Mechanisms governing autophagosome biogenesis

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Abstract | Autophagosomes are double-membrane vesicles newly formed during autophagy to engulf a wide range of intracellular material and transport this autophagic cargo to lysosomes (or vacuoles in yeasts and plants) for subsequent degradation. Autophagosome biogenesis responds to a plethora of signals and involves unique and dynamic membrane processes. Autophagy is an important cellular mechanism allowing the cell to meet various demands, and its disruption compromises homeostasis and leads to various diseases, including metabolic disorders, neurodegeneration and cancer. Thus, not surprisingly, the elucidation of the molecular mechanisms governing autophagosome biogenesis has attracted considerable interest. Key molecules and organelles involved in autophagosome biogenesis, including autophagy-related (ATG) proteins and the endoplasmic reticulum, have been discovered, and their roles and relationships have been investigated intensely. However, several fundamental questions, such as what supplies membranes/lipids to build the autophagosome and how the membrane nucleates, expands, bends into a spherical shape and finally closes, have proven difficult to address. Nonetheless, owing to recent studies with new approaches and technologies, we have begun to unveil the mechanisms underlying these processes on a molecular level. We now know that autophagosome biogenesis is a highly complex process, in which multiple proteins and lipids from various membrane sources, supported by the formation of membrane contact sites, cooperate with biophysical phenomena, including membrane shaping and liquid–liquid phase separation, to ensure seamless segregation of the autophagic cargo. Together, these studies pave the way to obtaining a holistic view of autophagosome biogenesis.

Macroautophagy (hereafter autophagy) — a process of degradation of intracellular components, including cytoplasmic content and (parts of) organelles and membranes as well as proteins and nucleic acids — was originally discovered by electron microscopy studies of mouse and rat cells in the late 1950s and early 1960s[1–6]. However, its physiological functions and molecular mechanisms had remained enigmatic for a long time until yeast genetic studies identified genes responsible for autophagy in the 1990s and in this century[7–9]. These genes, named 'Atg (autophagy-related) genes'[7], were also found in most eukaryotes, including higher plants and animals[10]. By knocking out or knocking down ATG genes, researchers began to study the impact of autophagy on various physiological functions in different cells and organisms, including adaptation to nutrient starvation, turnover of cellular constituents, antiageing, organelle homeostasis and immune response, as well as the relationship between autophagy and human diseases such as neurodegenerative diseases, diabetes, cancer and microbial infection[11,12]. Identification of the ATG genes and their products, ATG proteins, also set the stage for molecular dissection of the autophagy mechanism. Reliable methods for analysis of autophagy, including fluorescent protein tagging of ATG proteins, that are more accessible than electron microscopy have been established[13], accelerating the expansion of this research field. Consequently, autophagy has developed into one of the hottest topics in basic life science and medical research today.

Cellular materials to be degraded by autophagy are sequestered into double-membrane vesicles called 'autophagosomes' and transported to lysosomes in mammalian cells or vacuoles in yeast and plant cells. The biogenesis of autophagosomes is a hallmark of the autophagy process (FIG. 1) and constitutes the essence of autophagic engulfment of cargo. Briefly, during autophagosome biogenesis, a small flattened membrane structure called the 'isolation membrane' or 'phagophore' appears in the cytoplasm, expands, curves and becomes spherical, and following pore closure, formation of the double-membrane vesicular autophagosome

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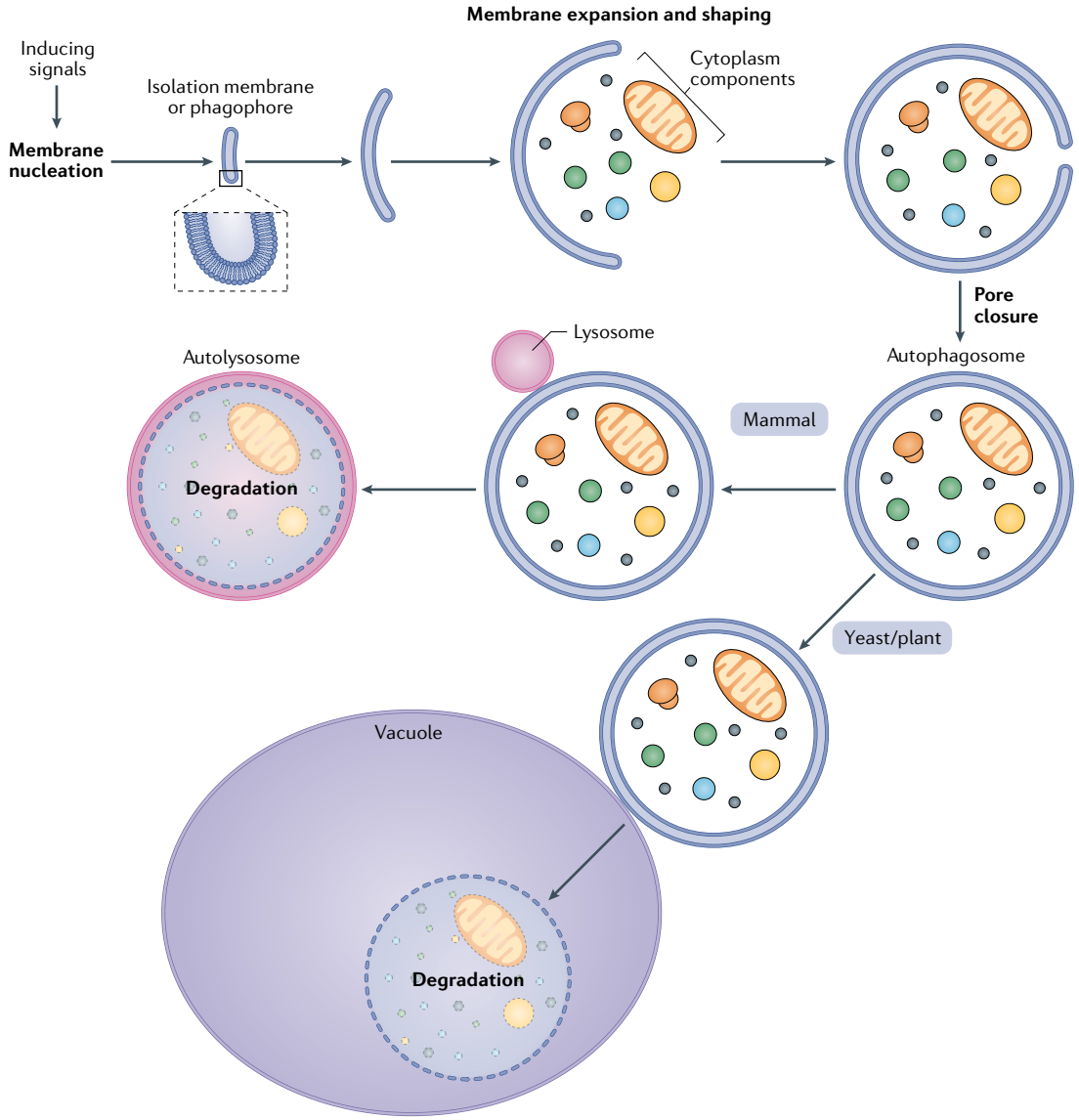

Fig. 1 | **Overview of the process of autophagic degradation.** In both yeasts and mammals, autophagosome biogenesis begins with the process of 'membrane nucleation' to generate the autophagosome precursor. Subsequently, the precursor is transformed into the isolation membrane or phagophore, which expands and bends into a spherical shape. Finally, the pore in the isolation membrane closes to complete the formation of the autophagosome. During this process, various intracellular materials are sequestered into the autophagosome. To complete autophagy, the outer autophagosomal membrane fuses with the lysosome in mammalian cells or with the vacuole in yeast and plant cells to allow disintegration of the inner autophagosomal membrane and degradation of the sequestered materials.

is completed. The autophagosome is then transported to the lysosome/vacuole, followed by membrane fusion between the outer autophagosomal membrane and the lysosomal/vacuolar membrane, establishing the autolysosome (BOX 1). The inner autophagosomal membrane and sequestered materials are then degraded in these lytic organelles containing many hydrolases, such as lipases, proteases, nucleases and glycosylases. Degradation products are transported back to the cytoplasm and recycled for different cellular purposes[14–17].

There are two main types of autophagy, which differ in the mode of sequestration of degradation targets. In non-selective autophagy, cytoplasmic components that happen to be at sites for autophagosome formation are randomly sequestered into autophagosomes. By contrast, in selective autophagy, certain molecules, structures and organelles are recognized by specific proteins called 'autophagy receptors' and are actively engulfed by autophagosomes[18–20]. Cells use these different types of autophagy according to the situation to maintain their homeostasis or regulate their functions[11,12].

The unique and dynamic process of autophagosome biogenesis has attracted many researchers in different fields for decades. Most ATG proteins identified by early studies were found to be involved in this process, and the molecular roles, structures and intracellular dynamics of these proteins have been studied intensely to elucidate the mechanisms that govern autophagosome

Target of rapamycin complex 1
(TORC1). A serine/threonine kinase complex that controls various cellular activities, including autophagy, according to nutrient availability.

Recycling endosomes
Organelles that receive proteins from early endosomes and mediate their recycling to the plasma membrane.

biogenesis. In addition, researchers have pursued cellular sources that supply membranes/lipids to nucleate and expand the autophagosomal membrane. Moreover, the mechanism of membrane shaping, which is important to bend the isolation membrane into a spherical shape and determine the size of the autophagosome, has also been investigated. This Review summarizes our current knowledge of the mechanisms of autophagosome biogenesis and discusses remaining issues in this key area of molecular cell biology. An enormous number of studies have reported mechanisms related to autophagy, including regulations upstream and downstream of autophagosome biogenesis and the relationships between autophagy and other endomembrane systems. This Review places special emphasis on core mechanisms that drive the biogenesis of the autophagosomal membrane irrespective of the autophagy type involved and will not address the specifics of selective autophagy, which can be found elsewhere[21,22].

Basic information about ATG proteins

To date, about 20 ATG proteins involved in autophagosome biogenesis have been reported (FIG. 2; TABLE 1). These 'core' ATG proteins constitute six functional groups: (1) the Atg1/ULK protein kinase complex, (2) Atg9/ATG9-containing vesicles, (3) the autophagy-specific phosphatidylinositol 3-kinase (PI3K) complex, (4) the Atg2–Atg18/ATG2–WD repeat protein interacting with phosphoinositides (WIPI) complex, (5) the Atg12–Atg5–Atg16/ATG12–ATG5–ATG16L1 complex and (6) the Atg8-family protein lipidation system. This section briefly outlines the basic information on these proteins. Of note, the nomenclature for these proteins often differs between yeasts and metazoans. When general mechanisms are being referred to, yeast and mammalian counterparts are given separated by a solidus. For species-specific references, relevant nomenclature is given.

Atg1/ULK protein kinase complex. The Atg1/ULK complex is an initiator kinase complex for autophagy that serves as a scaffold to recruit downstream factors and regulate their functions via phosphorylation of serine or threonine residues. In the budding yeast *Saccharomyces cerevisiae*, the Atg1 complex is composed of five subunits (Atg1, Atg13, Atg17, Atg29 and Atg31), and its formation is facilitated on inactivation of target of rapamycin complex 1 (TORC1)[23–28]. Under nutrient-replete conditions, active TORC1 phosphorylates Atg13 to block its interaction with Atg1 and the Atg17–Atg31–Atg29 complex[29–32] (FIG. 2a). When TORC1 activity is attenuated — which prominently occurs in response to a shortage of nutrients or the specific inhibitor rapamycin, but also downstream of other signals such as growth factors — protein phosphatases 2A and 2C dephosphorylate Atg13 to allow its interaction with Atg1 and Atg17, resulting in the formation of the Atg1 complex and activation of Atg1 kinase via its autophosphorylation[33–35]. The mammalian ULK complex is composed of ATG13, FIP200, ATG101 and ULK1 or ULK2. Unlike the Atg1 complex of *S. cerevisiae*, the ULK complex is constitutively formed, but instead, mammalian TORC1 (mTORC1) modulates ULK1 kinase activity via the phosphorylation of ULK1 and ATG13 on association with the complex in its active form[36–41] (FIG. 2a). To initiate autophagosome biogenesis, multiple copies of the Atg1/ULK complex are assembled, and this assemblage functions as a platform for the recruitment of other ATG proteins, as described later, and also facilitates intermolecular autophosphorylation of Atg1/ULK, resulting in activation of kinase activity[42–44]. The Atg1/ULK complex directly phosphorylates a number of proteins, including Atg4 and Atg9 in yeast[45–47] and ATG4B, ATG9, ATG14L, subunit of the PI3K complex, beclin 1 (BECN1), activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) and the component of coat protein complex II (COPII) SEC23B in mammals[48–55], to drive autophagosome biogenesis (see further details later). In addition to protein phosphorylation, mammalian ULK complex activity is regulated by other post-translational modifications: acetylation and ubiquitylation of ULK1 (REFS[56–60]).

