The origin of the autophagosomal membrane

Sharon A. Tooze and Tamotsu Yoshimori

Macroautophagy is initiated by the formation of the phagophore (also called the isolation membrane). This membrane can both selectively and non-selectively engulf cytosolic components, grow and close around the sequestered components and then deliver them to a degradative organelle, the lysosome. Where this membrane comes from and how it grows is not well understood. Since the discovery of autophagy in the 1950s the source of the membrane has been investigated, debated and re-investigated, with the consensus view oscillating between a *de novo* assembly mechanism or formation from the membranes of the endoplasmic reticulum (ER) or the Golgi. In recent months, new information has emerged that both the ER and mitochondria may provide a membrane source, enlightening some older findings and revealing how complex the initiation of autophagy may be in mammalian cells.

Autophagy is an evolutionarily conserved catabolic process in which cytosolic constituents, including organelles and macromolecules, are sequestered into a double-membrane structure and delivered to the endosome and lysosome. Efficient initiation and completion of autophagy is essential for cell health: malfunctioning or loss of Atg (autophagy-related) proteins or decreased efficiency at any step can cause alterations in cell growth and damage the cell, leading to cell death. Autophagy has an important role in a number of diseases, including cancer and neurodegeneration, as well as being an essential component in ageing and immunity[1].

The first crucial event in autophagy is the induction or nucleation of the membrane that will become an autophagosome, which is also the least understood step. This membrane has been called a phagophore or an isolation membrane and it has the ability to expand and grow, selectively and non-selectively engulfing organelles and proteins during its expansion. It is a double-membrane structure that finally closes to sequester its acquired content away from the cytosol, and become an autophagosome (Fig. 1). The autophagosome can then fuse both homotypically with other autophagosomes, and/or heterotypically with endosomes and lysosomes, during the maturation process. Maturation requires protein and lipid complexes that are thought to overlap with the known endocytic machinery in yeast and mammalian cells. During maturation, the autophagosome becomes an acidic, degradative autolysosome in which the sequestered content is degraded and recycled back to the cytosol as amino acids and macromolecules.

How the phagophore forms is central to understanding the process of autophagy. It is widely accepted that some unknown machinery must dock at, or be delivered to, an assembly point or platform that has been called the PAS (the preautophagosomal structure or the phagophore assembly site), and it is at this site that the phagophore forms. Recent discoveries in mammalian cells have markedly increased our knowledge of these first steps, and the site of phagophore formation. Surprisingly, these discoveries point to two different sources, the ER[2,3] and mitochondria[4]. Here, we summarize and discuss the implications of the new data supporting phagophore formation from the ER and the results demonstrating a role for mitochondria.

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The identification of molecular markers

In the late 1950s, morphologists working in mammalian cells first recognized autophagosomes as a unique compartment related to the lysosome, and in 1963 the term 'autophagy' was coined by de Duve. Over the last 40–50 years, the origin of the autophagosome has remained an unresolved question. Before addressing the most recent data about the origin of the autophagosome, it is worth considering why this remains an outstanding issue and why progess in this area is so exciting. Before the 1990s there were no specific molecular markers of the autophagosome, and because its content reflects the composition of the cytosol it was difficult to employ the standard technique of the time, subcellular fractionation, to isolate and characterize the membrane source or indeed any early intermediates. Morphological analysis combined with immunohistochemical stains provided data suggesting, for example, that membranes from the GERL (Golgi–ER–lysosome) could be involved in the formation of the autophagosome[5]. A notable advance was made by researchers who identified the phagophore in mammalian cells by fixation with reduced osmium[6] and by the observation that the autophagsosomal membrane was a relatively protein-poor membrane[7–10]. These seminal observations gave rise to the notion that the phagophore was a unique membrane, and it was hypothesized that it assembled *de novo* in the cytosol, a hypothesis later confirmed by the absence of known organelle markers in purified fractions[11]. However, contradictory data from several laboratories based on the presence or absence of ER and Golgi markers in the phagophore and autophagosome has been accumulating to support the notion

