Reconstruction of destruction – in vitro reconstitution methods in autophagy research

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Summary Statement

Autophagy is a versatile recycling system that ensures turnover of cytoplasmic material. This review highlights *in vitro* reconstitution studies that helped to discover fundamental mechanisms of the pathway.

Abstract

Autophagy is one of the most elaborative membrane remodeling systems in eukaryotic cells. Its major function is to recycle cytoplasmic material by delivering it to lysosomes for degradation. To achieve this, a membrane cisterna is formed that gradually captures cargo such as organelles or protein aggregates. The diversity of cargo requires autophagy to be highly versatile to adapt the shape of the phagophore to its substrate. Upon closure of the phagophore, a double-membrane surrounded autophagosome is formed that eventually fuses with lysosomes. In response to environmental cues such as cytotoxicity or starvation, bulk cytoplasm can be captured and delivered to lysosomes. Autophagy thus supports cellular survival under adverse conditions. During the past decades, groundbreaking genetic and cell biological studies identified the core machinery involved in the process. In this review, we are focusing on *in vitro* reconstitution approaches to decipher the details and spatiotemporal control of autophagy and how they contributed to our current understanding of the pathways in yeast and mammals. We highlight studies that revealed the function of the autophagy machinery at a molecular level with respect to its capacity to remodel membranes.

Introduction

Eukaryotic cells have evolved a highly conserved and sophisticated degradation and recycling system for damaged or superfluous organelles, termed macroautophagy (autophagy in the following) (Dikic and Elazar, 2018). This system requires cargo selection in a spatiotemporally defined and regulated fashion (Khaminets et al., 2016) and the degradation of cargo with very different sizes and shapes, demanding versatility. The complexity of these requirements is reflected in the complexity of autophagy, which involves the coordinated action of more than 40 dedicated autophagy-related (Atg) proteins (Mercer et al., 2018) that collaborate and are intertwined with a large number of cellular signaling, trafficking, and stress response pathways (Davis et al., 2017).

Autophagy was first characterized at a morphological and ultrastructural level as a pathway that delivers cytoplasm to lysosomes (Ashford and Porter, 1962; De Duve, 1963) and seminal work identified a set of Atg proteins using a genetic screen in yeast (Tsukada and Ohsumi, 1993), followed by related screens that expanded the repertoire of Atg proteins (Harding et al., 1995; Thumm et al., 1994). The identification of human homologs of yeast Atg proteins established that autophagy is conserved in higher eukaryotes (Kabeya et al., 2000; Mizushima et al., 1998). Furthermore, autophagy is involved in the onset of various human diseases such as cancer and neurodegeneration that revealed the importance of the pathway in maintaining cellular homeostasis under normal conditions and in response to cytotoxic stress (Liang et al., 1999; Ravikumar et al., 2002).

Although yeast and human autophagy pathways are conserved, there are significant mechanistic differences and the human autophagic machinery is much more elaborated compared to its yeast counterpart. In yeast, autophagy is initiated at the phagophore assembly site (PAS) at the vacuole and involves activation, assembly and recruitment of the Atg1-kinase complex (**Fig. 1A**) (Cheong et al., 2005; Kabeya et al., 2005; Kabeya et al., 2009). Following its activation, the Atg1-kinase complex recruits Atg9 vesicles to the PAS (**Fig. 1A**) (Mari et al., 2010; Rao et al., 2016; Sekito et al., 2009). These vesicles fuse to give rise to a precursor membrane, termed phagophore (Nair et al., 2011; Yamamoto et al., 2012). The expansion of these membrane cisternae allows cytoplasm to be sequestered and enclosed. The high demand of lipids that are needed for phagophore expansion requires a constant supply with membranes of various origins including the ER, the Golgi, the ER-Golgi intermediate compartment and mitochondria (Graef et al., 2013; Meiling-

Wesse et al., 2005; Ohashi and Munro, 2010; Reggiori et al., 2005). Moreover, the early phagophore is enriched in phosphatidylinositol(3)phosphate (PtdIns(3)P) through the action of an autophagy-specific class III PtdIns(3)-kinase complex (**Fig. 1B**). The recruitment of this lipid-kinase to the PAS is mediated by an interaction of Atg13 with the PtdIns(3)-kinase subunit Atg14 (Jao et al., 2013; Suzuki et al., 2007).

