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Mechanisms of Autophagosome Biogenesis

Minireview

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Autophagy is a unique membrane trafficking process whereby newly formed membranes, termed phagophores, engulf parts of the cytoplasm leading to the production of double-membraned autophagosomes that get delivered to lysosomes for degradation. This catabolic pathway has been linked to numerous physiological and pathological conditions, such as development, programmed cell death, cancer, pathogen infection, neurodegenerative disorders, and myopathies. In this review, we will focus on recent studies in yeast and mammalian systems that have provided insights into two critical areas of autophagosome biogenesis — the source of the autophagosomal membranes, and the mechanisms regulating the fusion of the edges of the double-membraned phagophores to form autophagosomes.

Introduction

(Macro)autophagy is a bulk degradation process that mediates the clearance of long-lived proteins and organelles [1]. Initially, double-membraned cup-shaped structures called phagophores engulf portions of cytoplasm. After fusion of the membranes (closing the 'cup'), the autophagosome is formed. These vesicles appear randomly throughout the cytoplasm, then traffic along microtubules with their movement biased towards the microtubule-organising centre, where lysosomes are concentrated [1]. This brings autophagosomes close to lysosomes, enabling fusion and degradation of the contents of the resulting autolysosomes by lysosomal hydrolases.

The biology of autophagy was revolutionised by the discovery of so-called Atg (autophagy) genes in yeast, many of which are conserved in humans. The identification of Atg genes allowed for the assessment of the importance of this pathway in various contexts, as well as a detailed dissection of its mechanism of action [2–4]. Autophagy has numerous roles in physiology and disease. Its primordial function from yeast to man is to act as a buffer against starvation by liberating building blocks from macromolecules. It has additional physiological roles, however, including permitting early embryonic development, removal of apoptotic corpses, antigen presentation, protection against cell death insults, and as a degradation route for various aggregate-prone proteins and infectious agents [5]. Abnormalities in autophagy may contribute to pathologies such as tumourigenesis, various neurodegenerative diseases, and certain muscle diseases [5].

The signals that regulate autophagy are diverse. The induction of autophagy in response to starvation is mediated in part via inactivation of the mammalian target of rapamycin (mTOR) and activation of Jun N-terminal kinase (JNK), while energy loss induces autophagy by activation of AMP kinase (AMPK). Other pathways regulating autophagy are regulated by calcium, cyclic AMP, calpains and the inositol trisphosphate (IP_3) receptor [6].

In this review, we will focus on recent studies in yeast and mammalian systems that have provided insights into two critical areas of autophagosome biogenesis. We will discuss the possible sources of the autophagosomal membranes, and the mechanisms regulating the fusion of the edges of the double-membraned phagophores to form autophagosomes.

Autophagosome Formation

A conventional view is that autophagosome formation starts at phagophore assembly sites (PAS) [7]. This concept is derived from studies in yeast which observed that a number of the key proteins involved in autophagosome formation colocalise at a single site in the cell [7,8]. This operational definition led to statements in the literature describing autophagosome formation as an event that occurred '*de novo*'. In mammalian cells, there are multiple PAS at any one time. The formation of phagophores requires the class III phosphoinositide 3-kinase (PI3K) Vps34, which acts in a large macromolecular complex, along with Beclin-1 (mammalian Atg6), Atg14 and Vps15 (previously known as p150), to form PI 3-phosphate (PI(3)P) [1]. The activity of this complex is dependent on upstream autophagy regulators, including the mammalian Atg1 orthologues ULK1 and ULK2, Atg13 and focal adhesion kinase (FAK)-family interacting protein of 200 kDa (FIP200) [1].

The elongation of membranes that evolve into autophagosomes is regulated by two ubiquitination-like reactions. First, the ubiquitin-like molecule Atg12 is conjugated to Atg5 by Atg7, which acts like an E1 ubiquitin-activating enzyme, and by Atg10, which is similar to an E2 ubiquitin-conjugating enzyme. The Atg5–Atg12 complex then interacts non-covalently with Atg16L1 and this resulting ternary complex associates with phagophores but dissociates from completed autophagosomes [9].