Atg9/ATG9-containing vesicles. Atg9/ATG9 is a multimembrane-spanning protein. Yeast Atg9 cycles between the Golgi apparatus and endosomes, but most of Atg9 localizes to cytoplasmic mobile vesicles of ~50 nm named 'Atg9 vesicles'[61] or to tubulovesicular clusters named 'Atg9 reservoirs'[62] in a manner dependent on Atg23 and Atg27 (REFS[61–69]) (FIG. 2b). In mammalian cells, ATG9 is trafficked through the endomembrane system, including the *trans*-Golgi network and recycling endosomes, and also localizes to cytoplasmic vesicles or tubules[70–72], which are likely to correspond to Atg9 vesicles or reservoirs in yeast (FIG. 2b).

Box 1 | After autophagosome biogenesis

Following its biogenesis, the autophagosome fuses with the lysosome or vacuole to achieve degradation of the contents. In yeast, the autophagosomes are formed at the endoplasmic reticulum (ER)–vacuole contact site and fuse with the vacuolar membrane immediately after their formation (the free autophagosomes are therefore rarely found in the yeast cytoplasm). In mammals, the autophagosomes are formed on the ER, which widely spreads throughout the cytoplasm, and are later transported to lysosomes along the microtubule by the dynein–dynactin motor complex, which is linked to the autophagosome via Rab-interacting lysosomal protein (RILP), an effector of RAB7 (REFS[273–276]). Fusion of the autophagosome with the lysosome/vacuole is mediated by the SNARE complex (STX17–SNAP29–VAMP8 and YKT6–SNAP29–STX7 in mammals and Ykt6–Vam3–Vti1–Vam7 in yeast) in association with RAB7/Ypt7, its guanine nucleotide exchange factor (the HOPS complex)[269–271,277–282], and other factors such as PLEKHM1 (REFS[283,284]), EPG5 (REF.[285]), ATG14L[286], TECPR1 (REF.[287]) and Atg17 (REF.[288]). In mammals, lysosomes fused with the autophagosome are called 'autolysosomes', in which degradation of autophagosome contents proceeds. During prolonged starvation, mammalian target of rapamycin complex 1 (mTORC1) is reactivated by nutrient supply via autophagic degradation of cellular components, leading to termination of autophagy[289,290]. Reactivated mTORC1 also promotes re-formation of lysosomes, in which tubules are extruded from autolysosomes along microtubules depending on clathrin and the kinesin KIF1B to form vesicles of 'protolysosomes'[291,292]. It is noteworthy that a mutation in *BECN1* (encoding the phosphatidylinositol 3-kinase (PI3K) complex I subunit beclin 1 (BECN1)), which impairs interaction of BECN1 with the negative regulator B cell lymphoma 2 (BCL-2) and thereby stimulates autophagy, extended the lifespan and healthspan in mice[293]. Similarly, knockdown or knockout of the gene that encodes Rubicon, which acts to impede autophagosome–lysosome fusion by inhibiting the function of PI3K complex II (REFS[84,85]), promoted longevity in worms and flies and also suppressed age-related phenotypes in mice[294]. This suggests that the maintenance of efficient autophagic degradation capacity confers longevity.

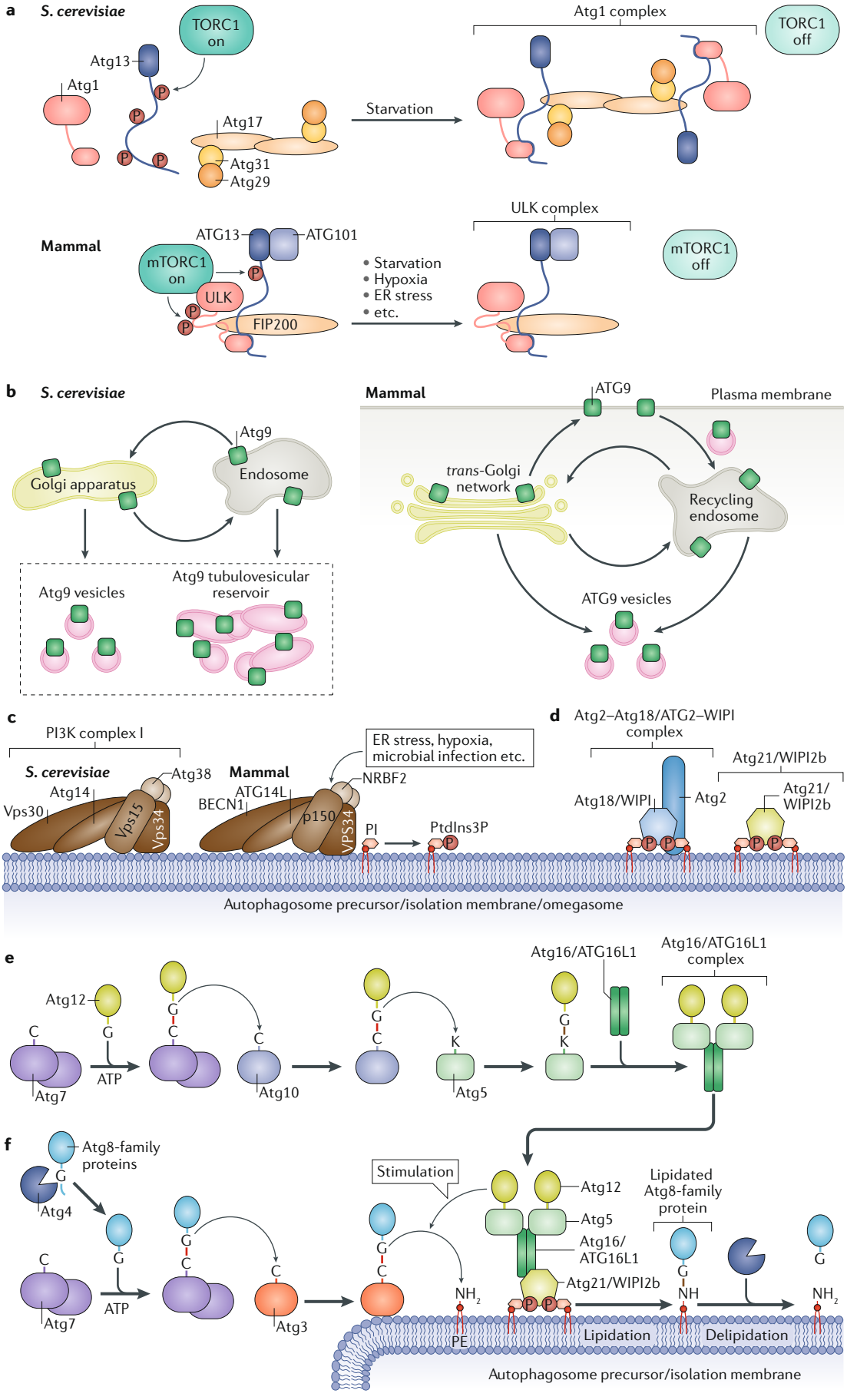
a
S. cerevisiae
TORC1 on
Atg13
Atg1
P
Atg17
Atg31
Atg29
Starvation
Atg1 complex
TORC1 off
Mammal
ATG13
ATG101
mTORC1 on
ULK
FIP200
• Starvation
• Hypoxia
• ER stress
• etc.
ULK complex
mTORC1 off
b
S. cerevisiae
Atg9
Golgi apparatus
Endosome
Atg9 vesicles
Atg9 tubulovesicular reservoir
Mammal
ATG9
Plasma membrane
trans-Golgi network
Recycling endosome
ATG9 vesicles
c
PI3K complex I
S. cerevisiae
Atg38
Atg14
Vps30
Vps15
Vps34
Mammal
ATG14L
BECN1
p150
VPS34
NRBF2
ER stress, hypoxia, microbial infection etc.
PI
PtdIns3P
Autophagosome precursor/isolation membrane/omegasome
d
Atg2–Atg18/ATG2–WIPI complex
Atg21/WIPI2b
Atg18/WIPI
Atg2
Atg21/ WIPI2b
e
Atg12
C
G
Atg7
ATP
Atg10
K
Atg5
Atg16/ATG16L1
Atg16/ATG16L1 complex
f
Atg8-family proteins
Atg4
Atg7
ATP
Atg3
Stimulation
Atg12
Atg5
Atg16/ ATG16L1
Atg21/WIPI2b
Lipidated Atg8-family protein
NH2
PE
Lipidation
Delipidation
Autophagosome precursor/isolation membrane

Fig. 2 | **Core ATG proteins and their complexes. a** | Atg1 complex (in yeast) and ULK complex (in mammals) are the initiator complexes for autophagy that on activation phosphorylate various targets. In the budding yeast *Saccharomyces cerevisiae*, the Atg1 complex is composed of Atg1, Atg13 and the Atg17–Atg31–Atg29 complex. Atg13 is subject to phosphorylation (P) by target of rapamycin complex 1 (TORC1), which blocks its interaction with Atg1 and the Atg17–Atg31–Atg29 complex. Starvation inhibits TORC1, thereby allowing the components to interact to induce autophagy. The mammalian ULK complex comprises ATG13, FIP200, ATG101 and ULK1 or ULK2. ULK1 and ATG13 are targets of mammalian TORC1 (mTORC1), which by phosphorylating these components inhibits ULK1 activity. Various stresses inhibit mTORC1, allowing ULK1 activation. **b** | Intracellular trafficking of Atg9/ATG9. In *S. cerevisiae*, the multimembrane-spanning protein Atg9 shuttles between the Golgi apparatus and the endosome, but most of this protein resides in small vesicles (Atg9 vesicles) or tubulovesicular clusters (Atg9 reservoirs) in the cytoplasm. On autophagy induction, these membrane structures localize to the autophagosome formation site and are likely to contribute to autophagosome precursor formation. In mammals, ATG9 is trafficked through the *trans*-Golgi network, recycling endosome and plasma membrane. ATG9-positive vesicles derived from the *trans*-Golgi network and plasma membrane are both thought to be involved in autophagosome precursor formation. **c** | Phosphatidylinositol 3-kinase (PI3K) complex I. PI3K complex I is composed of Vps34/VPS34, Vps15/p150, Vps30 (also known as Atg6)/beclin 1 (BECN1), Atg14/ATG14L and Atg38/NRBF2. This complex phosphorylates phosphatidylinositol (PI) to produce phosphatidylinositol 3-phosphate (PtdIns3P) in autophagosome biogenesis-related membranes, such as the autophagosome precursor, isolation membrane and omegasome, to recruit multiple PtdIns3P-binding proteins that regulate different steps in autophagosome formation. **d** | Atg2–Atg18/ATG2–WD repeat protein interacting with phosphoinositides (WIPI) complex and Atg21/WIPI2b. Atg2/ATG2 in association with the PtdIns3P-binding protein Atg18/WIPI localizes to autophagosome precursors/isolation membranes depending on PtdIns3P produced by PI3K complex I, and exerts its membrane tethering and lipid transfer activities in endoplasmic reticulum (ER)-associated isolation membrane expansion. Atg21/WIPI2b interacts with the Atg16/ATG16L complex and targets this complex to autophagosome precursors/isolation membranes. **e** | The ubiquitin-like conjugation reaction of Atg12/ATG12 and the formation of the Atg16/ATG16L1 complex. The carboxyl group of the C-terminal glycine residue (G) in the ubiquitin-like protein Atg12/ATG12 is activated by the E1 enzyme Atg7/ATG7 using ATP and forms a thioester bond (red line) with the catalytic cysteine residue (C) of Atg7/ATG7. Via the E2 enzyme Atg3/ATG3, it finally forms an isopeptide bond (brown line) with a lysine residue (K) in Atg5/ATG5. The resulting conjugate interacts with Atg16/ATG16L1 to form the Atg16/ATG16L1 complex. **f** | The ubiquitin-like reaction that conjugates Atg8-family proteins to phosphatidylethanolamine (PE). Ubiquitin-like Atg8-family proteins (microtubule-associated protein light chain 3 (LC3) isoforms and GABA receptor-associated proteins (GABARAPs) in mammals) are first cleaved by the cysteine protease Atg4/ATG4 in their C termini to expose a glycine residue essential for the conjugation reaction. By a conjugation reaction similar to that for Atg12/ATG12 conjugation to Atg5/ATG5, the E1 enzyme Atg7/ATG7 and the E2 enzyme Atg3/ATG3 conjugate Atg8-family proteins to the amino group (NH_2) of PE in autophagosome precursors/isolation membranes in collaboration with the Atg16/ATG16L complex, which acts as an E3 enzyme to stimulate Atg8-family protein transfer from Atg3/ATG3 to PE (lipidation). Atg4/ATG4 also catalyses delipidation of Atg8-family proteins. In parts **e**,**f** when homologous proteins share the same name, yeast nomenclature is used for simplicity.

