of Atg18 is essential for the recruitment of the Atg2–Atg18 complex to the PAS during autophagic processes (Obara et al., 2008b). Atg18 assumes a seven-bladed β-propeller fold that resembles a doughnut ring. Two blades (blades 5 and 6), which lie next to one another and share a conserved FRRG motif belonging to the PROPPIN family, harbor a phosphoinositide-binding site each (Baskaran et al., 2012; Krick et al., 2012, 2006; Nair et al., 2010; Watanabe et al., 2012). Both the arginine residues in the FRRG motif constitute a distinct phosphoinositide-binding site and are thought to interact with the phosphorylated inositol rings of PI(3)P and PI(3,5)P2. Arginine-to-threonine substitutions in the FRRG motif cause loss of phosphoinositide binding and defects in autophagy (Krick et al., 2006). In blade 6, β-strands C and D are connected by the long CD loop. Contact between Atg18 and membranes containing PI(3,5)P2 and/or PI(3)P results in reorganization of part of the CD loop into an AH, whose aligned hydrophobic residues penetrate the outer leaflet of the lipid bilayer (Gopaldass et al., 2017). The binding of PROPPIN Hsv2 (an Atg18 paralog) to liposomes was abolished by replacing the CD loop with a glycine-rich linker or substituting the aromatic residues in the AH with alanine (Baskaran et al., 2012; Busse et al., 2015). Moreover, deleting the CD loop in Atg18 resulted in the concentration of Atg18 on vacuolar membranes, although Atg18 lacking the amphipathic properties of the AH was less efficiently recruited to vacuoles than wild-type Atg18 (Gopaldass et al., 2017). These results suggest that AH formation within the CD loop acts as a positive regulator of membrane binding by PROPPIN proteins. Furthermore, the AH of Atg18 plays a crucial role in vacuole fission, but contributes to a much smaller degree to autophagic processes (Gopaldass et al., 2017). It is possible that Atg2 compensates for the loss of the membrane-binding function of the Atg18 mutants in autophagy, since Atg2 itself can also bind membranes. A recent structural and biophysical study of Atg18 revealed in detail its interaction with membranes (Scacioc et al., 2017). After initially binding to membranes through electrostatic interactions, Atg18 uses two phosphoinositide-binding sites to interact with PI(3)P or PI(3,5)P2 and maintain membrane residence. Membrane phosphoinositides, especially PI(3,5)P2, promote the oligomerization of Atg18, which exists as a monomer in solution. It is unclear whether Atg18 oligomerization is physiologically relevant and whether this also occurs when Atg18 is in a complex with Atg2. It has been proposed that during membrane binding, a doughnut-shaped Atg18 molecule is oriented perpendicularly to membranes, with both phosphoinositide-binding sites and the CD loop oriented toward membrane, and it simultaneously makes contact with other Atg18 molecules through its rim, leading to Atg18 oligomerization. Hsv2 shows much higher affinity for SUVs than LUVs (Busse et al., 2015), indicating that PROPPIN-family proteins prefer to bind membranes with high curvature. The edge of the IM, where the Atg2–Atg18 complex localizes during the autophagic process, is highly curved (~30 nm diameter) (Nguyen et al., 2017), suggesting that the

sensing of membrane curvature by Atg18 is probably important for autophagy (Michaillat et al., 2012). As AHs prefer highly curved membranes with lipid packing defects (Bigay and Antonny, 2012; Nguyen et al., 2017), Atg18 might efficiently bind the IM by sensing lipid packing defects via its CD loop in addition to electrostatic interactions and the specific recognition of PI(3)P.