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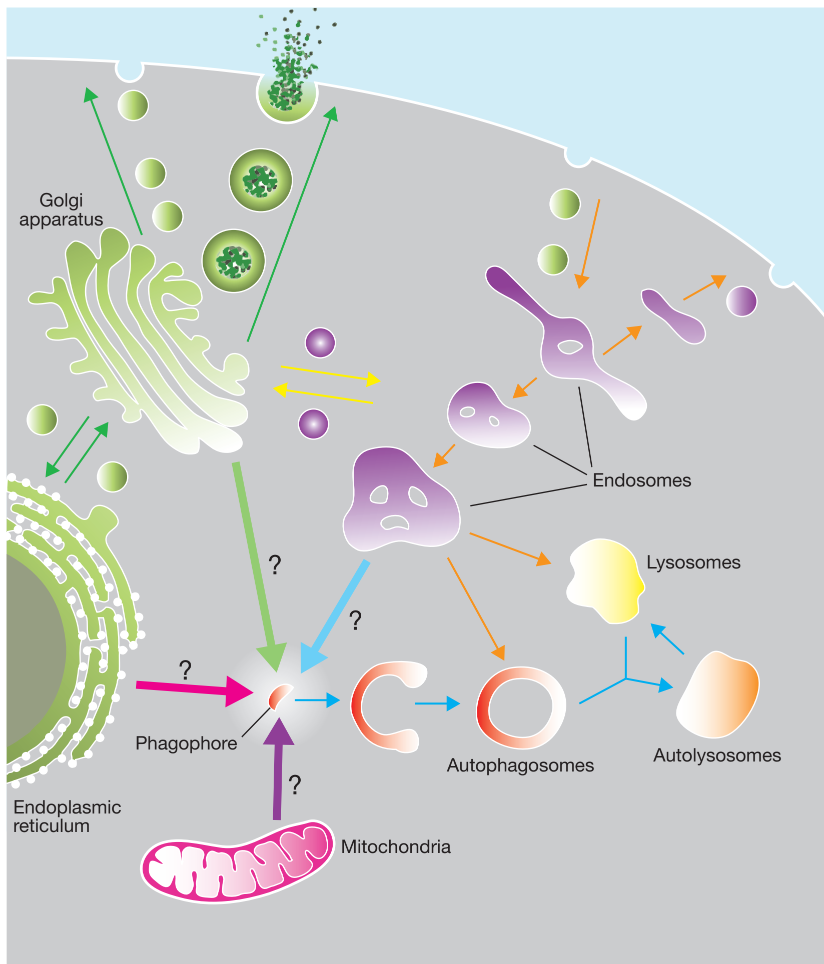

Figure 1 Potential sources for the phagophore include the Golgi complex, endosomes, ER and mitochondria. Previous data have supported the hypothesis that the ER and/or Golgi complex are the source of the phagophore (for review see ref. 34). However, evidence has emerged in the past year supporting the generation of the phagophore from the ER[3,19] and mitochondria[4].

that, although unique, the phagophore arises from a pre-existing compartment, probably the ER or the Golgi complex (for review, see refs 12 and 13).

Following on from their discovery in yeast[12,14], the Atg proteins, numbering over 30 in yeast and now almost 20 in mammalian cells, provided the markers needed to facilitate progress in understanding the molecular components that direct autophagosome formation. Most Atg proteins function primarily at early stages of autophagosome formation, up to the step where the phagophore becomes an autophagosome. In mammalian cells, the key components seem to be the kinase-containing ULK1 complex (Atg1 in yeast), the class III phosphatidylinositol 3-kinase (PtdIns3K) complex Vps34, the ubiquitin-like conjugation systems producing Atg5–Atg12–Atg16 and LC3-II (the phosphatidylethanolamine-containing LC3 conjugate), the sole multi-spanning membrane protein mammalian Atg9 (mAtg9, also known as Atg9a) and the phosphatidylinositol 3 phosphate (PtdIns(3)P)-binding protein WIPI2 (Atg18 in yeast). The molecular properties of most of these are broadly known, but the finer details of how they function and when they act in the early formation stages are not yet understood.

Of these Atg proteins, the best studied in mammalian cells is LC3-II, an Atg8 homologue, which has been tagged with green fluorescent protein (GFP) to enable the identification of early forming and closed autophagosomes in both live and fixed cells. In mouse models, Atg5 knockout provided the first evidence that autophagy is essential for development and post-natal survival *in vivo*[15]. However, the subcellular localization of the Atg5 and LC3 ubiquitin-like conjugates does not inform us about the source of the membrane as these are initially cytosolic, and only associate with the phagophore after it has started to form.