Ubiquitin-like protein
Small protein similar to ubiquitin that is conjugated to the amino group of target molecules at their C termini.

E1 enzyme
Enzyme that activates the C-terminal carboxyl group of ubiquitin or ubiquitin-like proteins using ATP.

E2 enzyme
Enzyme that receives ubiquitin/ubiquitin-like proteins from E1 enzymes and conjugates them to target molecules directly or via E3 enzymes.

This ATG9 trafficking is regulated by Rab GTPases, such as RAB11 and RAB1, their regulators and other proteins[70,73–78]. In addition, a small population of mammalian ATG9 resides in the plasma membrane[79]. In both yeast and mammals, Atg9/ATG9-containing membranes have been suggested to contribute to the generation of autophagosome precursors (see later).

Autophagy-specific PI3K complex. A complex of the class III PI3K Vps34/VPS34 involved in autophagosome biogenesis contains Vps15/p150, Vps30 (also known as Atg6)/BECN1 and the autophagy-specific subunits Atg14/ATG14L and Atg38/NRBF2 and hereafter will be referred to as PI3K complex I (another class III PI3K Vps34/VPS34 complex containing Vps38/UVRAG instead of Atg14/ATG14L and Atg38/NRBF2 is called 'PI3K complex II' and regulates endosomal function)[80–85]. During autophagy, PI3K complex I produces phosphatidylinositol 3-phosphate (PtdIns3P) in membranes related to autophagosome biogenesis[86–91] (FIG. 2c) to regulate a number of processes by recruiting different PtdIns3P-binding proteins. In mammals, several proteins, such as B cell lymphoma 2 (BCL-2), Bax-interacting factor 1 (BIF-1) and AMBRA1, have been reported to regulate this complex to control autophagy[92–95].

Atg2–Atg18/ATG2–WIPI complex. Among core ATG proteins, Atg18 and Atg21 are PtdIns3P-binding proteins in yeast and four WIPIs (WIPI1–WIPI4) are PtdIns3P-binding proteins in mammals, which all belong to PROPPIN (β-propellers that bind polyphosphoinositides) family proteins[96–100] (FIG. 2d). Whereas yeast Atg18 forms a complex with Atg2 (REF.[101]), mammalian WIPIs all interact with ATG2, for which WIPI4 has the highest affinity[102,103]. These complexes localize to the site of autophagosome formation depending on the PtdIns3P-binding ability of Atg18/WIPIs[104–107]. Recent studies have revealed that the Atg2–Atg18/ATG2–WIPI complex acts as a membrane tether and also has lipid transfer activity, providing insights into the mechanism of membrane expansion during autophagosome biogenesis, as described later.

Atg12–Atg5–Atg16/ATG12–ATG5–ATG16L1 complex. The ubiquitin-like protein Atg12/ATG12 forms an isopeptide bond with a lysine residue in Atg5/ATG5 via reactions catalysed by the E1 enzyme Atg7/ATG7 and the E2 enzyme Atg10/ATG10 (REFS[108,109]) (FIG. 2e). The resulting conjugate interacts with Atg16/ATG16L1 to form the Atg12–Atg5–Atg16/ATG12–ATG5–ATG16L1 complex (hereafter the Atg16/ATG16L1 complex)[110–112]. Since Atg16/ATG16L1 forms a dimer, the Atg16/ATG16L1 complex has a 2:2:2 stoichiometry of the subunits[113,114]. This complex interacts with Atg21/WIPI2b (a WIPI2 isoform) via Atg16/ATG16L1 and localizes to the autophagosome precursor/isolation membrane depending on PtdIns3P produced by PI3K complex I (REFS[115,116]). At the forming autophagosome the Atg16/ATG16L1 complex exerts E3 enzyme activity that promotes the lipid conjugation reaction of Atg8-family proteins[117–120].

Atg8-family protein lipidation system. Atg8-family proteins (Atg8 in yeast and microtubule-associated protein light chain 3 (LC3) isoforms and GABA receptor-associated proteins (GABARAPs) in mammals) are unique ubiquitin-like proteins, the C termini of which are conjugated to not a lysine residue in proteins but the amino group of the lipid phosphatidylethanolamine, resulting in their anchoring to membranes (FIG. 2f; TABLE 1). For this conjugation, Atg4/ATG4, which is structurally similar to ubiquitin-specific processing proteases, first cleaves Atg8-family proteins in the C-terminal tail to expose their glycine residues at the C termini[121–123]. Then, Atg7/ATG7, Atg3/ATG3 and the Atg16/ATG16L1 complex act as E1, E2 and E3 enzymes, respectively, to conjugate these proteins to phosphatidylethanolamine in the autophagosome precursor/isolation

Table 1 | **Atg proteins involved in autophagosome biogenesis**

Yeast	Mammal	Function in autophagosome biogenesis
Atg1 complex	***ULK complex***	***Recruiting and regulating autophagosome formation-related proteins***
Atg1	ULK1 and ULK2	Ser/Thr kinase
Atg13	ATG13	Complex formation and bridging complexes
Atg17	FIP200	Scaffold protein
Atg11		
Atg29	ATG101	Complex stabilization
Atg31		
Atg9/ATG9-containing vesicles		***Membrane source to generate autophagosome precursors and to drive isolation membrane expansion***
Atg9	ATG9A	Targeting the vesicles to autophagosome formation sites
PI3K complex I		***Producing PtdIns3P in autophagosome biogenesis-related membranes***
Vps34	VPS34	PI3K
Vps30 (also known as Atg6)	BECN1	Complex stabilization and a hub for Vps34/VPS34 regulation
Vps15	p150	Ser/Thr kinase required for Vps34/VPS34 activity
Atg14	ATG14L	Targeting the complex to autophagosome biogenesis-related membranes
Atg38	NRBF2	Complex stabilization
Atg2–Atg18 complex	***ATG2–WIPI complex***	***Tethering the autophagosome precursor/isolation membrane to the ER and transferring lipids***
Atg2	ATG2A and ATG2B	Membrane tethering and lipid transfer
Atg18	WIPI1, WIPI2, WIPI3 and WIPI4	Targeting the complex to the autophagosome precursor/isolation membrane by binding to PtdIns3P
Atg16 complex	***ATG16L1 complex***	***Stimulating Atg8-family protein lipidation***
Atg12	ATG12	Ubiquitin-like protein
Atg7	ATG7	E1 for Atg12/ATG12 and Atg8-family proteins
Atg10	ATG10	E2 for Atg12/ATG12
Atg5	ATG5	Conjugation target of Atg12/ATG12
Atg16	ATG16L1	Bridging Atg5/ATG5 and Atg21/WIPI2b
Atg8-family protein lipidation system		***Exerting multiple roles for autophagy***
Atg8	LC3A, LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2	Ubiquitin-like protein conjugated to phosphatidylethanolamine
Atg4	ATG4A, ATG4B, ATG4C and ATG4D	C-terminal processing/delipidation of Atg8-family proteins
Atg7	ATG7	E1 for Atg12/ATG12 and Atg8-family proteins
Atg3	ATG3	E2 for Atg8-family proteins

Atg/ATG, autophagy-related (yeast/mammal); BECN1, beclin 1; ER, endoplasmic reticulum; FIP200, focal adhesion kinase family interacting protein of 200 kDa; GABARAP, GABA receptor-associated protein; LC3, microtubule-associated protein light chain 3; NRBF2, nuclear receptor-binding factor 2; p150, protein of 150 kDa; PI3K, phosphatidylinositol 3-kinase; PtdIns3P, phosphatidylinositol 3-phosphate; ULK, unc-51-like kinase; Vps/VPS, vacuolar protein sorting; WIPI, WD repeat protein interacting with phosphoinositides.

membrane[121,122,124,125]. Atg8-family proteins anchored to the membranes are likely to drive membrane expansion in different ways, as described later. In addition, these proteins also play pivotal roles in transport and fusion of complete autophagosomes to lysosomes as well as in cargo capture by binding autophagy receptors in selective autophagy[126–128]. Atg4/ATG4 also catalyses delipidation of Atg8-family proteins for the reuse of the Atg8-family proteins for the next round of autophagosome formation as well as efficient autophagosome formation through an unknown mechanism[123,129–134].

Initiation of autophagosome biogenesis

Autophagosome biogenesis is induced by a wide variety of signals from the inside and the outside of cells, including the shortage of nutrients (amino acids, glucose, iron, zinc, phosphate and so on), stresses (endoplasmic reticulum (ER) stress, oxidative stress, hypoxia, DNA damage and so on), emergence of aberrant protein aggregates or damaged organelles and microbial infection[135–139]. These signals are transmitted via different signalling pathways, but many of them converge at TORC1, which is a hub for nutrient sensing and accordingly regulates cell growth and functions[140]. As already mentioned, because TORC1 phosphorylates Atg1/ULK complex subunits to block downstream events for autophagosome biogenesis, signals that attenuate TORC1 activity will generally induce autophagy (FIG. 2a). In addition, as shown in yeast, TORC1-mediated phosphorylation of other Atg proteins may also contribute to autophagy regulation[47]. In yeast, cAMP-dependent protein kinase A also negatively regulates autophagosome biogenesis by phosphorylating Atg1 and Atg13 (REFS[141,142]). In mammals, AMP-activated protein kinase (AMPK) plays a major role in autophagy induction in response to low glucose levels through not only mTORC1 inactivation but also direct phosphorylation of ULK1 (REFS[143,144]). The yeast AMPK homologue, Snf1, was also reported to positively regulate autophagy[145]. Moreover, a number of signals, such as ER stress, hypoxia and microbial infection, stimulate PI3K complex I to upregulate autophagosome formation probably via PtdIns3P production[135,137]. These are only a fraction of examples, and the initiation of autophagosome biogenesis seems to be regulated in a more complicated manner involving crosstalk between upstream signalling pathways[135–139].

As mentioned already, generally TORC1 inactivation triggers Atg1/ULK assemblage formation and kinase activation, which is the key mechanism in the initiation of non-selective autophagy. By contrast, in selective autophagy, degradation targets themselves appear to emit signals that stimulate autophagosome biogenesis. Specifically, autophagy receptors, which recognize selective autophagy cargo, mediate the assembly of multiple Atg1/ULK complexes on degradation targets (independently of TORC1 inhibition) via the interaction with Atg11/FIP200; Atg11 associates with the Atg1 complex and is especially important for selective autophagy initiation[146–151]. In any case, the assembly of multiple Atg1/ULK complexes is the common first step for the initiation of autophagosome biogenesis.