The second of the ubiquitin-like reactions involves the conjugation of ubiquitin-like molecules of the Atg8 family, which comprises the three subfamilies LC3, GABARAP and GATE-16 [10], to the lipid phosphatidylethanolamine. Microtubule-associated protein 1 light chain 3 (MAP-LC3/Atg8/LC3), the most well-characterised member of the Atg8 protein family, is conjugated to phosphatidylethanolamine by Atg7 (E1-like) and Atg3 (E2-like), resulting in autophagosome-associated LC3-II. The Atg5–Atg12 complex may be able to enhance LC3 conjugation to phosphatidylethanolamine by acting in an E3-like fashion. In this way, the Atg5–Atg12–Atg16L complex may determine the sites of autophagosome synthesis by regulating the targeting of LC3 to Atg5–Atg12-associated membranes.

Although the Atg5–Atg12–Atg16L1 complex localises to phagophores and pre-phagophore structures and dissociates from fully formed (completed) autophagosomes, LC3-II remains associated with autophagosomes until after their fusion with lysosomes. The LC3-II inside the autolysosomes is degraded, while the LC3-II on the cytoplasmic

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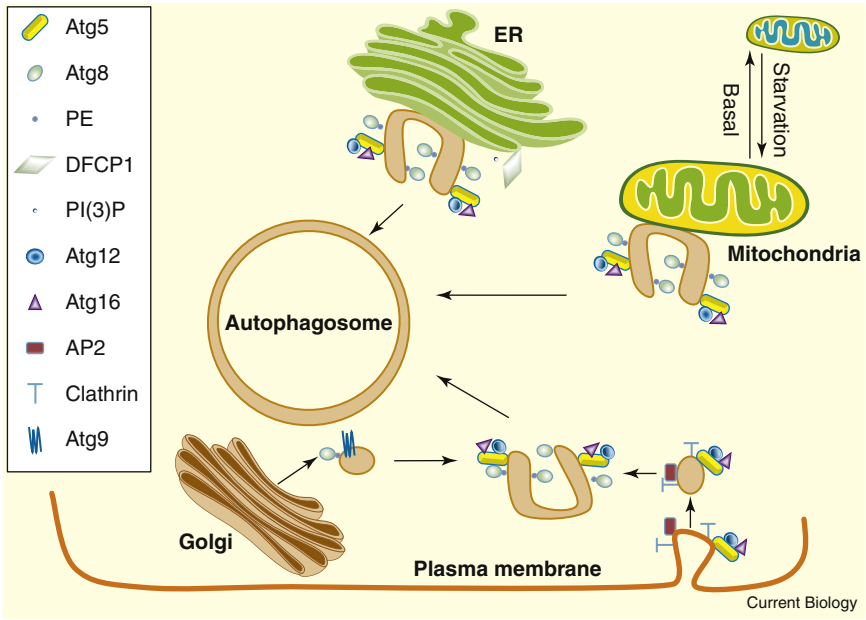

Figure 1. Membrane sources for phagophores.

Phagophores require lipids and proteins to mature into autophagosomes. The endoplasmic reticulum is the site of formation of omegasomes, which are essential for phagophore formation and elongation. Mitochondria grow upon starvation and supply lipid vesicles to the phagophore and the Golgi is essential for the trafficking of Atg9-containing vesicles to the phagophore. The plasma membrane contributes membranes to phagophores and autophagosomes under both basal and starvation conditions.

surface can be delipidated and recycled. Thus, Atg5–Atg12–Atg16L1-positive LC3-negative vesicles represent pre-autophagosomal structures (pre-phagophores and possibly early phagophores), Atg5–Atg12–Atg16L1-positive LC3-positive structures can be considered to be phagophores, and Atg5–Atg12–Atg16L1-negative LC3-positive vesicles can be regarded as completed autophagosomes [11]. Note that these definitions do not consider intermediate structures and therefore may end up being imprecise as our understanding of the structure, shape and protein composition of autophagy-related membranes evolves. Also, the PAS, at least in mammalian cells, may include some of these distinct structures.

Membrane Sources for Phagophores

For much of the history of the autophagy field, the origins of phagophore membranes have been elusive. However, recent data suggest that membranes from distinct locations may contribute lipid (and possibly key proteins) to evolving autophagosomes (Figure 1).