Recent studies on mammalian Atg2 orthologs have shed light on the membrane-binding mode of Atg2 and its function in autophagy. There are two mammalian Atg2 orthologs, ATG2A and ATG2B, both of which are essential for mammalian autophagy (Velikkakath et al., 2012). ATG2A localizes to IMs, which appears to depend on four regions of ATG2A. Three of the four regions, which are evolutionally conserved among Atg2 orthologs, are registered in the Pfam database: Chorein_N (residues 14–111), ATG2_CAD (residues 1116–1251), and ATG_C (residues 1840–1932) (residue numbers corresponding to human ATG2A). The fourth region consists of residues 1723–1829 and includes a conserved AH-forming sequence (residues 1750–1767) (Chowdhury et al., 2018; Tamura et al., 2017). To simplify its description, this AH-containing region is hereinafter referred to as AHCR. The amphiphilicity of the AHCR's AH is essential for the localization of ATG2A to the IM as well as for autophagic activity. Interestingly, AHCR alone binds to SUVs and LUVs *in vitro*, suggesting that the AHCR AH is dispensable in recognizing membrane curvature. The N-terminal region containing Chorein_N is also essential for autophagic activity, whereas the ATG_C-containing region is dispensable for that (Tamura et al., 2017). Although the relationship between ATG2_CAD and autophagy remains elusive, ATG2_CAD appears to act as a sensor for highly curved membranes. Three-dimensional reconstructions of the ATG2A–WIPI4 (also known as WDR45) and ATG2B–WIPI4 complexes (Atg2–Atg18 homologs) using negative-staining EM revealed the relative positions of the four conserved regions (Chowdhury et al., 2018; Tamura et al., 2017; Zheng et al., 2017). ATG2A and WIPI4 form a rod-shaped heterodimer in which ATG2A takes on an elongated bar-like shape and WIPI4 binds to one end of the bar. These heterodimers are formed by interactions between a Y/HF motif conserved in mammalian ATG2s and a loop at the distal side of the phosphoinositide-binding sites of WIPI proteins (Chowdhury et al., 2018; Rieter et al., 2013; Watanabe et al., 2012; Zheng et al., 2017). Chorein_N and ATG2_CAD near the WIPI4-binding region are located at the opposite ends of the bar-shaped ATG2A, whereas ATG_C and AHCR could be observed at both ends of the rod due to the flexibility of the C-terminal region. Both ends of the bar-shaped ATG2A bind to SUVs independently of PI(3)P, enabling ATG2A to bridge the neighboring SUVs (Chowdhury et al., 2018). By contrast, efficient tethering of LUVs by ATG2A occurs only in the presence of PI(3)P and WIPI4. These *in vitro* experiments indicate that one end of the bar-shaped ATG2A containging Chorein_N can recognize various degrees of membrane curvature, while the other end of the bar (containing ATG2_CAD) binds specifically to highly curved membranes (Chowdhury et al., 2018). In this model, Atg2 ATG2_CAD and Atg18 enable one end of the rod-shaped complex to make specific contact with the IM edge by recognizing membrane curvature and PI(3)P. The relationship between in vitro membrane binding and in vivo functions was recently studied in detail for yeast Atg2. The N-terminal region in Chorein_N (residues 1–46 of ScAtg2) fused to GFP can target to the ER, whereas the C-terminal region containing an AH (residues 1347-1373 of ScAtg2) is necessary for targeting Atg2 to the PAS. Importantly, both regions were shown to be required for tethering liposomes in vitro, confirming that membrane binding and tethering activities observed in vitro are important for autophagy, possibly through tethering ER to the IM (Kotani et al., 2018). In yeast, the expanding edge of the IM, where Atg2-Atg18 complex and Atg9 colocalize, is in contact with the ER. Mutation in Atg2 that impairs the interaction with Atg9 changed the localization of Atg2 to the entire IM membranes and caused tethering of the ER to the entire IM, suggesting that Atg2 and Atg9 cooperatively form the contact site between the IM and the ER (Gomez-Sanchez et al., 2018). These observations collectively suggest that the Atg2-Atg18 complex tethers the ER (especially the ER exit site) to the expanding edge of the IM for autophagosome formation. Very recently, the Chorein_N domain of Vps13, which functions at organelle contact sites, was shown to possess lipid transfer activity *in vitro* (Kumar et al., 2018). Although Vps13 is not related to autophagy and the sequence homolog of Vps13 with Atg2 is quite low, it is tempting to speculate that Atg2 transfers lipids from ER to IM for autophagosome formation. Structural study of the Atg2-Atg18 complex at an atomic resolution will give us important insights into such fascinating mechanisms.