E3 enzyme
Enzyme that determines target specificity and stimulates or mediates the conjugation reaction by E2 enzymes.

cAMP-dependent protein kinase A
A protein kinase that is activated on increase in cAMP levels and regulates various metabolic enzymes.

AMP-activated protein kinase
(AMPK). A protein kinase that is activated in response to low ATP levels and acts to maintain cellular energy homeostasis.

Contact sites
Sites where two different organelles contact with each other for interorganellar communication, including transfer of molecules such as lipids.

Intrinsically disordered region
Protein region that does not adopt a defined 3D structure even under physiological conditions.

Exocyst complex
A multimeric protein complex that tethers secretory vesicles to the plasma membrane in exocytosis.

Membrane nucleation

On autophagy induction, otherwise cytoplasmic ATG proteins are recruited to sites for autophagosome biogenesis to generate an 'autophagosome precursor', which is called the 'pre-autophagosomal structure' (PAS) in yeast[8,152] (FIG. 3). In yeast, autophagosome formation sites are localized to the vacuole at places where it is in close contact with the ER (vacuole–ER contact sites)[152–154], with Vac8 tethering the Atg1 complex assemblage to the vacuole[155]. In mammals, autophagosomes formation initiates at ER subdomains, mostly those associated with mitochondria (ER–mitochondrion contact sites)[88,106,156]. Generating the autophagosome precursor at these initiation sites requires the interplay of multiple core ATG proteins and membrane sources, which engage in elaborate protein–protein and protein–lipid interactions. This section describes these intricate mechanisms.

Scaffold assembly by the Atg1/ULK1 complex. Autophagosome precursor formation starts with the assemblage of multiple copies of the Atg1/ULK complex, which forms a scaffold for the recruitment of other ATG proteins[106,157–159]. As described earlier, in *S. cerevisiae*, TORC1 inactivation leads to the formation of the Atg1 complex via Atg13 dephosphorylation. Moreover, Atg13 in an Atg1 complex uses a binding site in its long intrinsically disordered region to capture Atg17 in another Atg1 complex[32]. In this way, dozens of Atg1 complexes are crosslinked with each other via Atg13, resulting in a superassembly[32,160] (FIG. 3a, step 1). A recent study revealed that assembly of Atg1 complexes is driven by the principle of liquid–liquid phase separation[161]. The resulting liquid droplets were shown to be sensitive to phosphorylation, linking their formation to TORC1-mediated regulation of Atg1. A flexible nature of the liquid droplets may be advantageous for immediate assembly and disassembly of the PAS in response to cellular nutrient availability. It is noteworthy that liquid–liquid phase separation also plays important roles in cargo condensation and sequestration during selective autophagy. In worms and yeast, PGL proteins and the aminopeptidase Ape1, which, respectively, serve as autophagic cargoes in these model organisms, are assembled into liquid droplets via phase separation, and their liquidity determines the efficiency of their autophagic sequestration[162,163]. In mammals, the ubiquitin-binding autophagy receptor/adaptor p62 undergoes phase separation in association with autophagic cargoes such as ubiquitylated proteins and the oxidative stress sensor KEAP1, and this process is regulated by post-translational modifications of p62 and another ubiquitin-binding receptor/adaptor NBR1 (REFS[149,164–168]).

In mammals, multiple ULK complexes appear to assemble on an ER subdomain enriched in the ER membrane protein complex VMP1–TMEM41B and phosphatidylinositol synthase (PIS) in response to autophagy induction[106,159,169–172] (FIG. 3b, step 1). It was reported that ER recruitment of the ULK complexes is mediated by the ER transmembrane proteins VAPA and VAPB via interaction with FIP200. These proteins were also shown to interact with WIPI2, establishing isolation membrane–ER contacts and thereby contributing to membrane expansion (see later)[173]. PIS-mediated phosphatidylinositol production in this region is important for the recruitment of downstream proteins such as WIPI2 (REF.[169]), and the VMP1–TMEM41B complex is likely important at later stages of autophagosome biogenesis to mediate the detachment of the isolation membrane from the ER to release the autophagosome[170–172,174]. It was also reported that the mammalian exocyst complex associated with RalB GTPase directly interacts with mTORC1 and several ATG proteins, serving as a platform to regulate the initiation of autophagosome formation on nutrient starvation[175]. However, how this model is integrated with the ER-associated assembly model discussed above is unknown.

Recruitment of Atg9/ATG9-containing membranes. In the next step, in yeast, Atg9 vesicles are recruited to the Atg1 complex assemblage via the interaction of Atg9 with the HORMA domain of Atg13 and Atg17 in starvation-induced non-selective autophagy[176,177] (FIG. 3a, step 2) or with Atg11 in selective types of autophagy[178]. Immunoelectron microscopy detected Atg9 in the autophagosomal membrane, suggesting that these vesicles contribute to establishment of the autophagosomal membrane. Notably however, once several Atg9 vesicles have localized to the autophagosome formation site at an early stage of PAS formation, Atg9 vesicles are not additionally recruited during expansion of the isolation membrane[61]. These results are consistent with the idea that in yeast Atg9 vesicles serve as a seed to form the autophagosome precursor rather than as a source for membrane expansion and also raise the possibility that several Atg9 vesicles coalesce with each other to form the autophagosome precursor (FIG. 3a, steps 3 and 4). Atg9 reservoirs, that is, Atg9-containing tubulovesicular clusters, were also suggested to become autophagosome precursors[62]. Thus, Atg9 vesicles and Atg9 reservoirs are different in their morphological characteristics but functionally are likely to be the same structures. The difference in morphology might be explained by differences in experimental conditions, including expression levels of Atg9 and methods for morphological analysis[61,62].

In mammalian cells, *trans*-Golgi network-derived ATG9 vesicles may also be involved in autophagosome precursor formation in a similar manner. Mammalian ATG9 can localize to the autophagosome formation site early on, in a manner independent of the ULK complex[179,180] (FIG. 3b, step 1). A recent biochemical study revealed that the ULK complex first associates with the ER and then becomes associated with ATG9 vesicles in an ATG14 (PI3K complex I)-dependent manner[169] (FIG. 3b, step 2). The same study also showed that at least part of the ATG9 pool is incorporated into the isolation membrane. A series of studies proposed a different model for ATG9 engagement in autophagosome biogenesis, involving ATG9-positive vesicles derived from the plasma membrane, rather than the *trans*-Golgi network. It was shown that ATG9 and ATG16L1 reside at the plasma membrane and are internalized into vesicles via clathrin-mediated endocytosis. Both vesicles are trafficked intracellularly via different routes, with ATG9 vesicles showing pronounced residence in