Enrichment of the phagophore with PtdIns(3)P allows PtdIns(3)P-binding proteins such as the yeast β-propellers that bind phosphoinositides (PROPPIN)-repeat containing Atg18 and Atg21 to be recruited (Nascimbeni et al., 2017). Atg21 initiates the transition from phagophore initiation to phagophore expansion by recruiting Atg16 (Juris et al., 2015), whereas Atg18, in conjugation with its interaction partner Atg2, stimulates phagophore expansion by establishing ER-phagophore contact sites (Gómez-Sánchez et al., 2018). The expansion of the phagophore membrane is regulated by a ubiquitin (Ub)-like conjugation machinery that catalyzes the covalent attachment of the Ub-like protein Atg8 to phosphatidylethanolamine (PE) within the phagophore (**Fig. 1B**) (Ichimura et al., 2000). This reaction is initiated by an Atg4-dependent priming of Atg8 through proteolytic cleavage of its C-terminal residue (Kirisako et al., 2000). The conjugation of Atg8 itself involves sequential activity of the E1-like Atg7 and the E2-like Atg3 enzymes as well as the E3-like ligase complex Atg12–Atg5-Atg16 (Geng and Klionsky, 2008). Apart from its function during phagophore expansion, Atg8 also coordinates selection of cargo through its interaction with cargo receptors such as Atg19 or Atg32 (**Fig. 1C**). Moreover, Atg8 promotes sealing of phagophores and fusion with the yeast vacuole (**Fig. 1D, E**) (Abeliovich et al., 2000; Kaufmann et al., 2014; Kondo-Okamoto et al., 2012; Nakatogawa et al., 2007; Noda et al., 2010; Suzuki et al., 2010; Xie et al., 2008). Shortly before fusion of the two organelles occur, the cytosolic accessible pool of Atg8 is cleaved from the membrane by Atg4 (**Fig. 1D**) (Abreu et al., 2017; Kirisako et al., 2000; Yu et al., 2012). This 'uncoating reaction' is followed by tethering of autophagosomes to the vacuole through the homotypic fusion and vacuole protein sorting (HOPS) complex and SNARE-mediated fusion of the outer autophagosomal and the vacuolar membranes (**Fig. 1E**) (Nair et al., 2011; Rieder and Emr, 1997). Although autophagy is well understood at a molecular level in yeast, mechanistic insights into the process as well as its spatiotemporal regulation are still limited. The core autophagy machinery is highly conserved and close homologs of the yeast proteins are expressed in human cells (Yang et al., 2017) (see **Box 1**). Significant mechanistical differences between the pathways in these species reflect regulatory requirements that are needed for

cooperation of cells in multicellular organisms (Boya et al., 2018) as well as autophagy-unrelated functions of ATG proteins in higher eukaryotes (Schaaf et al., 2016).

Many insights into the nucleation of autophagosomes, the selection of cargo and its tethering to autophagic membranes as well as autophagosome completion and fusion with lysosomes have been revealed by *in vitro* reconstitution methods. The advantages of *in vitro* approaches in characterizing complex biological pathways were first recognized by Günter Blobel, who developed a cell-free system to recapitulate the translocation of proteins across the ER membrane (Blobel and Sabatini, 1971). A similar approach was used in seminal studies by Randy Schekman, which led to the identification of components of the secretory pathway (Rexach and Schekman, 1991). Advances in membrane physics and recombinant protein production made it possible to reconstitute entire biological pathways from purified components in the test tube. Here, we highlight such reconstitution approaches and summarize how they contributed to our current understanding of autophagy in yeast and humans.

Out of nowhere - nucleation of the phagophore in yeast and humans

The coordinated activity of the Atg1 (Ulk1)-kinase complex, the PtdIns(3)-kinase complex and the Atg9 compartment is required to initiate autophagy in yeast and humans. The two subunits Atg17 and Atg11 are major scaffolds for the assembly of two distinct Atg1-kinase complexes that function in selective (Atg11) or non-selective (Atg17) autophagy in yeast (Kabeya et al., 2005; Kabeya et al., 2009; Kamber et al., 2015; Yorimitsu and Klionsky, 2005). Moreover, both proteins recruit the transmembrane protein Atg9 to the PAS, linking autophagy initiation to nucleation of the phagophore (He et al., 2006; Sekito et al., 2009). First insights into membrane-binding properties of the Atg1-kinase complex came from *in vitro* reconstitutions of a minimal complex, comprising Atg17, Atg31, Atg29 as well as fragments of Atg1 and Atg13 (Ragusa et al., 2012). Using Folch liposomes with different diameters as membrane substrate (see **Box 2**), the C-terminal domain of Atg1 was found to bind membranes in a curvature-dependent manner, preferring liposomes with high curvature that corresponds to vesicle diameters of ~30 nm (Ragusa et al., 2012). Atg9 vesicles that are recruited by the Atg1-kinase complex *in vivo* possess similar diameters, suggesting that curvature sensing by Atg1 contributes to the selective recruitment of these vesicles.