4.2. The Atg20–Atg24 complex

The Atg20 and Atg24 sorting nexins form a heterodimer that binds to the scaffold protein Atg11, an event that is essential for selective autophagy including the cytoplasm-to-vacuole targeting (Cvt) pathway (Nice et al., 2002; Reggiori and Klionsky, 2013). The precise roles of these proteins in selective autophagy remain elusive, but their membrane-binding regions have been defined (Popelka et al., 2017). Similar to other sorting nexins, both Atg20 and Atg24 contain a PX domain within their N-termini that acts as a PI(3)P-recognition module. The PX domain was first identified in the N-terminal regions of the $p40^{phox}$ and $p47^{phox}$ subunits of the NADPH oxidase complex (Bravo et al., 2001; Hiroaki et al., 2001). The PX domain folds into a wedge-like structure with a single pocket for PI(3)P-binding. Recognition of PI(3)P by the PX domains is mediated by two arginine residues and a tyrosine residue that recognize the phosphate groups and the inositol ring, respectively (Bravo et al., 2001; Lenoir et al., 2018; Xing et al., 2004; Zhou et al., 2003). Although not structurally characterized, the PX domains of Atg20 and Atg24 probably recognize PI(3)P contained in autophagic membranes similarly with other PX domains (Pylypenko et al., 2007; Wang et al., 2008). Besides the PX domain, Atg20 and Atg24 encode a BAR domain within their C-terminal regions. The BAR domain, which is composed of a three-helix bundle, dimerizes to form a well-known crescent shape (Frost et al., 2007). Heterodimer formation between Atg20 and Atg24 appears to be required for interactions between their BAR domains, although this is yet to be shown by direct evidence (Popelka et al., 2017). Except for the inverse BAR family, the concave surfaces of BAR domain dimers are positively charged and bind to membranes by electrostatic interactions. The BAR domains are involved in sensing membrane curvature using their unique shapes, and some domains contain an AH (Peter et al., 2004; Pylypenko et al., 2007). The BAR domain of Atg20 is thought to harbor an AH within a region connecting two helices within the helix bundle (Popelka et al., 2017). Membrane binding and the physiological role of the Atg20–Atg24 complex depend on the AH of Atg20, because mutations of Atg20 that affect the AH decrease the affinity of the heterodimer for liposomes and cause a significant defect in the Cvt pathway. Understanding the detailed functions of the Atg20–Atg24 complex together with Atg11 is important to understand the molecular mechanisms of selective autophagy.

4.3. The Atg conjugation system

The Atg conjugation system comprises eight Atg proteins constituting two ubiquitin-like conjugation systems essential for autophagy (Mizushima et al., 2011; Noda and Inagaki, 2015). In the first system, the ubiquitin-like protein Atg8 is processed by a cysteine protease, Atg4, to expose a glycine at its C-terminus. Subsequently, an E1-like enzyme, Atg7, and an E2-like enzyme, Atg3, catalyze the conjugation of Atg8 to the amine terminus of PE (Ichimura et al., 2000). In the second system, another ubiquitin-like protein, Atg12, is conjugated to a lysine residue in Atg5 through reactions catalyzed by Atg7 and another E2-like enzyme, Atg10 (Mizushima et al., 1998). The Atg12–Atg5 conjugate interacts with Atg16, giving rise to the Atg12–Atg5–Atg16 complex (Mizushima et al., 1999). The Atg12–Atg5–Atg16 complex functions as an E3-like enzyme to accelerate the conjugation of Atg8

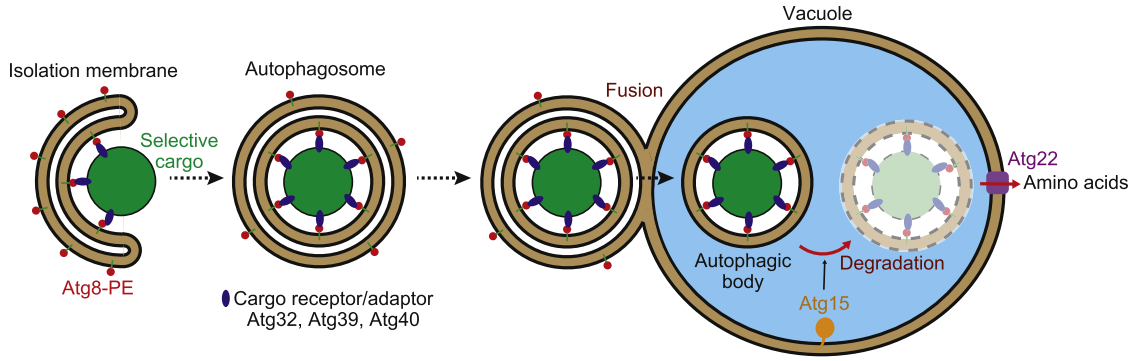

Fig. 2. Schematic drawing of selective autophagy and last steps of recycling mediated by transmembrane proteins.
Selective cargos such as mitochondria, ER, and nucleus possess a cargo receptor/adaptor with a transmembrane helix on the cytosolic side of their membrane. Atg8-PE on the IM recognizes the receptor and tethers the cargo to the concave surface of the IM, thereby facilitates selective incorporation of the cargo into the autophagosome. Autophagosomes then fuse with the vacuole and release their inner membrane (autophagic body), which is then degraded by a phospholipase Atg15 and other hydrolases. Amino acids from degraded proteins are transported to the cytoplasm through Atg22 for recycling.