Endoplasmic Reticulum

The reconsideration of the endoplasmic reticulum (ER) as an autophagosome membrane source was initiated by Ktistakis and colleagues [12], who studied a protein called DFCP1, which has two FYVE domains that account for its ability to bind PI(3)P. These authors were intrigued that DFCP1, unlike many other PI(3)P-binding proteins, localises to the ER and Golgi but not to endosomes. Upon starvation, DFCP1 moved to punctuate structures at the ER in a PI(3)P-dependent fashion, and these structures, which they called ‘omegasomes’, colocalised with LC3 and Atg5 [12].

This study noted that the ER normally contains very little PI(3)P in non-starved cells, and suggested that Vps34 may be delivered to the ER by late endosomes and lysosomes. Subsequent 3D electron tomography studies have revealed interconnections between the ER and forming autophagosomes (Atg16L positive), and have provided further support for a role for the ER by showing that Atg14L targeting to the ER may be important for autophagosome formation [13–15].

However, some questions remain. First, it is not clear whether the ER is an important source of autophagosomal membranes under non-starvation conditions. Second, the topology of the isolation membranes in the 3D electron tomography experiments suggests that there may be ER membrane closely apposed to both sides of the isolation membrane. It would be interesting to understand the next steps of the process that enable the isolation membrane to be freed to engulf cytoplasm and organelles like mitochondria. Alternatively, it is possible that these autophagosomal membranes may show some preference for degradation of ER and its contents, a process known as ER-phagy.

Mitochondria

The Lippincott-Schwartz lab [16] reported that starvation-induced autophagosomes could emerge from sites on mitochondria, via elegant live-cell microscopy studies tracking markers for LC3 and Atg5. Their data suggest that the colocalisation of mitochondria and autophagosomal membranes is not due to mitophagy (autophagy of mitochondria) and that lipids are delivered from mitochondria to autophagosomes that form close to the mitochondria. Importantly, these phenomena appeared to be seen only in glucose-starved cells and were not associated with either basal autophagy or ER-stress-induced autophagy. The idea that mitochondria may be a membrane source for autophagosomes specifically during starvation is interesting to consider in the light of recent data showing that mitochondria are protected from autophagic degradation during starvation because starvation signals cause these organelles to elongate and become refractory to autophagic engulfment [17].

Plasma Membrane

Our own recent data suggest that the plasma membrane contributes to early autophagosomal precursor structures, as we saw the rapid colocalisation of different plasma membrane markers with autophagosomal precursor structures after shifting cells from 4°C to 37°C to enable endocytosis [18]. Importantly, the plasma membrane markers associated with structures that were Atg5–Atg12–Atg16L1-positive but LC3-negative (pre-phagophore structures), structures that contain both Atg5–Atg12–Atg16L1 and LC3 (phagophores), and structures that were LC3-positive but Atg5–Atg12–Atg16L1-negative (completed autophagosomes), suggesting a simple itinerary for plasma membrane from