Furthermore, mutational analysis and immunoprecipitation analysis suggested that both Atg17 and Atg13 bind Atg9 (Sekito et al., 2009; Suzuki et al., 2015), although which of the two proteins directly interacted with Atg9 was unclear. It was addressed by *in vitro* reconstitutions using artificial Atg9 vesicles and purified subunits of the Atg1-kinase complex: Atg9 vesicles were generated by incorporating recombinant and purified $Atg9_{core}$, which comprised the highly conserved transmembrane region of Atg9 but lacked the unstructured N- and C-termini, into small unilamellar vesicles (SUVs) (see **Box 2**). Floatation experiments of these vesicles with purified Atg1-kinase complex subunits (see **Box 3**) revealed that Atg17 physically interacts with Atg9, suggesting that binding of Atg9 by the Atg1-kinase complex mainly depends on Atg17 (Rao et al., 2016). Furthermore, the crystal structure of the Atg17-Atg31-Atg29 subcomplex revealed two interesting properties: first, Atg17 is a highly elongated crescent-shaped molecule that forms tail-to-tail dimers; second, Atg31 directly binds Atg17 and both Atg31 and Atg29 occupy the central region of the Atg17 crescent (Ragusa et al., 2012). A plausible explanation for this unusual architecture was, again, revealed by reconstitution approaches (Rao et al., 2016). The Atg17 dimer was found to bind two Atg9 vesicles independently and electron microscopy revealed that these vesicles were tightly tethered to each other (**Fig. 1A**). The Atg17-Atg31-Atg29 subcomplex was, however, not able to tether such vesicles, although the subcomplex retained a limited capacity to bind Atg9. This suggests that the prominent localization of Atg31 and Atg29 that has been observed in the crystal structure inhibits binding of Atg17 to Atg9.

Upon induction of autophagy, the pentameric Atg1-kinase complex assembles at the PAS *in vivo* (Kabeya et al., 2005). In agreement with this observation, interaction studies with purified components revealed that this complex binds Atg9 vesicles with a 2:2 stoichiometry - as it has been observed for Atg17 - and tethers Atg9 vesicles *in vitro* (**Fig. 1A**) (Rao et al., 2016). The combination of *in vivo* experiments, crystal structures and *in vitro* reconstitution of the Atg1-kinase complex thus provided a detailed model for its function during autophagy initiation. However, the high demand of lipids for nucleation and expansion of autophagosomes cannot be fulfilled by just two Atg9 vesicles, suggesting early PAS organization by more than one Atg1-kinase complex and its multiple tethered Atg9 vesicles. A combinatorial *in vitro* and *in vivo* approach revealed that Atg13 has two Atg17 binding sites. One of these sites activates Atg17-Atg31-Atg29 within the same complex whereas the other binding site links two Atg1-kinase complexes together by binding Atg17 of another complex (Yamamoto et al., 2016). This crosslinking allows the formation of

larger super-molecular assemblies and provides thus the means to recruit and tether multiple Atg9 vesicles in order to nucleate the phagophore. This model is supported by fluorescence-based quantifications *in vivo*, which demonstrated that twenty to sixty Atg1-kinase complexes are recruited to the PAS upon induction of autophagy (Geng et al., 2008; Köfinger et al., 2015; Lin et al., 2018).