and PE (Hanada et al., 2007). Since all eight Atgs are soluble proteins, they must interact with membranes in order to promote the conjugation of Atg8 and PE in the membrane. As described above, Atg8–PE and Atg12–Atg5–Atg16 are targeted to autophagic membranes in a PI(3)P-dependent manner (Suzuki et al., 2007); however, there have been no reports of direct interactions between them. Here, we summarize the membrane-binding properties of these proteins.

Atg8 is the only known protein that can be tightly bound to both the concave and convex sides of both forming and complete autophagosome membranes (Kirisako et al., 1999), and plays critical roles in the processes of autophagosome formation and selective cargo recognition (Fig. 2) (Nakatogawa et al., 2007; Noda et al., 2010). The tight membrane binding of Atg8 is mediated through covalent conjugation of the C-terminal glycine with PE (Ichimura et al., 2000). Although *in vitro* experiments showed that Atg8 can be conjugated with several lipids other than PE, including phosphatidylserine under high pH conditions (> 7.0) (Oh-oka et al., 2008), PE is considered to be the sole lipid that conjugates with Atg8 *in vivo*. *In vitro* experiments also revealed that the addition of acidic lipids aided the conjugation reaction between Atg8 and PE (Oh-oka et al., 2008). Since the acidic lipid PI(3)P is produced at the PAS and plays a critical role in recruiting Atg8 to the PAS, PI(3)P might serve to accelerate the Atg8 conjugation reaction *in vivo* (Krick et al., 2006; Obara et al., 2008a; Stromhaug et al., 2004; Suzuki et al., 2007). The molecular function of Atg8-PE conjugates in autophagosome formation has been a mystery. Atg8-PE was shown to be important for autophagic membrane expansion in yeast (Nakatogawa et al., 2007; Xie et al., 2008), but not essential in mammals, where mammalian Atg8-PE homologs were proposed to be important for the closing step of membranes or their degradation step after lysosomal fusion (Sou et al., 2008; Tsuboyama et al., 2016). PE has a small head group and thus has a cone shape, which is in contrast with cylindrical shapes of other phospholipids. Atg8 conjugation dramatically affects the shape of PE, which might be important for some steps in autophagy. Further biophysical characterization of Atg8-PE is important for unveiling the functions of this unique protein-lipid conjugate in autophagy.

Atg3 mediates the conjugation reaction between Atg8 and PE (Ichimura et al., 2000). The N-terminal region of Atg3 was predicted to contain an AH that was shown to be essential for the conjugation reaction between Atg8 and PE both *in vitro and in vivo* (Hanada et al., 2009; Nath et al., 2014). Atg3 binds directly to highly curved liposomes in an AH-dependent manner, especially those incorporating negatively charged lipids, such as cardiolipin and phosphatidic acid (Nath et al., 2014). The positively charged residues Lys9 and Lys11 in the AH of human Atg3 were shown to be important for this recognition (Hervas et al., 2017). The high content of the cone-shaped lipid PE in liposomes accelerated the conjugation between Atg8 and PE *in vitro*, even in the absence of the E3-like Atg12–Atg5–Atg16 complex (Nath et al., 2014). This effect could be attributed to the AH of Atg3, which senses lipid packaging defects in membranes and thereby anchors Atg3 to membranes. Among autophagic membranes, the edges of IMs are considered to be highly curved and thus to have lipid packing defects; therefore, it might be speculated that Atg3 binds in these regions. However, recent studies demonstrated that Atg3 is localized to entire IMs and not specifically to their edges (Ngu et al., 2015; Sakoh-Nakatogawa et al., 2015). It was also observed that the localization of Atg3 to the IMs depended on the Atg8-family interacting motif of Atg3, suggesting that Atg3 binds to Atg8–PE on the IMs rather than to the membranes directly (Sakoh-Nakatogawa et al., 2015; Yamaguchi et al., 2010). Very recently, the acetylation of Lys19 and Lys48 of Atg3 was reported to promote interactions with liposomes containing a physiological ratio of PE of 20% and to accelerate the conjugation between Atg8 and PE *in vitro* (Li et al., 2017). It is important to carefully relate the *in vitro* membrane-binding activities of Atg3 to its *in vivo* localization and functions in future studies.