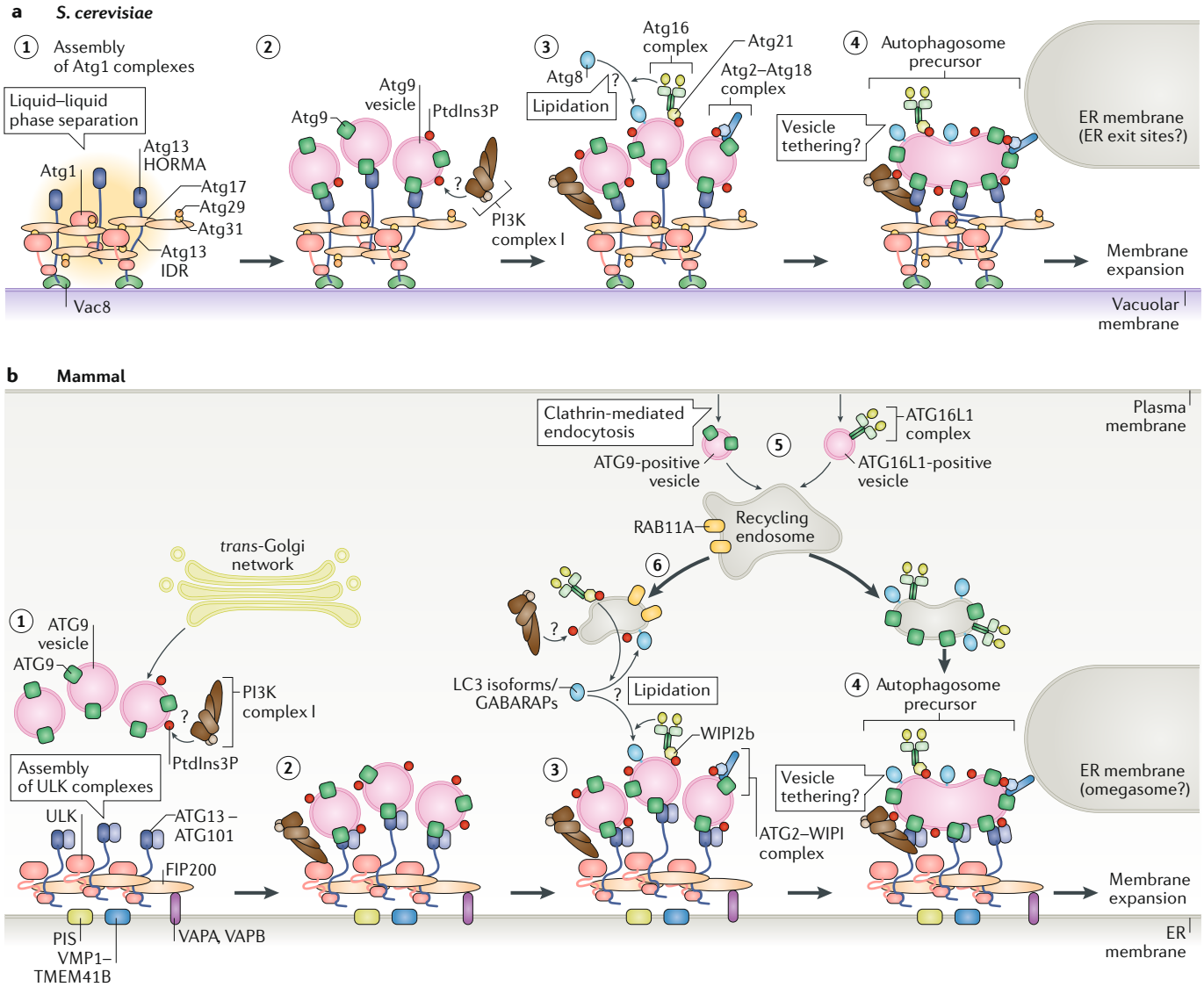

Fig. 3 | **A model for the process of membrane nucleation in autophagosome biogenesis. a** | In *Saccharomyces cerevisiae*, autophagosomes are formed at the vacuole, close to the endoplasmic reticulum (ER). Dozens of Atg1 complexes (see FIG. 2a) are assembled to form a scaffold for autophagosome biogenesis. Vac8 tethers the assemblage to the vacuolar membrane. These assemblages possess liquid-like properties driven by liquid–liquid phase separation of Atg13, owing to the presence of intrinsically disordered regions (IDRs) (step 1). Atg9 vesicles (see FIG. 2b) are recruited via the interaction of Atg9 with the HORMA domain of Atg13 (and with Atg17, not shown), and phosphatidylinositol 3-kinase (PI3K) complex I starts producing phosphatidylinositol 3-phosphate (PtdIns3P) probably in the Atg9 vesicle membrane (indicated by a question mark) (step 2). PtdIns3P recruits the Atg16 complex and Atg2 via the PtdIns3P-binding proteins Atg21 and Atg18, respectively. The Atg16 complex promotes Atg8 lipidation (attachment to phosphatidylethanolamine) in the growing membrane (step 3). Atg9 vesicles fuse with each other — which could be supported by lipidated Atg8 (question mark) — to generate the autophagosome precursor. The Atg2–Atg18 complex tethers the precursor membrane to the ER (probably (question mark) at ER exit sites), leading to membrane expansion (step 4). **b** | In mammals, autophagosomes form at the ER, prominently at ER contact sites with mitochondria (not shown). Sites for autophagosome biogenesis are marked with phosphatidylinositol synthase (PIS) and VMP1–TMEM41B complex and harbour the membrane-tethering proteins VAPA and VAPB. Multiple ULK complexes (see FIG. 2a) are assembled at this ER domain supported by the interaction of FIP200 with VAPA/VAPB. ATG9-containing vesicles can localize to the autophagosome formation site early on, in a manner independent of the ULK complex (step 1). Furthermore, the ULK complex becomes associated with ATG9-containing vesicles in a PI3K complex I-dependent manner (step 2). As with *S. cerevisiae*, ATG9 vesicles are a candidate site for PtdIns3P generation (step 1). The ATG16L1 complex and the ATG2–WIPI complex are recruited via WD repeat protein interacting with phosphoinositides (WIPIs) binding to PtdIns3P, and lipidation of Atg8-family proteins (microtubule-associated protein light chain 3 (LC3) isoforms and GABA receptor-associated proteins (GABARAPs)) proceeds (step 3). Fusion of ATG9-containing vesicles occurs to generate the autophagosome precursor, which can be tethered via ATG2–WIPI to the ER (potentially (question mark) to specific ER subdomains known as omegasomes that could support lipid transfer from the ER to the autophagosome) (step 4) (see also FIG. 4c). The main source of ATG9 vesicles was ascribed to the *trans*-Golgi network (step 1). However, ATG9-positive vesicles as well as ATG16L1-positive vesicles can also be generated from the plasma membrane via clathrin-mediated endocytosis. These vesicles coalesce at the recycling endosome to serve as membrane sources for the autophagosome precursor (step 5) (see also FIG. 2b). A membrane compartment containing the late endosomal protein RAB11A can also serve as the site for PI3K complex I recruitment, PtdIns3P generation and WIPI2 recruitment (step 6).

Early endosomes
Organelles that serve as a sorting platform for proteins endocytosed from the plasma membrane or transported from the Golgi apparatus.

SNARE
A family of proteins that mediate most membrane fusion events within cells. For fusion, SNAREs on two opposed membranes tightly associate with each other, bring the membranes in close proximity and induce their fusion.

Sorting nexin
A group of proteins that contain the PX and BAR domains and function in membrane trafficking.

early endosomes and ATG16L1 vesicles showing minimal overlap with this endosomal compartment, but expanding their size via homotypic fusion to give rise to tubulovesicular structures. Nevertheless, both ATG9 vesicles and ATG16L1 structures eventually coalesce at the recycling endosomes, where they undergo VAMP3 SNARE-mediated heterotypic fusion, which could be important for the exit of these heterotypic structures from the endosome to contribute to the establishment of the autophagosome precursor[79,181,182] (FIG. 3b, step 5). In line with this model, it was reported that the sorting nexin SNX18 remodels recycling endosomes to supply ATG16L1-positive membranes to autophagosome precursors[183]. Recently, it was also reported that mammalian ATG9-containing vesicles deliver phosphatidylinositol 4-kinase IIIβ to the autophagosome formation site, which promotes the recruitment of ATG proteins to the site via an unknown mechanism[184].

These observations in yeast and mammals are consistent in that Atg9/ATG9-containing membranes contribute to autophagosome precursor formation. However, the stable localization of ATG9 to the isolation membrane and autophagosome is not microscopically observed in mammals[71], leaving the possibility that ATG9-containing membranes participate in autophagosome biogenesis in a manner different from that in yeast. Furthermore, the plasma membrane localization of Atg9 and Atg16 has not been reported in yeast. Future studies should scrutinize these apparent contradictions and clarify whether the mechanism of autophagosomal membrane nucleation indeed differs between yeast and mammals. Finally, it is also possible that other membrane sources, such as ER-derived COPII vesicles (see the next section), contribute to autophagosome precursor formation via heterotopic fusion. Overall, further dissection of the membrane sources for autophagosome precursor establishment (and growth), their potential interplay and their context-dependent contributions is an interesting avenue for future studies.

Membrane modifications for autophagosome precursor formation. Studies in yeast and mammals consistently suggest that following recruitment of Atg9/ATG9 vesicles, PI3K complex I is targeted to the autophagosome formation site[106,185], although the targeting mechanism is still unclear. PtdIns3P is detected in the isolation membrane and autophagosomal membrane[86,87]. In addition, a specific ER domain called the 'omegasome', which is closely associated with isolation membrane expansion, is also enriched in PtdIns3P (see the next section). These observations suggest that PI3K complex I produces PtdIns3P in these membranes. In addition, because PI3K complex I and PtdIns3P-binding proteins, including Atg18/WIPIs, are required for the formation of the autophagosome precursor[101,106,185], PI3K complex I should also produce PtdIns3P during this process. Atg9/ATG9-containing membranes are a potent candidate for a site of this reaction (FIG. 3a, step 2 and FIG. 3b, step 1), although further validation is required to confirm this model. In mammals, it was also proposed that PI3K complex I recruitment, PtdIns3P generation and WIPI2 recruitment occur on a membrane compartment containing the late endosomal protein RAB11A[186]. This is consistent with the findings described earlier that plasma membrane-derived ATG16L1 and ATG9 vesicles that are trafficked via endosome-related organelles serve as sources of membranes for autophagosome precursors.

Subsequent to PtdIns3P generation, the Atg16/ATG16L1 complex bound to Atg21/WIPI2b is recruited depending on PtdIns3P binding of Atg21/WIPI2b[115,116] (FIG. 3a, step 3 and FIG. 3b, step 3). The Atg16/Atg16L1 complex then interacts with the E2 enzyme Atg3/ATG3 to stimulate lipidation of Atg8-family proteins[117,118,187]. This lipidation reaction probably occurs on Atg9/ATG9-containing vesicles, but in mammals the role of the RAB11A-positive compartment in LC3 lipidation has also been demonstrated (FIG. 3a, step 3 and FIG. 3b, steps 3 and 6). The lipidated forms of Atg8-family proteins are likely to exert different functions during autophagosome formation, including membrane tethering and fusion (see later sections). How these functions of Atg8-family proteins drive autophagosome biogenesis remains to be addressed, but they may also be involved in the process of autophagosome precursor formation. An intriguing possibility is that Atg8-family proteins tether Atg9/ATG9-containing vesicles together to facilitate their homotypic fusion (FIG. 3a, steps 3 and 4 and FIG. 3b, steps 3 and 4). The Atg16/ATG16L1 complex is also recruited in a PtdIns3P-independent manner in both yeast and mammals via the Atg1/ULK complex. Whereas ATG16L1 interacts with FIP200 in mammals[188,189], Atg12 interacts with Atg17 in yeast[190]. Thus, the Atg1/ULK complex recruits the Atg16/ATG16L1 complex at an early stage of membrane nucleation before the recruitment by the PtdIns3P-dependent mechanism. This early recruitment of the Atg16/ATG16L complex is indispensable for autophagosome biogenesis in mammals, and it collaborates with the PtdIns3P-dependent mechanism in a partly redundant manner in yeast. The yeast Atg16 complex was also suggested to promote the superassembly of the Atg1 complex via Atg12–Atg17 interaction, a non-E3 function of the Atg16 complex[190].

The Atg2–Atg18/ATG2–WIPI complex also localizes to PtdIns3P-positive pre-autophagosomal membranes (FIG. 3a, step 3 and FIG. 3b, step 3). In addition to Atg18, yeast Atg2 was reported to have affinity for PtdIns3P, which may contribute to its localization to these membranes as well[191,192]. The recruitment of the yeast Atg2–Atg18 complex also involves interaction of Atg2 with Atg9, which is positively regulated by Atg9 phosphorylation by Atg1 (REFS[46,192]). Similarly, mammalian ATG9 interacts with ATG2 (REF.[193]). As described in the next section in detail, recent studies proposed that the Atg2–Atg18/ATG2–WIPI complex tethers the autophagosome precursor to the ER, triggering ER-associated membrane expansion (FIG. 3a, step 4 and FIG. 3b, step 4).

Membrane expansion

In this section, our current knowledge of what supplies membranes/lipids for expansion of the isolation membrane and how these processes are achieved are reviewed with a particular focus on the mechanisms associated with the ER.

ATG protein distributions and functions in membrane expansion. In yeast, comprehensive fluorescence microscopy analyses revealed that core Atg proteins exhibit different distribution profiles in the growing isolation membrane[153,154]. Whereas the Atg1 complex and PI3K complex I are retained at the vacuole–isolation membrane contact site, Atg8, the Atg16 complex and Atg1 (not as the Atg1 complex) are relatively evenly distributed in the isolation membrane, and Atg9 and the Atg2–Atg18 complex are localized to contacts between the isolation membrane edge and the ER[153,154,194] (FIG. 4a). This Atg2 localization depends on its interaction with Atg9 (REF.[192]). In mammals, TRAPPC11 was proposed to be involved in the recruitment of the ATG2–WIPI complex to the isolation membrane[195]. Atg8-family proteins appear to spread throughout the isolation membrane in mammalian cells as well as yeast cells. In addition, immunoelectron microscopy revealed the precise localization of the ATG16L1 complex; it predominantly localizes to the convex surface of the isolation membrane[121,196], suggesting that lipidation of Atg8-family proteins more actively occurs on this side of the isolation membrane and that the lipidated proteins diffuse to the other side. In vitro studies revealed that Atg8-family proteins conjugated to phosphatidylethanolamine in different membranes interact with each other, thereby tethering the membranes together[197–199]. Moreover, yeast Atg8–phosphatidylethanolamine mediated hemifusion of liposomes (fusion between the outer leaflets of the membranes with the inner leaflets intact)[197] and mammalian and nematode Atg8-family proteins crosslinked to phosphatidylethanolamine caused complete liposome fusion[198,199]. Atg8-family protein mutants deficient in these in vitro functions are defective in autophagosome biogenesis. In addition, small autophagosomes were formed in yeast Atg8 mutants partially defective in the membrane-tethering and hemifusion functions[197]. Similarly, lowering Atg8 levels results in a reduction in the size of the autophagosome[200]. These results suggest that Atg8-family proteins are involved in the expansion of the isolation membrane, likely via mediating the tethering and fusion of membrane-supplying vesicles to the isolation membrane. However, membrane hemifusion by Atg8 required a high phosphatidylethanolamine concentration, which is unusual in typical endomembranes[201]. Therefore, if Atg8-family proteins indeed mediate vesicle fusion during autophagosome biogenesis, they most likely do so assisted by other fusogenic factors, such as proteins like SNAREs, high membrane curvature and adequate lipid compositions of the membrane structures.

Atg8-family proteins also promote membrane expansion by recruiting other proteins to the isolation membrane (FIG. 4a). After initiating autophagosome formation, Atg1 (individually, not within the Atg1 complex) and the ULK1 complex associate with the isolation membrane by binding to Atg8-family proteins via the Atg8-family protein interacting motif (AIM) or LC3-interacting region, and the association is important for autophagosome formation[202–204]. In yeast and mammals, Atg1/ULK1 phosphorylates Atg4/ATG4 to inhibit its activity for Atg8-family protein delipidation, promoting isolation membrane expansion[45,48]. Because the localization of Atg4/ATG4 to the isolation membrane has not been reported, cytoplasmic Atg4/ATG4 may be locally phosphorylated by Atg1/ULK1 around isolation membranes. A recent study in mammalian cells reported that phosphorylation of the Atg8-family isoforms LC3C and GABARAPL2 by TANK-binding kinase 1 (TBK1) prevents ATG4-mediated delipidation and hence premature removal of these proteins by ATG4, thereby ensuring efficient cargo engulfment by the autophagosome[205]. In this connection, an earlier study reported that reactive oxygen species modify a cysteine residue proximal to the ATG4 catalytic site and thereby attenuate its activity to stimulate autophagosome formation[206]. Other factors binding to lipidated Atg8-family proteins could also prevent their delipidation by ATG4 (REF.[105]). Yeast Atg3 (Atg8 lipidation enzyme) also localizes to the isolation membrane via its AIM to further promote isolation membrane expansion[207,208].