More information could be obtained from a membrane-associated Atg protein, but only one has been described so far. Atg9 has six transmembrane domains and its amino- and carboxy-terminal domains are present in the cytosol. It has not been as well studied and is less understood than other Atg proteins. In yeast, Atg9 cycles between the PAS and a peripheral pool, which comprises clusters of vesicles and tubules, and are often adjacent to mitochondria[16]. mAtg9 has been shown to transit between the endosome and a juxtanuclear Golgi compartment in fed cells and relocalize to a peripheral compartment after starvation[17]. As found for yeast Atg1, ULK1 is required for mAtg9 cycling between the two pools in normal and starved cells[17], and the cycling also requires p38 MAPK-interacting protein, p38IP[18]. So far there is no known function for yeast or mAtg9, but mAtg9 knockout mice are unable to survive after birth[19], an apparently identical phenotype to mice lacking Atg5 and the E1 and E2-like proteins, Atg7 and Atg3. In contrast, homozygous loss of *beclin1* (a subunit of the PtdIns3K complex, and the homologue of yeast Atg6/Vps30) in mice results in loss of viability at an earlier stage, E5–6 (refs 20 and 21). This is a more severe phenotype, which perhaps highlights the role of the Beclin1–PtdIns3K complex at multiple stages of endocytosis as well as in autophagy, whereas the more restricted phenotype of the mAtg9 knockout mice strongly suggests its main role is in autophagy. Although further information about mAtg9 is needed, its localization supports a role for a juxtanuclear Golgi and/or endosomal compartment in phagophore formation (Fig. 1). Recent data from yeast studies strongly support this hypothesis: a subset of COG (conserved oligomeric Golgi) subunits[22], Golgi ADP ribosylation factors (ARFs) and sec proteins[23,24], all known to control exit from the Golgi, are involved in early stages of autophagy. In addition, a newly discovered tethering complex, called TRAPP complex III, was shown to be required for autophagy and recruitment of Atg proteins to the PAS along with a yeast Rab GTPase, Ypt1 (ref. 25). These new results lend further support to models that suggest a role for post-ER Golgi compartments in phagophore formation.

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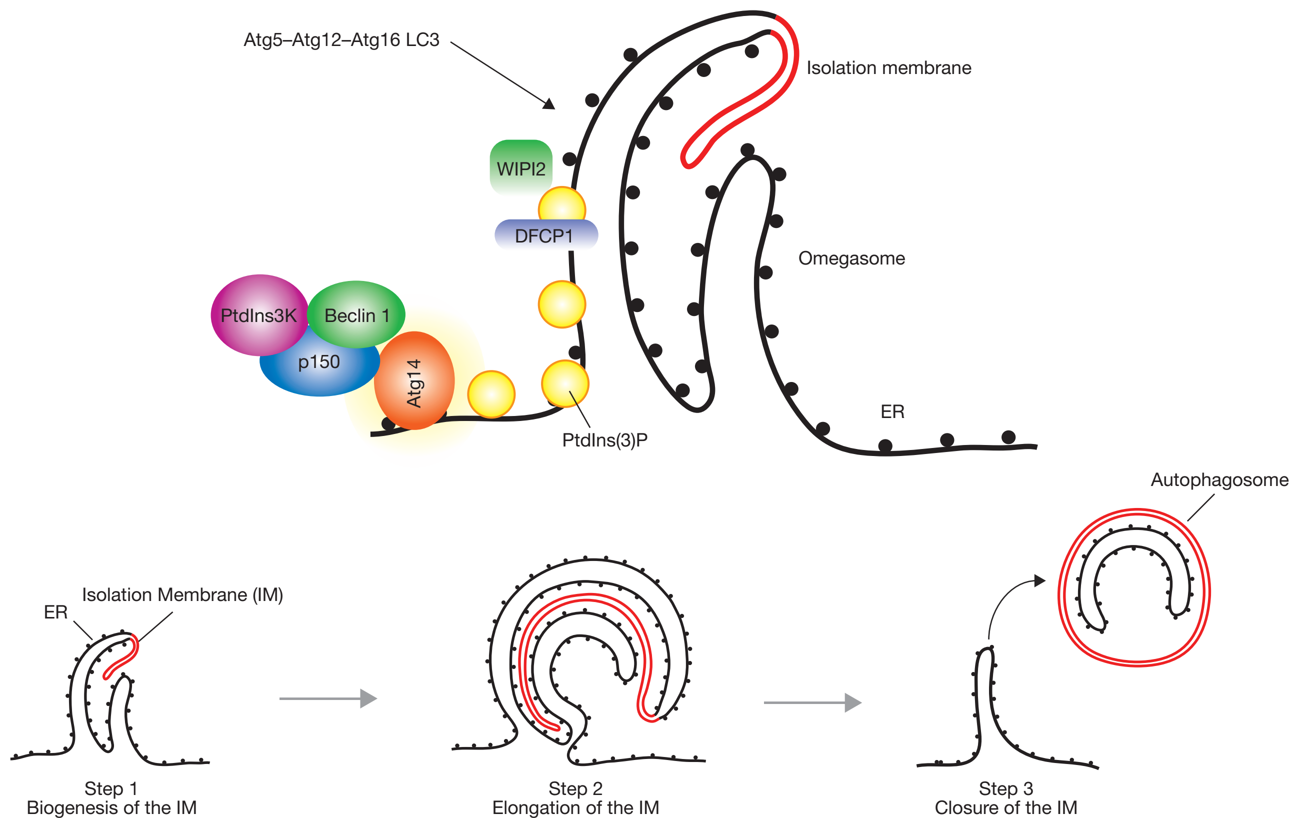