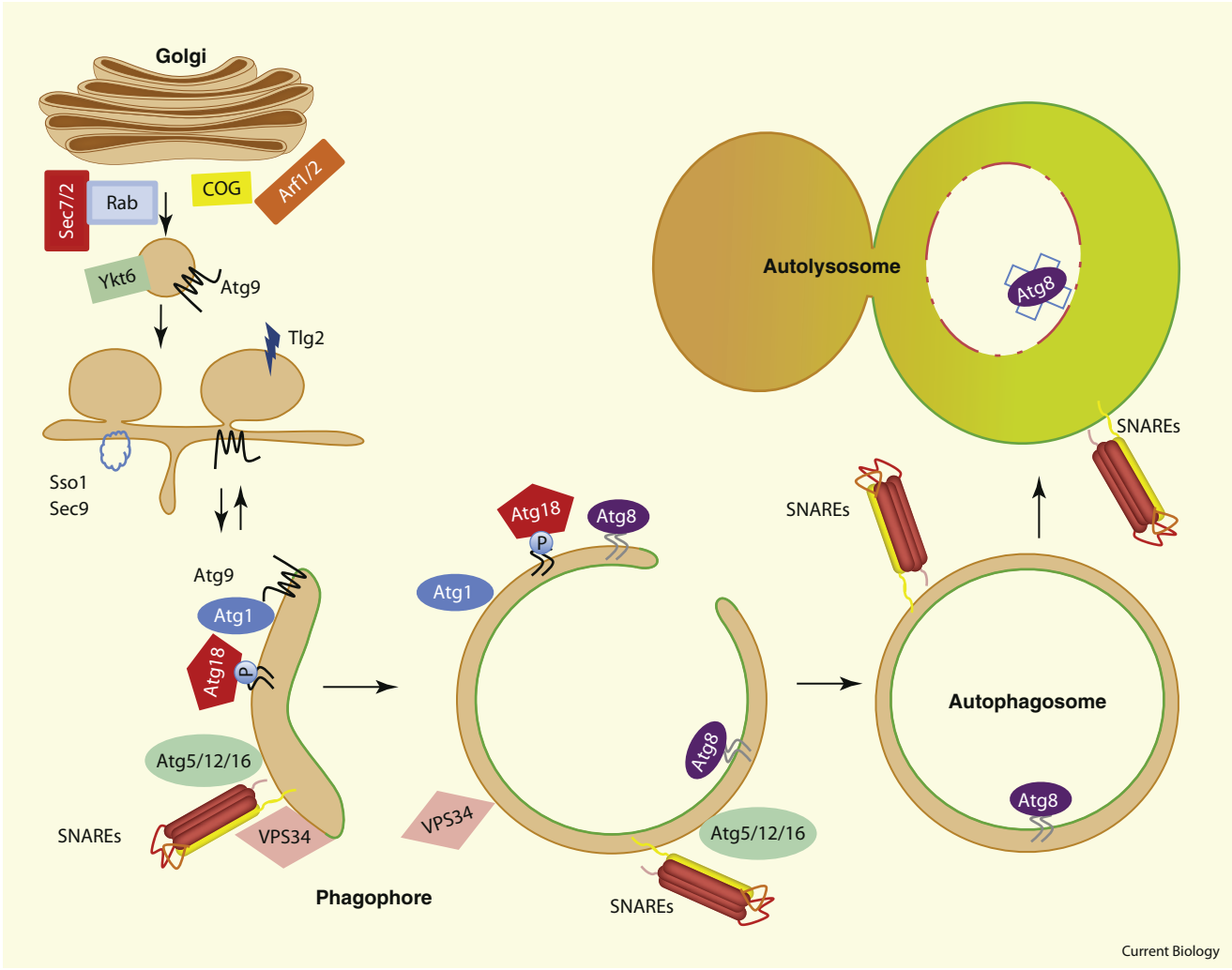

Figure 2. Autophagosome biogenesis in yeast.

Atg9-containing vesicles accumulate in tubulovesicular structures and are delivered to the pre-autophagosomal structure along with SNARE proteins to form the phagophore. Atg8 mediates phagophore elongation, and SNARE proteins mediate the fusion of the autophagosome with the lysosome.

autophagosome precursor to fully-formed autophagosome. The ability of the plasma membrane to contribute to autophagosome formation was associated with the localisation of Atg16L1 at the plasma membrane via Atg16L1–AP-2–clathrin heavy chain interactions. Scission of the Atg16L1-associated clathrin coated pits, leading to the formation of early endosomal-like intermediates, is a crucial step that enables the liberation of these Atg16L1 vesicles and subsequent maturation into autophagosomes. Although they contain Atg16L, Atg5 and Atg12, these autophagosome precursors appear to be membrane structures that precede the phagophore stage [18].

Our data suggest that this process is essential for autophagosome formation under both basal and induced conditions (e.g. starvation), since blocking clathrin-dependent endocytosis attenuated autophagosome biogenesis at the phagophore stage. Although endocytosis appears to be important for autophagosome formation under basal and starvation-induced conditions, it is possible that the ability of the plasma membrane to contribute to autophagosome formation may be particularly important during periods of increased autophagy. At these times, the large surface area of the plasma membrane may serve as an extensive membrane reservoir that allows cells to undergo periods of autophagosome synthesis at much higher rates than under basal conditions, without compromising other processes.