The inhibition of Atg17 by Atg31-Atg29 limits its function to conditions under which autophagy is induced. However, selective cargo such as the vacuolar aminopeptidase 1 (Ape1) is delivered to the vacuole even under non-induced conditions and independently of Atg17 (Shintani et al., 2002). The initiation complex in selective autophagy consists of Atg1, Atg13 and the scaffolding protein Atg11 (Kim et al., 2001). *In vitro* reconstitution experiments using Atg11 that was purified from yeast cell lysates indicated that the major function of Atg11 involves activation of Atg1. Using an engineered Atg1-kinase that metabolized an ATPγS analog instead of conventional ATP, this study revealed that the activation of Atg1 depends not only on Atg11, but requires the formation of a complex of Atg1 with cargo, cargo receptor, and Atg11 (Kamber et al., 2015). However, the kinase activity of Atg1 was previously found to be important for autophagy expansion, but dispensible for the nucleation of autophagosomes (Cheong and Klionsky, 2008). This suggests that the Atg1-Atg11 complex has two functions in autophagy: promoting autophagy initiation and regulating phagophore expansion. The function of Atg11 during nucleation of selective autophagosomes might depend on properties that are similar to those of Atg17, including the recruitment of Atg9 vesicles to the PAS (Backues and Klionsky, 2012).

A lipid diet for autophagy - expansion of the phagophore membrane

A key step during the phagophore initiation to expansion transition is the acquisition of Atg8/LC3, a process that involves recruitment of the conjugation machinery to the phagophore. Key factors in this are the two PtdIns(3)P-binding proteins WIPI2 (human) and Atg21 (yeast) that recruit ATG16L1/Atg16 to the phagophore (**Fig. 2A**). Both proteins thus promote expansion by coupling Atg8/LC3 lipidation to PtdIns(3)P-containing phagophores (Dooley et al., 2014; Juris et al., 2015). In order to identify the membrane source that becomes substrate for ATG8 conjugation, a cell-free reconstitution approach combined cytosol fractions of wildtype cells with membrane fractionation from ATG5-knockout cell lysates (Ge et al., 2013). Due to the absence of ATG5, these membranes

were devoid of LC3-PE. Combining them with wildtype cytosol complemented ATG5 and initiated lipidation of LC3. The fraction that was enriched in membranes from the ER-Golgi intermediate compartment was found to be the best substrate for LC3-lipidation, implicating that this compartment donates membrane for autophagosome biogenesis (Ge et al., 2013). By applying a similar approach, coat protein complex II (COPII) has been found to bud vesicles from the ER-Golgi intermediate compartment upon autophagy induction, suggesting that such vesicles contribute to the formation of autophagosomes (Ge et al., 2014). Furthermore, several other studies reported that COPII is required for initiation and expansion of autophagosomes (**Fig. 2B**) (Ge et al., 2017; Graef et al., 2013; Stadel et al., 2015). Whether COPII vesicles transport membranes from the ER to autophagosomes remains, however, to be investigated.

The most likely region of the phagophore to which COPII and other donor vesicles might fuse is its highly bent membrane at the phagophore rim (Nguyen et al., 2017). This suggests that factors that are involved in this process are targeted to this membrane region by sensing membrane curvature (**Fig. 2B,D**). The localization of Atg proteins at the phagophore in yeast was analyzed by inducing the assembly of a giant ApeI substrate through overexpression of ApeI. Under these conditions, a large spherical ApeI structure is formed in the cytoplasm at which the phagophore membrane could be visualized. It was shown that subunits of the Atg1-kinase and PtdIns(3)-kinase complexes as well as Atg9 colocalize with the edges of the phagophore, whereas Atg1 itself and the Ub-like conjugation system cover the entire membrane (Suzuki et al., 2013). Thus, autophagy initiation complexes are found exclusively at the edges of phagophores, whereas the phagophore expansion machinery is more or less evenly distributed on yeast phagophores. The spatially restricted localization of Atg17 and Atg9 implies that Atg17 tethers Atg9 vesicles not only to each other upon autophagy induction, but also to the phagophore rim during expansion (**Fig. 2B**).

The localization of the PtdIns(3)-kinase complex to the rim of phagophores can be explained by curvature sensing motifs in VPS34 and ATG14 that have been identified *in vitro* using large unilamellar vesicles (LUVs) with different sizes (Fan et al., 2011; Miller et al., 2010). The curvature sensing property of ATG14 was found to be counteracted by increasing concentrations of PtdInsPs. This suggests that during autophagy initiation, a positive feedback loop enriches ATG14 at the PtdIns(3)P-containing omegasome, whereas during phagophore expansion, incoming PtdIns from donor vesicles are phosphorylated in a concerted manner at the phagophore rim (Fan et al., 2011) **(Fig. 2C)**.