The Atg12–Atg5–Atg16 complex functions as an E3-like enzyme to accelerate the conjugation between Atg8 and PE (Hanada et al., 2007). This ternary complex is important for Atg8–PE formation *in vivo* (Suzuki et al., 2001), whereas the Atg12–Atg5 conjugate (in the absence of Atg16) is sufficient for E3-like activity *in vitro* (Fujioka et al., 2008; Hanada et al., 2007). Atg12 mediates direct interaction with Atg3 (Metlagel et al., 2013; Noda et al., 2013; Otomo et al., 2013), whereas the Atg5–Atg16 subcomplex is responsible for localization to the PAS (Matsushita et al., 2007; Suzuki et al., 2001). The catalytic site of Atg3 is proposed to remain in an inactive conformation in the free form and is reorganized into an active conformation when the Atg12–Atg5 conjugate binds Atg3 (Sakoh-Nakatogawa et al., 2013). The Atg12–Atg5–Atg16 complex localizes to the PAS in a PI(3)P-dependent manner (Suzuki et al., 2007), although the mechanisms underlying this event are yet to be established. These observations suggest two distinct functions of the Atg12–Atg5–Atg16 complex: activating Atg3 and defining the site of Atg8–PE formation in cells. *In vitro* studies suggested some membrane-binding activities of this complex. A study using giant unilamellar vesicles (GUVs) suggested that Atg5 has binding activity with negatively charged membranes, which is impaired by the conjugation with Atg12 but reactivated by complex formation with Atg16 (Romanov et al., 2012). The recognition of negative charges in membranes was also shown to be mediated by Lys160 and Arg171 of Atg5. Another study using GUVs proposed a two-step membrane-binding model for the Atg12–Atg5–Atg16 complex: the complex alone can bind membranes, but with weak affinity yielding Atg8–PE on the membrane, which then recruits additional Atg12–Atg5–Atg16 complexes and forms

a two-dimensional meshwork over GUVs together with the Atg12–Atg5–Atg16 complexes (Kaufmann et al., 2014). The meshwork formation model of Atg8–PE and Atg12–Atg5–Atg16 is attractive as a framework for autophagosome formation, but requires further validation *in vivo*. These observations do not explain the necessity of PI(3)P for the localization of the Atg conjugation system at the PAS. Two groups independently reported a similar model that could explain the necessity of PI(3)P: the PI(3)P-binding PROPPIN-family proteins, Atg21 and WIPI2b, recruit Atg16 and ATG16L1 to the PAS in yeast and to the autophagosome formation site in mammalian cells, respectively (Dooley et al., 2014; Juris et al., 2015). Collectively, these observations suggest that the targeting mechanism of the Atg12–Atg5–Atg16 complex to the membrane is complicated and may be mediated through a combination of several distinct mechanisms. Structural studies of this complex with other Atgs and membranes are required for a more definitive understanding of its membrane interactions.

5. Atg proteins with a transmembrane region

As described in the introduction, nine Atg proteins (Atg9, 15, 22, 27, 32, 33, 37, 39, and 40) are transmembrane proteins. Here we briefly introduce the reported functions of these proteins (some of them are summarized in Fig. 2).

Atg9 is predicted to possess six transmembrane helices and is incorporated into a small vesicle (Atg9 vesicle) together with Atg27, which possesses one transmembrane helix (Kakuta et al., 2012; Yamamoto et al., 2012). Atg9 interacts with various Atg proteins that include Atg2, Atg11, Atg13, Atg17 and Atg18 (He et al., 2006; Reggiori et al., 2004; Sekito et al., 2009; Suzuki et al., 2015). Atg9 is essential for both bulk and selective autophagy, whereas Atg27 is less important for bulk autophagy (Yen et al., 2007). As mentioned above, Atg9 vesicles are considered to provide initial membranes for generating IMs. However, the molecular functions of Atg9 and Atg27 remain to be established.