Golgi, Atg9 and Autophagosome Formation in Yeast

Atg9 is the only known multipass-membrane protein that regulates autophagy [19]. Since it cycles between the PAS and different cytoplasmic membranes [20], it has reasonably been assumed to be associated with membrane that contributes to autophagosomes. Understanding its exact intracellular trafficking routes and characterisation of the vehicles that mediate its trafficking is expected to provide valuable information on the cellular mechanism involved in autophagosome biogenesis. Recent studies in yeast suggested that tubulovesicular structures containing Atg9 are delivered to the PAS, a trafficking step essential for autophagosome formation [21]. Klionsky and colleagues [22] identified a set of SNARE molecules that enable the organisation of these tubulovesicular Atg9-containing structures and showed that these SNAREs, in turn, regulated autophagy (Figure 2).

This specific function for SNAREs has not yet been studied in mammalian autophagy.

The cycling of yeast Atg9 not only involves specific autophagic factors, but also implicates more general factors involved in intracellular trafficking, including Sec12 and Vps52 [23], Sec7 and Sec2 (guanine nucleotide exchange factors for Arf and Rab GTPases, respectively), Arf1/2 and the Rab Sec4 [24,25], all of which are essential for autophagosome formation via the regulation of Atg9 cycling. Manipulations of the conserved oligomeric Golgi (COG) complex and genes involved in Golgi–endosomal traffic affect autophagosome formation and this is associated with mislocalisation of the autophagy proteins Atg9 and Atg8 [26]. Thus, it is likely that this Golgi route contributes to autophagosome biogenesis in yeast, although it is not clear if this pathway is important in mammalian systems.

Mammalian Atg9 (mAtg9) was more recently implicated in autophagy. Under normal growth conditions, this transmembrane protein, which is synthesised in the ER, localises to the Golgi, the trans-Golgi network, and late endosomes. Following amino-acid starvation, this protein translocates to LC3-labeled autophagosomes in an ULK1- and PI3K-dependent manner [27]. Cycling of mAtg9 is negatively regulated by p38α MAPK, which competes with mAtg9 for binding to p38IP [28]. In addition, Rab1a and α-synuclein (a protein that causes forms of Parkinson's disease) participate in autophagosome biogenesis, possibly by regulating the localisation of mAtg9 and DFCP1 to omegasomes [29].

When considering the possibility that Atg9 cycling may deliver membrane needed for autophagosome biogenesis, it is important to take into account the fact that Atg9 shuttles back and forth from various cytoplasmic membranes into the pre-autophagosomal membrane (possibly both to phagophores and pre-phagophores). To allow the formation and elongation of the phagophore, the vesicles reaching this membrane should be larger than those carrying Atg9 in the opposite direction. Another non-mutually exclusive possibility is that Atg9 delivers a set of lipids into the autophagosome precursors, while removing other lipids when it departs — this would not require inbound Atg9-containing vesicles to be larger than those returning from the autophagosome precursors. It is also possible that the Atg9-associated membranes are not important for autophagosome formation; perhaps Atg9 has transient structural or catalytic functions at the PAS (or other sites) that regulate autophagy. These are some of the issues that await resolution through further study.

Transition from Autophagosome Precursor to Phagophore

The data described above reveal that there are autophagosome precursor structures that precede phagophores. It is possible that such structures from various sources coalesce prior to phagophore formation or meet at the phagophore itself, thereby increasing the size of the membranes at the phagophores. In mammalian systems, we have observed that the pre-phagophore Atg5–Atg12–Atg16L1-positive (and LC3-negative) precursors undergo SNARE-mediated homotypic fusion [30]. This process is important for autophagosome formation and autophagy flux, since the homotypic fusion results in vesicles that are larger. Our data suggest that the increase in vesicle size may be a prerequisite for optimal acquisition of LC3 and progression from the autophagosome precursor stage to a nascent phagophore (Figure 3).

Transition from Phagophore to Autophagosomes — Roles of Atg8 Proteins

In order for a phagophore to seal its edges and become an autophagosome the membranes probably require elongation and then they need to fuse. In yeast, lipidated Atg8 was found to be essential for elongation of the autophagic membrane [31,32]. This role of Atg8 was further assessed in an *in vitro* liposome-based system, where it was shown to promote membrane fusion [33,34]. Importantly, mutations within Atg8 proteins identified in these systems were found to cause defective autophagosome biogenesis in *Saccharomyces cerevisiae*. However, more direct experiments are needed to determine the mechanism by which Atg8 promotes membrane fusion, including the fusion of Atg9-containing vesicles with the phagophore membrane. For Atg8 (as well as other Atg proteins), it is important to consider other roles in autophagy and cell biology. For instance, Atg8 also binds to other autophagic factors, such as Atg1, which might regulate its recruitment and activity on the autophagic membrane [35]. Other more general trafficking factors interact with Atg8, including the AAA ATPase Cdc48/p97 and its substrate-recruiting cofactor Shp1. Although these interactions were suggested to control the formation of autophagosomes [36], they may also enable Atg8 to link classical autophagic machinery with general trafficking factors that are crucial for autophagy.