It was reported that the two subfamilies of mammalian Atg8-family proteins, LC3 isoforms and GABARAPs, are involved in different steps of autophagosome biogenesis, membrane expansion and a later step (probably membrane closure), respectively[209]. In support of this, a comprehensive structure–function relationship study on LC3 and GABARAP homologues from *Caenorhabditis elegans*, LGG-2 and LGG-1, respectively, revealed that these proteins adopt closed and open conformations that differ in their binding partners and membrane fusion activity[199]. The distinct roles for LC3 and GABARAP subfamilies in autophagosome formation in mammals may be attributed to these different structural and functional properties. It is also possible that Atg8-family proteins regulate membrane expansion by affecting isolation membrane shaping, as discussed later.

It is obvious that Atg8-family proteins play important roles in different stages during autophagy. However, several studies in mammalian cells have reported that these proteins and the lipidation system are not absolutely indispensable for autophagosome biogenesis. Nevertheless, they are important for efficient development of the isolation membrane as well as later for the disintegration of the inner autophagosomal membrane in the autolysosome[210–213].

Membrane sources and mechanisms of membrane/lipid supply. As already hinted in the context of autophagosome initiation, studies to date have proposed several membrane sources for autophagosome biogenesis, and researchers have engaged in intense debate about proposed models and the possible interplay and redundancy between different membrane sources[79,88,91,181,214–216]. As already mentioned, the Golgi apparatus and the plasma membrane/recycling endosome are thought to supply membranes to form autophagosome precursors. By contrast, recent studies have highlighted the ER as the most promising candidate for a membrane source in isolation membrane expansion, as described below.

Lipids may be delivered from the ER to the isolation membrane either locally, at the sites of close apposition of membranes (in mammals these sites are associated

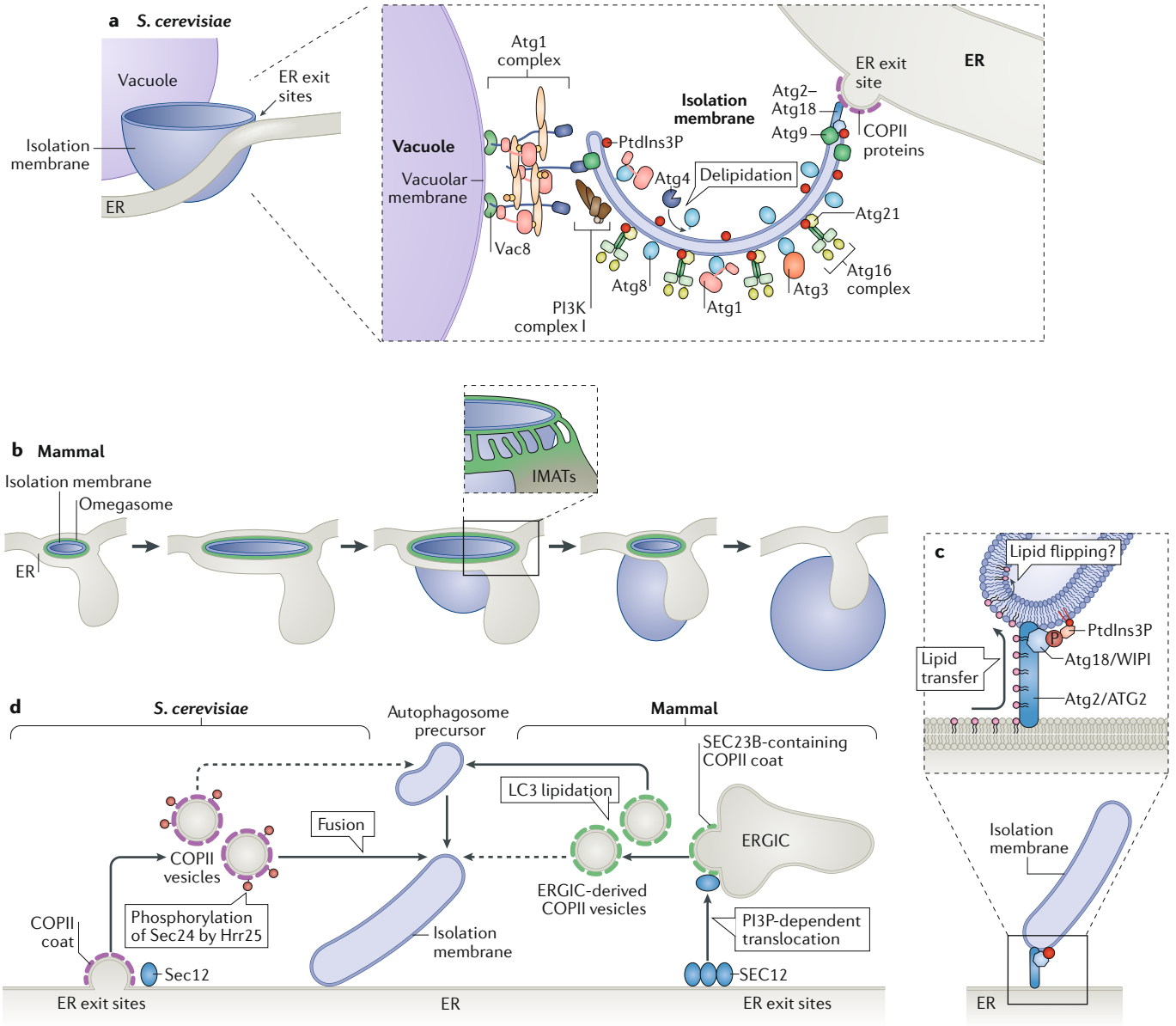

Fig. 4 | **Mechanisms of ER-associated membrane expansion in autophagosome biogenesis. a** | Atg protein distributions in the isolation membrane in *Saccharomyces cerevisiae*. While the Atg1 complex and phosphatidylinositol 3-kinase (PI3K) complex I are retained at the contact site between the isolation membrane and the vacuolar membrane, Atg9 and the Atg2–Atg18 complex reside at isolation membrane–endoplasmic reticulum (ER) exit site contacts. Given results in mammalian cells, the yeast Atg16 complex could be expected to localize to the convex surface of the isolation membrane. Lipidated Atg8 is distributed throughout the isolation membrane. By binding to Atg8, Atg1 and Atg3 also localize to the isolation membrane. A pool of membrane-bound Atg8 at the isolation membrane is delipidated via the activity of Atg4, allowing Atg8 recycling. **b** | Mammalian cell isolation membrane expansion in association with the omegasome/isolation membrane-associated tubular structures (IMATs), which connect the ER and the opening edge of the isolation membrane, likely allowing direct lipid transfer between the ER and autophagosomal membranes (see FIG. 4c). **c** | The Atg2–Atg18/ATG2–WD repeat protein interacting with phosphoinositides (WIPI) complex tethers the autophagosome precursor/isolation membrane to the ER, in which Atg2/ATG2 associates with the ER, while Atg18/WIPI binds phosphatidylinositol 3-phosphate (PtdIns3P) in the autophagosome precursor/isolation membrane. This complex has been shown to mediate direct lipid transfer from the ER to these membranes. To expand the autophagosomal membrane through this direct lipid transfer, flipping of ER-supplied lipids to the inner leaflet of the isolation membrane would be required, but the mechanisms of this putative flipping have not been elucidated (question mark). **d** | In *S. cerevisiae*, coat protein complex II (COPII) vesicles formed at the ER contribute to isolation membrane expansion, in which the COPII coat protein Sec24 is phosphorylated by Hrr25 to interact with Atg9. These vesicles are also suggested to be involved in the formation of the autophagosome precursor (but this remains to be formally shown (dashed line)). In mammalian cells, COPII vesicles are unusually formed at the ER–Golgi intermediate compartment (ERGIC) depending on PI3K complex I and the specific COPII coat protein SEC23B. In this process, a COPII vesicle biogenesis factor, SEC12, is transferred to the ERGIC depending on PI3K complex I activity, where it initiates the formation of COPII vesicles. These vesicles have been primarily proposed to form autophagosome precursors, and may also contribute to isolation membrane expansion (which awaits further validation (dashed line)).

with specific ER subdomains known as omegasomes; see further discussion) or from more distant ER locations via COPII vesicles (of note, these mechanisms are certainly not mutually exclusive). Regarding the former mechanism, in mammals, autophagy-inducing signals trigger the formation of a PtdIns3P-enriched domain in the ER depending on PI3K complex I. This domain, which can be visualized with use of double FYVE domain-containing protein 1 (DFCP1), is developed into a ring-shaped structure, which has been called the 'omegasome'[88] (FIG. 4b). By fluorescence microscopy it appears that as the omegasome ring enlarges, a flat isolation membrane forms and expands inside the ring. Then the isolation membrane curves, protrudes from the ring and finally closes concomitantly with the contraction of the omegasome ring. While the first electron tomography studies reported that isolation membranes, in their both concave and convex surfaces, are closely associated with ER sheets and these membranes are connected with thin tubules[214,217], a closer examination revealed the fine structure of the omegasome as isolation membrane-associated tubular structures (IMATs), which connect the ER and the opening edge of the isolation membrane[218]. These observations allow us to speculate that lipids are transferred from the ER into the isolation membrane through the omegasome/IMATs. DFCP1 is the sole protein known to localize to the omegasome but is dispensable for autophagosome biogenesis[88]. Identification of an omegasome-specific protein required for membrane expansion, and investigation of defects caused by the absence of the protein will help our understanding of the involvement of the omegasome/IMATs in autophagosome biogenesis.

A structure equivalent to the omegasome/IMATs has not been observed in yeast. In yeast cells, isolation membrane–ER contacts seem to be poorly developed during autophagosome biogenesis (a few spots per isolation membrane)[153,154] compared with mammalian cells (FIG. 4a,b). However, it is possible that an omegasome/IMAT-like structure is formed at these contact sites but is less well defined or its visualization may necessitate other, specific markers. It is also noteworthy that a nuclear membrane (perinuclear ER) domain likely corresponding to the omegasome was recently described in yeast, but the details await further investigations[219].

Irrespective of the presence/nature of the omegasome in yeast, there is evidence that in both yeast and mammals autophagosomal membranes are tethered to the ER, and recent studies suggested direct lipid transfer from the ER to the isolation membrane involving Atg2/ATG2 (FIG. 4a,c). Both yeast Atg2 and mammalian ATG2 were shown to have a membrane-tethering function[102,192,220–222]. Whereas an amphipathic helix in the C-terminal region of Atg2/ATG2 binds the autophagosome precursor/isolation membrane edge in cooperation with Atg18/WIPIs binding to PtdIns3P[220,223], the N-terminal region is likely to be involved in the association with the ER, establishing contacts between these membranes[220]. Moreover, three groups reported that Atg2/ATG2 proteins have lipid transfer activity — without strict head group specificity — which is enhanced by Atg18/WIPIs[221,222,224,225]. Consistently, the structural determination of the N-terminal domain of Atg2 from the fission yeast *Schizosaccharomyces pombe* has revealed that this domain has a hydrophobic cavity, which accommodates phospholipid acyl chains[222]. Combined with the model that Atg2/ATG2 tethers the autophagosome precursor/isolation membrane to the ER, it was proposed that Atg2/ATG2 extracts lipid molecules from the ER and transfers them to the autophagosome precursor/isolation membrane through its long hydrophobic groove (FIG. 4c). This model fits with the observation by electron microscopy that the autophagosomal membrane is low in proteins[226,227]. However, lipid transfer to the cytoplasmic layer of the isolation membrane is not sufficient to expand the membrane; some mechanism that can flip the ER-supplied lipids into the lumenal layer is also required, but currently the mechanism is elusive. Future studies should also address how ER to isolation membrane unidirectional lipid transfer is achieved. Overall, it appears that the close apposition of the ER and isolation membranes can drive direct lipid transfer to fuel autophagosomal membrane expansion.