Figure 2 Model for recruitment of Atg proteins and DFCP1 to the ER-derived membrane and formation of the phagophore. (**a**) PtdIns(3)P is generated by the Vps34 kinase complex which is recruited to this site by Atg14. PtdIns(3)P is then recognized by the effectors DFCP1 and WIPI2. The phagophore shown in red extends from this PtdIns(3)P-rich structure and elongates, cupped between two ER cisternae[2,3]. Resident ER proteins are prevented from entering the phagophore, perhaps through a diffusion barrier. It is not known if the PtdIns(3)P produced is localized to the ER or the phagophore, and if localized to the ER whether it is also prevented from entering the phagophore by a diffusion barrier. (**b**) The final stages of elongation and closure of the phagophore and detachment from the ER is illustrated. To allow such a dramatic re-organization of the ER and detachment of the autophagosome other unidentified machinery must be involved[36].

Localized and novel PtdIns(3)P-mediated early events

Data from hierarchical analyses in yeast and biochemical and pharmacological experiments in mammalian cells have catalysed recent work on the Atg protein complexes that act upstream of the ubiquitin-like conjugation machinery: the essential ULK1 kinase complex (ULK1, Atg13 and FIP200) and the PtdIns3K complex (Beclin1, Vps34, p150 and the autophagy-specific subunit Atg14). In yeast, the ULK1-complex member Atg17 (FIP200 in mammals) is the most-upstream Atg in starvation-induced autophagy. In mammalian cells, ULK1 has been linked directly to mammalian target of rapamycin complex 1 (mTORC1) activity, as it has been found to associate with the mTOR-interacting partner, raptor (under fed conditions), and co-localize with Atg16 on autophagosomes (for review see ref. 26). Inhibition of the activity of the PtdIns3K complex using wortmannin, for example, has shown that it is essential for autophagy. Importantly, the activities of the ULK1 complex and the PtdIns3K complex are both thought to respond to nutrients and amino acids, although it is not yet clear how these responses are coordinated.

PtdIns(3)P production is essential for autophagosome formation (for review see ref. 27) and it is probable that this essential function is regulated by Atg14. On starvation, PtdIns(3)P production is induced and occurs ectopically in cellular compartments such as the ER, which is thought to not normally have high levels of PtdIns(3)P[28]. The observation that Atg14 localizes to the ER as well as the phagophore[29] supports the idea that the ER may be the site of phagophore formation. This is further supported by the observation that a mutated Atg14 unable to localise to the ER loses its ability to induce autophagosomes, whereas the addition of an ER-targeting motif to the mutated Atg14 restores its ability to localize to the ER and promote autophagy[30]. It is not known why PtdIns(3)P is required for phagophore formation. Proposed functions include alteration of the ER-derived lipid bilayer composition to create a phagophore, or simply recruitment of PtdIns(3)P-binding proteins that are required for the synthesis of the phagophore**.**

In yeast, the Atg effector-proteins recruited by PtdIns(3)P are Atg18 and Atg21; the mammalian homologues of Atg18 are WIPI2 and WIPI1–4 (WD-40 repeat-containing protein that interacts with PtdIns)[31,32]. WD-repeat domain-containing proteins specifically recognize PtdIns(3)P, and are recruited to the phagophore. Interestingly, although PtdIns(3)P is synthesized by the Vps34 PtdIns3K elsewhere in the cell (for example in endosomes), WIPI2 (the homologue present in all cells and shown to be required for autophagy[31,32]) responds and relocalizes to phagophores containing the pool of PtdIns(3)P produced by the autophagic PtdIns3K complex. WIPI2 forms punctate structures after starvation, which are sensitive to wortmannin and that partially localize with Atg16 and GFP–LC3, but also localize with ULK1, suggesting

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WIPI2 is recruited to very early autophagic membranes[31].