In contrast to yeast, where there is only one Atg8, there are multiple different mammalian Atg8 proteins found on autophagosomes [37–39], all of which undergo post-translational modifications mediated by mammalian orthologs of Atg4, Atg7 and Atg3 [40]. Recently, it has emerged that these are not simply redundant members of a protein family. Our studies indicate that the LC3 and the GABARAP/GATE-16 subfamilies are essential for autophagy and act at different stages of autophagosome formation: members of the LC3 subfamily are responsible for the elongation of the autophagic membrane, whereas GABARAP/GATE-16 family members act downstream in a step coupled to dissociation of the Atg12–Atg5–Atg16L complex [41]. Both mammalian LC3 and GABARAP/GATE-16 subfamilies assist with membrane fusion after conjugation to phosphatidylethanolamine [34]. This study revealed that the first amino-terminal α helix of LC3 and GABARAP/GATE-16 proteins was both essential and sufficient for this activity.

Synthesis, Challenges and Future Questions

Our belief (which will need to be tested) is that there are multiple membrane sources for autophagosomes. Indeed, this may explain why LC3-positive autophagosomes appear to be formed '*de novo*' and do not have the same protein compositions as other intracellular membranes, such as the ER. It will be interesting to consider whether different membrane sources have specific contributions in response to different autophagy-inducing stimuli, if and how the different membrane sources may act cooperatively, or whether these sources may have different contributions to specialised forms of autophagy, including autophagy of mitochondria (mitophagy), ER-phagy, and autophagy of bacteria.

Future studies should ideally strive to test the importance of different membrane sources under different conditions