COPII vesicles — the second proposed source of ER lipids for the growing isolation membrane — are formed in the ER to transport proteins and lipids from the ER to the Golgi apparatus. An early yeast study showed that proteins related to COPII vesicle formation are important for autophagosome biogenesis[228]. Two groups also reported independently that the PAS and the isolation membrane edge are closely associated with ER exit sites (ERES), where COPII vesicles are formed[153,154] (FIG. 4a). In addition, a recent study revealed that a transmembrane protein can be transferred from the ER to the isolation membrane by means of COPII vesicles[229]. These results strongly suggest that COPII vesicles contribute to the autophagosomal membrane (FIG. 4d). Although it is indisputable that COPII vesicles are an essential membrane source for autophagosome biogenesis, it remains unclear whether these vesicles are a major source for membrane expansion. An alternative possibility is that these vesicles supply specific ER proteins or lipids to the isolation membrane, which are important for membrane expansion. Of note, the localization of COPII vesicles to the PAS is also observed although to a lesser extent than that to isolation membranes/autophagosomes[229]. In addition, the PAS localization of some Atg proteins is perturbed in COPII-mutant cells[154]. These observations suggest that COPII vesicles are also involved in the formation of the autophagosome precursor (FIG. 4d).

Previous studies in yeast provided insights into how COPII vesicles, which inherently fuse with the Golgi apparatus, are targeted to the PAS/isolation membrane. First, physical proximity to ERES can facilitate COPII vesicle targeting to these membranes. In addition, the RAB1 homologue Ypt1, which localizes to COPII vesicles and targets them to the Golgi appratus, and its guanine nucleotide exchange factor complex TRAPPIII localize to the PAS and interact with Atg1 complex subunits and Atg9 (REFS[64,230–232]). Moreover, the COPII coat protein Sec24 binds Atg9 when phosphorylated by the casein kinase 1δ Hrr25, which is activated by Ypt1 at the PAS[233,234]. These protein–protein interactions can target COPII vesicles to the PAS/isolation membrane.

ER exit sites
(ERES). A domain in the endoplasmic reticulum where COPII proteins are assembled to form COPII vesicles.

Subsequently, COPII vesicles should fuse with these membranes depending on SNAREs. Previous studies showed that SNAREs responsible for COPII vesicle–Golgi apparatus fusion are important for autophagy[201,235]; however, whether these SNAREs also mediate COPII vesicle fusion to the PAS/isolation membrane has not been clarified. It should also be mentioned that there are contradictory results that SNAREs are dispensable for autophagosome biogenesis[228,236].

COPII vesicles participate in autophagosome biogenesis in mammals as well. However, these vesicles are formed in an unusual manner in the context of autophagy (FIG. 4d). ERES positive for SEC12 — a guanine nucleotide exchange factor, which functions to initiate COPII vesicle formation in the ER under normal conditions — are enlarged on starvation depending on the ERES protein cutaneous T cell lymphoma-associated antigen 5 (CTAGE5) and FIP200 but not on the other ULK complex subunits[89–91]. Then, SEC12 translocates to the ER–Golgi intermediate compartment (ERGIC) depending on PI3K complex I, where it initiates the formation of COPII vesicles. LC3 lipidation efficiently occurs in these vesicles. Collectively, it was proposed that these ERGIC-derived COPII vesicles are used to nucleate the autophagosomal membrane (FIG. 4d). It is possible that these vesicles also contribute to isolation membrane expansion. Given the intriguing observation that the ERGIC marker ERGIC53 colocalizes well with the omegasome marker DFCP1 (REF.[91]), it is tempting to speculate that the ERGIC is transformed into the omegasome under starvation conditions.

It was further revealed that mammalian cells use a specific COPII protein to generate COPII vesicles at the ERGIC (FIG. 4d). Under nutrient-replete conditions, the COPII protein SEC23B (one of the two SEC23 paralogues) is degraded by the ubiquitin–proteasome system in a manner dependent on the ubiquitin ligase subunit F-box protein FBXW5 (REF.[55]). Starvation-induced phosphorylation of SEC23B by ULK1 blocks its interaction with FBXW5, and the stabilized protein localizes to the ERGIC to participate in COPII vesicle formation.

In addition to ER sources, de novo lipid synthesis has also been considered as a possible mechanism for supplying lipids during autophagosome biogenesis. Although synthesis of lipids, such as monounsaturated fatty acids, phosphatidylinositol and phosphatidylcholine, was reported to be required for autophagosome biogenesis[169,237–239], it remains elusive whether these lipids are directly generated at the autophagosome precursors/isolation membranes or are supplied from other locations. A very recent study in yeast revealed that the acyl-CoA synthetase Faa1, which localizes to the isolation membrane, promotes fatty acid incorporation into Atg8-positive (autophagosome-related) membranes probably via phospholipid synthesis at contact sites between the isolation membrane and ERES, providing evidence for the contribution of de novo lipid synthesis to autophagosome biogenesis[240]. However, the authors proposed that the Atg2–Atg18 complex transfers these newly synthesized phospholipids from the ER to the isolation membrane as described above, rather than that phospholipids are produced in the isolation membrane itself. It is also conceivable that COPII vesicles mediate transport of these lipids. Overall, how and to what extent newly synthesized lipids support autophagosome biogenesis remains to be addressed.

Mitochondria were also proposed to supply lipids for isolation membrane expansion, in which phosphatidylserine is transferred from the ER to mitochondria, converted to phosphatidylethanolamine and then transferred to the isolation membrane for its expansion via lipidation (phosphatidylethanolamine conjugation) of Atg8-family proteins[215]. Given the observation that mammalian ATG proteins are assembled at contact sites between mitochondria and the ER[156], cooperation between these two organelles in lipid metabolism/transfer seems important for autophagosomal membrane formation. In addition to the ER and mitochondria, a recent electron microscopy study revealed contact sites between the isolation membrane and other organelles, including endosomes, lysosomes and the Golgi apparatus, in mammalian cells[241]. Further investigation is required to assess whether these organelles are functionally associated with the isolation membrane.

Finally, it was reported that yeast cells deficient for the biogenesis of lipid droplets cannot form autophagosomes under nitrogen starvation conditions[242,243], raising the possibility that lipid droplets serve as a direct lipid source for autophagosome formation. However, a recent study clearly showed that defective lipid droplet biogenesis affects the lipid composition and morphology of the ER and thereby indirectly impairs autophagosome formation under these conditions; autophagosomes can be formed even in cells lacking lipid droplets if they are treated with rapamycin (to inhibit TORC1) instead of nitrogen starvation[244]. The contribution of lipid droplets to autophagosome biogenesis in mammals remains to be investigated.

Membrane shaping

Autophagosomes are formed through unique membrane dynamics, involving transition from a small membrane seed to a spherical double-membrane vesicle. How does the isolation membrane curve and become spherical? What determines the final size of the autophagosome? Most researchers would have assumed that specific proteins play pivotal roles to govern these processes of membrane shaping. Indeed, studies have found many membrane curvature sensing or generating proteins involved in autophagosome biogenesis[245], among which proteins that are likely to contribute to isolation membrane shaping are focused on here.

In vitro experiments demonstrated that the yeast Atg16 complex bound to giant unilamellar vesicles (GUVs)[246] and was further assembled into a mesh-like structure in association with Atg8 lipidated on the GUV membrane, which might serve as a scaffold or coat to shape the isolation membrane[247] (FIG. 5a). Although fluorescence microscopy-based quantitative analysis suggested that the copy number of the Atg16 complex assembled at the autophagosome formation site is too small to cover the entire surface of an average-sized autophagosome[160], it is possible that patches of Atg16 complex–Atg8 assemblages affect the shaping of

ER–Golgi intermediate compartment
(ERGIC). A membrane compartment that mediates vesicle transport between the endoplasmic reticulum and the Golgi apparatus.

Acyl-CoA
Coenzyme that functions in fatty acid metabolism; it serves as an acyl chain donor in phospholipid synthesis.

Giant unilamellar vesicles
(GUVs). Artificial micrometre-sized vesicles bound by a single membrane that are used as a model for analysis of cellular membranes.

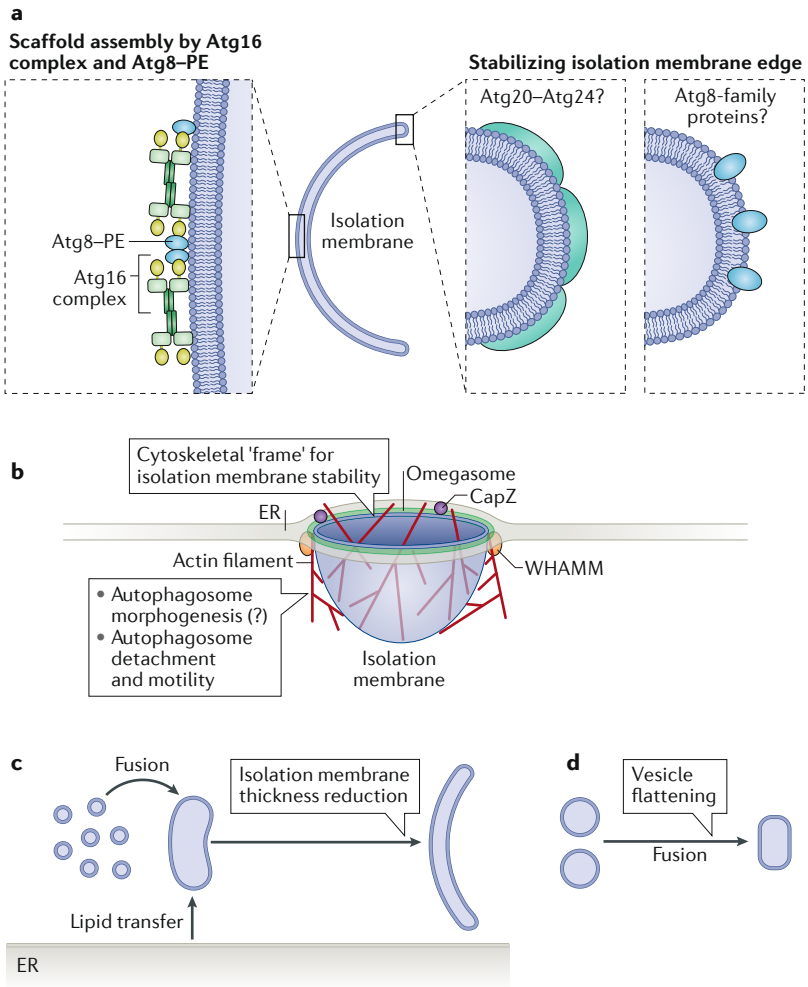

Fig. 5 | **Mechanisms of autophagosomal membrane shaping. a** | Possible contribution of Atg proteins to isolation membrane shaping (insights from yeast). The Atg16 complex could form a membrane scaffold in association with lipidated (phosphatidylethanolamine (PE)-conjugated) Atg8 on the convex surface of the isolation membrane, which may affect isolation membrane bending. The sorting nexin complex Atg20–Atg24, which has phosphatidylinositol 3-phosphate-binding PX domains and membrane curvature-sensing/generating BAR domains, could localize to and stabilize the isolation membrane edge. Lipidated forms of Atg8-family proteins can also stabilize highly curved membranes like the isolation membrane edge. **b** | The role of branched actin filaments in isolation membrane shaping. At the omegasome, the actin-capping protein CapZ and the actin-nucleation promoting factor WHAMM coordinate the formation of the actin filament network on the concave and convex sides of the isolation membrane, respectively. The concave actin network seems to serve as a 'frame' that prevents isolation membrane collapse. The convex network has been implicated in both the morphogenesis of the autophagosome (although the mechanisms involved here are elusive (question mark)) and the release and motility of complete autophagosomes via comet-tail propulsion. **c** | Small-vesicle fusion and direct lipid transfer can both result in a decrease in isolation membrane thickness, thereby increasing membrane curvature at the edge region that could contribute to isolation membrane shaping. **d** | Fusion between spherical vesicles results in a flattened vesicle, a shape associated with autophagosome precursors. ER, endoplasmic reticulum.

isolation membranes. In mammalian cells, blocking lipidation of Atg8-family proteins results in the accumulation of isolation membranes with abnormal morphology[210,211]. This may be owing to the absence of ATG16L1 complex–Atg8-family protein assemblages on the convex surface of the isolation membrane or a function of lipidated Atg8-family proteins itself. Further analysis is required to assess this hypothetical role for the Atg16/ATG16L1 complex in the shaping of the isolation membrane. As already mentioned, Atg8-family proteins can cause membrane tethering and hemifusion/fusion when anchored to small liposomes. However, lipidated Atg8 generates positive membrane curvature in GUV membranes[248], probably by inserting its protein part into the outer leaflet of the vesicle (FIG. 5a). This property of Atg8 could also stabilize highly curved membranes. In addition, Atg8-family proteins tend to be lipidated in and sorted to membrane regions with high curvature[248,249], increasing their local concentration in these regions and thereby boosting their membrane curvature-generating or membrane curvature-stabilizing effect. Therefore, Atg8-family proteins could also affect membrane shaping during isolation membrane expansion.