Omegasomes or mitochondria?

The progress made towards the identification of the membrane source of the phagophore using the Atg proteins is remarkable, but the discovery of a novel non-Atg protein that binds to the autophagic PtdIns(3)P pool has recently polarized this research. Axe *et al.*[25] have reported that a protein named DFCP1 (double FYVE-domain-containing protein 1) binds PtdIns(3)P through its FYVE domains. Although DFCP1 is associated with the Golgi complex in fed cells, on starvation it translocates to an ER-associated membrane called the omegasome by the authors. The omegasome is proposed to function as a platform for autophagosome formation because the Atg5 complex and LC3 were recruited to this structure after starvation, and Atg5- and LC3-positive membranes seem to emerge from the omegasome. ULK1, Atg14 and WIPI2 were all observed to be recruited to the omegasome (Fig. 2a). Importantly, Atg14 knockdown results in disappearance of the omegasome[30], whereas WIPI2 knockdown causes a massive accumulation of omegasome membranes[31].

Atg4 is an endopeptidase of Atg8, LC3 and GABARAP (γ-aminobutyric acid receptor-associated protein) and regulates the lipidation of these proteins. Expression of its catalytically-inactive mutant causes accumulation of open phagophores[33]. In starved NRK (normal rat kidney) cells[3], or NIH 3T3 cells and MEFs (mouse embryonic fibroblasts)[2], three-dimensional (3D)-electron microscopy tomography revealed phagophores juxtaposed and enwrapped by membrane that were in continuity with the ER (Fig. 2b), labelled with DFCP1 and thus related to omegasomes. The reconstructions from the 3D-electron microscopy tomograms show that the phagophore is cradled between the two ER membranes, that there are connections between the phagophore with the ER and that 50% of autophagic structures were associated with the ER[2,3].

These results provide compelling evidence for the hypothesis that the ER can provide membrane for the expansion of the phagophore in some cell lines. Several questions remain to be addressed: is a connection between the ER and phagophore required? Is there always an ER cisternae inside the autophagosome? How would mitochondria fit into the ER-derived membrane? What about selective autophagy processes? Do they use the ER as a source? Are the lipids and proteins required for phagophore growth and expansion derived from the ER, or could they be supplied from other sources? Regarding the final question, the evidence so far supports a role of post-ER Golgi compartments in the expansion of the phagophore but further identification of lipids or the proteins components involved is required.

In contrast to the proposal that the ER is the source of the phagophore, a recent paper points, surprisingly, to the mitochondria as a source of autophagososomal membrane[4]. So far, no direct experiments have demonstrated that the mitochondria are involved in the formation of the phagophore, although some indirect evidence, mostly based on the localization of Beclin 1 to mitochondria, are suggestive. However, the study shows co-localization of Atg5 and LC3 with mitochondria and that a protein anchored in the outer leaflet of mitochondria (but not outer or inner membrane proteins, or those inside the mitochondria) can be found on forming autophagosomes. The data also suggests that the mitochondrial outer leaflet may supply membrane to the expanding phagophore, and in addition, may be a source of newly synthesized phosphatidylethanolamine, which could then be conjugated to Atg8. However, the role of PtdIns(3)P and of PtdIns(3)P-binding proteins such as DFCP1 and WIPI2 in this model is not addressed. Whether recently characterized ER–mitochondrial connections[34] are involved in transfer of lipids or proteins from the mitochondria to the ER during the formation of the phagophore also remains to be investigated.

Future perspectives

Rapid advances in the past years have provided many molecular details about how the phagophore forms. But, even if the function of PtdIns(3)P in the process has been clarified, do we now know the answer to this central question: where is the membrane coming from? Probably not yet but we are getting closer all the time. Higher resolution light microscopy and identification and characterisation of more non-Atg proteins acting in this process will all contribute to progress in this field.

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COMPETING FINANCIAL INTERESTS
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