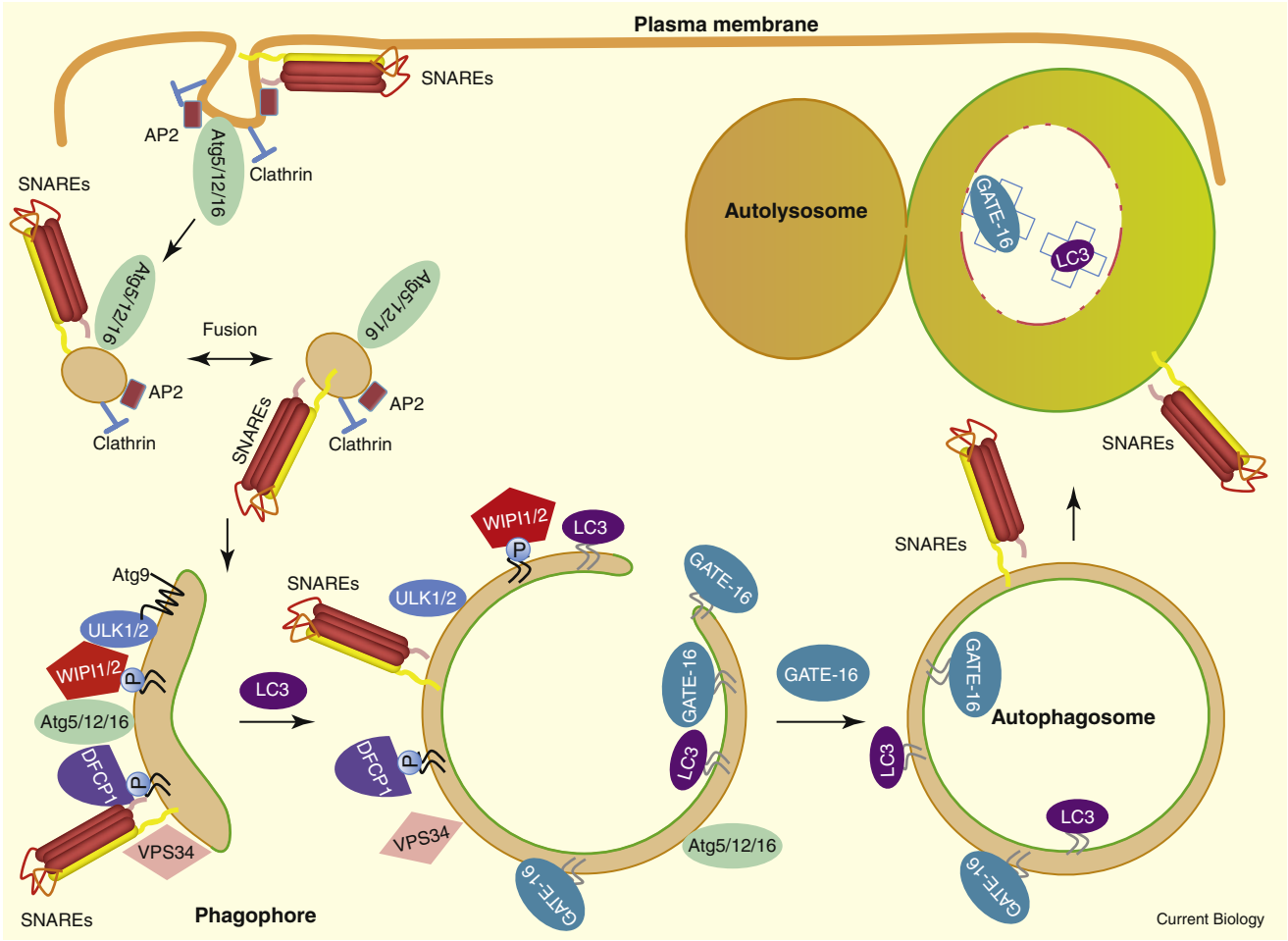

Figure 3. Autophagosome biogenesis in mammals, focusing on the plasma membrane as an origin of autophagosome membranes.

Atg16L1-containing vesicles derived from the plasma membrane undergo homotypic fusion, an essential step for phagophore formation. Phagophore elongation is mediated by LC3, and GATE-16 acts downstream in a step coupled to the dissociation of the Atg12–Atg5–Atg16L1 complex. Although SNAREs have been shown to be required for homotypic fusion of Atg12–Atg5–Atg16L1-positive phagophore precursors as well as for autophagosome–lysosome fusion, it is also possible that SNAREs may act at other points of the pathway.

and in different cell types. While it is easier to investigate the role of the plasma membrane than other sources by blocking endocytosis, we still need to try to rigorously test all pathways to exclude alternative possibilities, including the possibility that pre-phagophores/phagophores/autophagosomes may 'bump into' other compartments and exchange membrane in a manner that is neither productive nor deleterious, or that autophagosomes/autophagosome precursors may be involved in membrane repair of other organelles.

It is thought that autophagosomes are formed by the delivery of vesicles from different sources leading to the formation of phagophores varying in size between 300 and 900 nm that ultimately are sealed to form double-membraned vesicles. The outer membrane then fuses with the lysosomal membrane, whereas the inner vesicle containing the sequestered cargo is degraded within the lysosomal lumen. Importantly, the two membranes are expected to be asymmetric: the outer membrane is similar in its protein and lipid content to the lysosomal limiting membrane, while the inner membrane contains a small amount of membrane proteins and a lipid composition that renders it amenable to rapid lysosomal degradation. The mechanism responsible for the formation of such asymmetry is poorly understood. Such differential lipid compositions may help explain how phagophore membrane curvature is initiated (given that this is a double membrane). However, at the moment we cannot exclude the possibility that the membrane curvature may be initiated spontaneously and that this serves as a mechanism to enable subsequent inner versus outer membrane differentiation.

Finally, another key issue is to understand how yeast autophagy processes differ from those seen in mammalian cells. Despite the high degree of conservation between mammalian and yeast Atg proteins and conservation of key aspects of the core processes, certain important differences between yeast and mammalian systems are already known, including the fact that yeast has one PAS and one Atg8 whereas there are multiple PAS and a family of Atg8 proteins in mammalian cells. Thus, it would not be too surprising if there were differences in other aspects of the autophagy processes between yeast and mammalian systems. Likewise, various aspects of mammalian autophagy may even vary in different human cell types.

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