During selective autophagy, specific cargo is generally linked to the IM via a cargo receptor (or adaptor) and a lipidated form of Atg8-family proteins that decorates the IM. Atg32, Atg39, and Atg40 are receptors for selective autophagy of mitochondria (mitophagy), nucleus (nucleophagy), and ER (ERphagy), respectively (Kanki et al., 2009b; Mochida et al., 2015; Okamoto et al., 2009). They have a transmembrane helix that anchors each protein to each specific organelle membrane, thereby labeling each organelle for specific sequestration by the IM. Besides the labeling role, some of these receptors might have a role in organelle fragmentation. In the case of nucleophagy and ERphagy (and some cases in mitophagy), these organelles are too large to be sequestered by an autophagosome. Therefore, they must be fragmentated prior to the sequestration, for which these receptors might be involved. Atg33 is also responsible for mitophagy, but its molecular function remains to be established (Kanki et al., 2009a). Atg37 is responsible for selective autophagy of peroxisomes (pexophagy) (Nazarko et al., 2014). It was reported that Atg37 regulates the phosphorylation of Atg30, a pexophagy receptor, through recruiting the Hrr25 kinase that positively regulates pexophagy (Zientara-Rytter et al., 2018).

Atg15 and Atg22 function at the final steps of autophagy after fusion of autophagosomes with the vacuole. Atg15 is a vacuolar phospholipase that is responsible for degradation of autophagic bodies in the vacuole (Epple et al., 2001; Ramya and Rajasekharan, 2016; Teter et al., 2001). A transmembrane helix is predicted at the N-terminus of Atg15, which would anchor this protein to the luminal side of the vacuolar membrane. Since Atg15 is a lipase, it is speculated that Atg15 degrades the membrane of autophagic bodies using its lipase activity; however, the molecular mechanism remains to be elucidated. Atg22 is a vacuolar membrane protein that is predicted to possess 10–12 transmembrane helices (Suriapranata et al., 2000). Atg22 functions as a vacuolar effluxer that is proposed to mediate efflux of amino acids resulting from autophagic degradation, the final step of recycling mediated by autophagy (Yang et al., 2006).

6. Relationship between membrane binding domains in autophagy and membrane lipids

It is known that there are two membrane territories in the cell (Bigay and Antonny, 2012). One territory includes ER and cis-Golgi, where membranes are poorly charged on their cytosolic leaflet but have high lipid-packing defects due to the abundance of lipids with monounsaturated chains. The other territory includes trans-Golgi, endosome, and plasma membrane, where membranes are abundant with negative lipids on their cytosolic leaflet but are tightly packed due to the abundance of saturated lipids. As mentioned above, many Atg proteins possess an AH and utilize it for membrane binding. Since AHs prefer highly curved membranes with lipid packing defects (Bigay and Antonny, 2012; Nguyen et al., 2017), it can be speculated that autophagy-related proteins favor ER and cis-Golgi membranes. Consistently, in mammals, autophagy-related proteins are known to cluster on ER membranes (Axe et al., 2008; Itakura and Mizushima, 2010). Membranes with high lipid-packing defects are more easily deformed compared with those with low lipid packing defects and thus seem to be suitable for functioning as a membrane source for autophagosome formation.

Among phospholipid biosynthetic enzymes involved in the de novo synthesis and remodeling pathways, phosphatidylinositol synthase, cholinephosphotransferase 1, choline/ethanolaminephosphotransferase 1, phosphatidylserine synthase 1, and phospholipase D1A were shown to colocalize with the autophagy initiation complex in starved mammalian cells (Nishimura et al., 2017). These enzymes mediate de novo synthesis of PI, phosphatidylcholine, PE, phosphatidylserine, and phosphatidic acid, major components of endomembranes. Interestingly, phosphatidylinositol synthase forms puncta on the ER membranes, to which the autophagy-initiation complex is recruited. It is tempting to speculate that upon autophagy induction, de novo synthesis of PI and its conversion to PI(3)P procced at autophagosome formation sites, which are used for recruiting autophagy-related PI(3)P effectors. It is also important, but yet unresolved, issue that what percentage of phospholipids that are used as building blocks for autophagosomes are de novo synthesized after autophagy induction.

7. Conclusions and perspectives

In order to regulate membrane dynamics during autophagy, Atg proteins must interact with membranes in various ways. Most of these interactions, which are summarized here, appear to be weak and transient. Even the well-characterized interaction of PROPPIN-family proteins with PI(3)P is quite weak. It may be possible that the weakness of the membrane-binding activity is important for the regulation of complicated membrane dynamics during autophagy. The elucidation of weak protein–membrane interactions is a major challenge that must be overcome to unveil the mysteries of autophagy.

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