Studies have suggested that actin filaments play important roles in early steps during autophagosome biogenesis, such as the recruitment of ATG proteins to the autophagosome formation site[250]. Actin was also reported to contribute to isolation membrane shaping in mammals (FIG. 5b). A branched actin network was found to form on the concave side of the isolation membrane depending on the actin-branching Arp2/3 complex[251]. In this process, the actin-capping protein CapZ, the activity of which is reduced by its binding to PtdIns3P probably at the omegasome, is likely to spatiotemporally organize the formation of branched actin filaments. CapZ knockdown or Arp2/3 inhibition results in the collapse of isolation membranes. These results suggest that the branched actin filament network serves as a cytoskeletal 'frame' inside the isolation membrane for its development with normal morphology (FIG. 5b). It seems that actin filaments also participate in isolation membrane shaping in a different manner. On the basis of analysis of the actin nucleation-promoting factor WHAMM, it was proposed that this protein targets the Arp2/3 complex to the autophagosome formation site on nutrient starvation and initiates formation of branched actin filaments, promoting autophagosome biogenesis and its detachment from the ER and motility by an actin-comet tail mechanism[252] (FIG. 5b), resembling the role of WASL (another nucleation-promoting factor) in forming endocytic vesicles at the plasma membrane[253]. However, whereas the force generated by actin filament polymerization is likely to promote endocytic vesicle formation by simply pulling on and extending the plasma membrane, this may not be applicable to isolation membrane expansion, where large changes of the membrane surface driven by lipid supply are required. Pulling the isolation membrane is unlikely to promote supply of membranes/lipids from the ER by any of the mechanisms discussed above. In any case, actin filament networks on the convex surface as well as those on the concave side of the isolation membrane may support autophagosome morphogenesis.

In the context of membrane shaping it is also important to consider biophysical phenomena as drivers of shape changes. One theoretical study has addressed this problem, providing critical insights into the mechanism that shapes expanding isolation membranes from the viewpoint of the physical properties of lipid bilayers[254]. It was demonstrated that in a disc-like membranous

Actin-capping protein
Protein that binds to the end of actin filaments and blocks the polymerization and depolymerization of the filaments at the end.

Multivesicular bodies
Endosome-related organelles that contain lumenal vesicles that mediate lysosomal degradation of membrane proteins or secrete these vesicles as exosomes.

ESCRT
(Endosomal sorting complexes required for transport). Protein complexes (ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III) that mediate protein sorting and intralumenal vesicle formation in the endosome and are also responsible for different membrane fission events. Their main activity is driving membrane constriction, and hence they can be involved in different processes requiring membrane shaping in the cell.

vesicle representing an isolation membrane, the highly curved edge region is energetically unstable and therefore can be a factor that affects the shape of the vesicle. As the disc-like vesicle expands, the edge area increases, and the vesicle accordingly becomes more unstable. Calculation of the bending energy of that disc-like vesicle demonstrated that when the vesicle expands to a critical size, it bends and becomes spherical spontaneously (without proteins) to reduce the unstable edge area[254]. In light of this logic, an important force for isolation membrane shaping could be the physical properties of the membrane itself, and specific proteins could be dispensable. In another hypothetical point of view, stabilizing the isolation membrane edge by membrane-binding proteins or by altering lipid compositions can retard the timing of isolation membrane bending, resulting in the formation of larger autophagosomes and vice versa. Among yeast Atg proteins, Atg20 and Atg24 are sorting nexins, which have a PX domain, which binds PtdIns3P, and a BAR domain, which senses, stabilizes or generates membrane curvature[255]. These proteins form a heterodimer, and this complex is a potent candidate for such an edge-stabilizing factor (FIG. 5a). Consistent with this notion, smaller autophagosomes are formed in *S. pombe* cells lacking these proteins[256]. In addition, in both *S. cerevisiae* and *S. pombe*, selective autophagy of organelles was severely impaired in the absence of these proteins[255–257]. Since the isolation membrane has to enlarge its opening when engulfing organelles, edge stabilization by these proteins would become of particular importance. The possible curvature-stabilizing effect of lipidated Atg8-family proteins as discussed above[248] may also work to stabilize the isolation membrane edge (FIG. 5a); this idea is consistent with the results that the protein level and functionality of Atg8 correlate with autophagosome size[197,200].

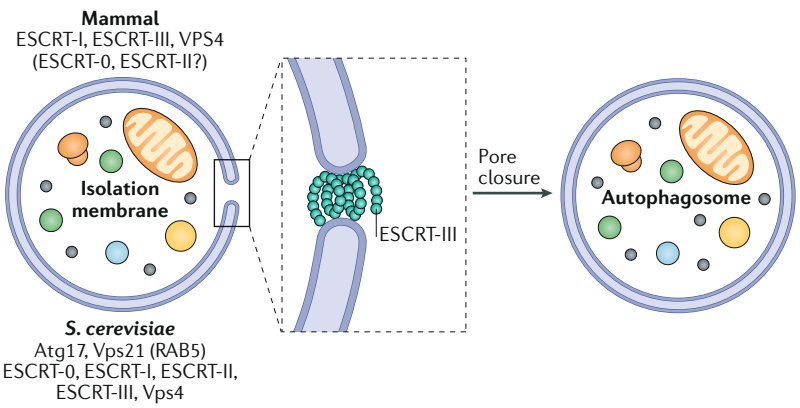

Fig. 6 | **Mechanism of autophagosomal pore closure.** ESCRT-III mediates pore closure in the isolation membrane. In *Saccharomyces cerevisiae*, the other ESCRT (endosomal sorting complexes required for transport) components (ESCRT-0, ESCRT-I and ESCRT-II), Vps4 and Vps21 (a RAB5 homologue) were also reported to be important for this process. In addition, the ESCRT-III component Snf7 interacts with Atg17, a possible mechanism for the recruitment of the ESCRT machinery to the isolation membrane. In mammals, it remains elusive whether this process involves ESCRT-0 and ESCRT-II. There is also evidence that in yeast autophagosome closure can proceed in the absence of Vps4 (which is a key factor for ESCRT-mediated membrane scission), and hence it is likely that autophagosome pore closure involves also factors other than ESCRT.

Another aspect to be considered is that membrane/lipid supply per se can also affect isolation membrane shaping. Fusion of small vesicles (such as ATG9 and COPII vesicles), which have a higher membrane area to lumen volume ratio than the isolation membrane, and direct lipid transfer would both result in the reduction of the isolation membrane thickness (FIG. 5c). This may also increase curvature at the isolation membrane edge and thereby expedite the timing of isolation membrane bending. A similar logic (membrane flattening on vesicle fusion) could be applied to the process of autophagosome precursor formation. If seed vesicles (for example, Atg9/ATG9-positive and ATG16L1-positive vesicles) fuse with each other, the resulting vesicle should be flattened rather than spherical (FIG. 5d). Thus, the flattened shape of the isolation membrane could be intrinsically attributed to the process of membrane nucleation and expansion per se.

Pore closure

After the isolation membrane bends into a spherical shape, a small pore remains, which needs to close to complete formation of the autophagosome (FIG. 6) and allow its release from the ER and transport to the lysosome. This process involves membrane fission (not, as could be expected, membrane fusion)[258], which is topologically identical to the formation of lumenal vesicles in multivesicular bodies. Therefore, the involvement of the ESCRT (endosomal sorting complexes required for transport) machinery, which mediates different membrane fission events (including multivesicular body lumenal vesicle formation[259]) had been assumed in isolation membrane pore closure. Indeed, recent studies revealed that ESCRT components localize to the isolation membrane and promote its closure in mammals[260–262] (FIG. 6). It was reported that isolation membrane closure in yeast also involves the ESCRT machinery in addition to Vps21 (related to RAB5 involved in early endocytic pathway) and Atg17 (REF.[263]). However, this report seems contradictory to the fact that a number of studies have observed autophagic bodies (inner autophagosomal membrane vesicles released in the vacuolar lumen after autophagosome–vacuole fusion) in yeast cells lacking the ESCRT component ATPase Vps4 (REF.[264]). Thus, other membrane shaping systems might also be involved in closing the isolation membrane.

Conclusions and perspectives

Identification of ATG genes marked the beginning of molecular dissection of autophagosome biogenesis. Molecules that function in other cellular processes, prominently including those functioning in the secretory pathway and membrane shaping, as well as several organelles and their membrane contacts were also shown to be differently involved in the process, and the interplays among these players have been intensely investigated. Molecular functions of most ATG proteins have been elucidated, and how autophagy-inducing signals are transmitted to these proteins and how these proteins are assembled at the site for autophagosome biogenesis have become better understood. Although the origins of autophagosomal membranes have been a long-standing question, recent

studies have shed new light on contributing membrane sources. Autophagosome initiation appears to depend on Golgi apparatus and plasma membrane-derived ATG9 vesicles as well as plasma membrane-derived ATG16L1 vesicles, whereas most studies strongly advocate that the ER is an essential membrane/lipid source for membrane expansion, in which different modes of membrane/lipid supply — either local, at ER–isolation membrane contacts or involving COPII vesicle-mediated transfer — have been proposed. Moreover, the ESCRT machinery was shown to mediate pore closure in the isolation membrane to complete autophagosome formation. Although these were not described here in detail, a great deal of information about phosphoinositides and Rab GTPases in the regulation of autophagosome formation has been obtained[265–268]. Furthermore, the mechanism of SNARE-mediated autophagosome–lysosome/vacuole fusion has also been elucidated[269–272] (BOX 1). Although it is obvious that details differ between yeast and mammals and that mammals have evolved more sophisticated or complicated systems that allow more flexibility in autophagy regulation, common or conceptually equivalent mechanisms appear to underlie the fundamental processes of autophagosome biogenesis.

Despite the recent progress that has been made, a number of critical issues remain to be addressed before we fully understand autophagosome formation. First, autophagosomes are formed on the vacuole and the ER in yeast and mammals, respectively, but it is unclear what determines the sites for autophagosome formation on these organelles. In the step of membrane nucleation, although ATG protein-positive vesicles are suggested to serve as seed membranes, how these vesicles are targeted to the appropriate location and transformed into autophagosome precursors remains to be addressed. Regarding autophagosomal membrane sources, future studies should investigate the interplay of the ER with other candidates for autophagosomal membrane expansion as well as the roles and contribution degrees of these different sources in autophagosome biogenesis. It is also important to know how different modes of membrane/lipid supply from the ER are coordinated to expand the isolation membrane. In vitro reconstitution of autophagosome biogenesis will be a powerful tool to precisely analyse these processes. Mechanisms of membrane shaping during autophagosome formation have also been discussed, but are so far only predictions or speculations. Addressing these questions will have important implications for controlling autophagy to fully harness its roles in the maintenance of cellular homeostasis.

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