Interestingly, membrane curvature sensing is not only restricted to components of the initiation machinery: an *in vitro* reconstitution of the human Ub-conjugation machinery identified ATG3 as a curvature sensor. As shown for ATG14, ATG3 contains an amphipathic lipid packing sensor (ALPS) motif which forms, upon contact with lipid bilayers, an amphipathic helix that partially inserts into membranes (Nath et al., 2014). The penetration of this helix is promoted by lipid packaging defects in the bilayer of highly curved membranes, explaining why such motifs are curvature sensors (Antonny, 2011; Bigay et al., 2005) **(Fig. 2D)**. The localization of ATG3 at the phagophore rim spatially restricts conjugation of ATG8, which allows incoming membranes during expansion to be decorated with ATG8 in a spatiotemporally concerted manner. Moreover, ATG8 is found on both membranes of the phagophore but the high curvature of its rim forms an effective diffusion barrier for lipids and thus for lipidated ATG8s as well (Domanov et al., 2011). Targeting ATG3 to the rim permits ATG8s to reach both faces of the phagophore after conjugation (**Fig. 2D**).

In contrast to these observations *in vitro*, yeast Atg3 has been found to be evenly distributed at the entire area of the phagophore *in vivo* (Ngu et al., 2015). This indicates that curvature sensing by ATG3 can be overruled by other factors such as the ATG12–ATG5-ATG16L1 complex, which is known to recruit ATG3 *in vivo* (Hanada et al., 2007). ATG16L1 also covers the surface of phagophores, but its localization is confined to the outer phagophore membrane (Mizushima et al., 2003). The restriction of ATG16L1 to one face of the phagophore might be a consequence of the asymmetric distribution of PtdIns(3)P in human cells (**Fig. 2C**). PtdIns(3)P is enriched in the outer membrane and bound by WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) (Cheng et al., 2014), which also physically interacts with ATG16L1 (Dooley et al., 2014). How the asymmetric distribution of PtdIns(3)P is achieved and maintained remains to be shown. Moreover, the functional consequence of the asymmetry is currently unclear since in yeast, PtdIns(3)P is enriched at the inner and not at the outer membrane (Cheng et al., 2014).

The components and activity of the autophagy-specific conjugation system were revealed by landmark *in vitro* reconstitutions from the laboratory of Yoshinori Ōsumi, using purified proteins to recapitulate the enzymatic conjugation of Atg12 to Atg5 by Atg7 and Atg10 (Mizushima et al., 1998). Subsequently, a second conjugation system was identified that targets Atg8 to membranes through its conjugation to PE (Kirisako et al., 2000). Using LUVs as model membranes, this conjugation could be recapitulated with purified Atg8, Atg7, and Atg3 (Ichimura et al., 2004) and

a functional connection of both conjugation reactions was revealed by the observation that Atg12–Atg5 promotes lipidation of Atg8 *in vivo*. Furthermore, reconstitutions of Atg8-lipidation in the presence of Atg12–Atg5 showed that the latter functions as E3-ligase and promotes the transfer of Atg8 from its E2-like enzyme Atg3 to PE (Hanada et al., 2007).

The observation that - at least in human cells - the ATG12–ATG5-ATG16L1 complex localizes at the outer membrane of the expanding phagophore raises the question why the E3-ligase complex is retained on the phagophore after conjugation of ATG8 took place. This was addressed by a reconstitution of the entire yeast conjugation machinery on model membranes (Kaufmann et al., 2014). Atomic force microscopy of supported lipid bilayers (SLBs) (**see Box 2**) to which Atg8 was conjugated in the absence of Atg16 revealed that Atg8 associates together with the Atg12–Atg5 conjugate into uniform particles composed of at least two subunits each. In the presence of Atg16, however, Atg8-Atg12–Atg5 became immobile and, instead of single particles, a continuous 12 nm thin membrane coat was detected on SLBs. The meshwork-like appearance of the coat was caused by the 16 nm long coiled-coil domain of Atg16 that spatially separated two Atg8-Atg12–Atg5 complexes (**Fig. 1C**). An Atg16 mutant, which could not self-assemble into tail-to-tail dimers caused a remarkable autophagy defect: cells that expressed this mutant produced large phagophore structures with unusual long persistence in the cytoplasm. Moreover, these phagophores did not transport autophagic cargo to the vacuole, implicating that the formed membrane structures correspond to immature and non-productive autophagosomes (Kaufmann et al., 2014). *In vivo* data from human cells supported this model by showing that deletion of all human ATG8 homologs led to a maturation and closure defect of autophagosomes, albeit autophagy progressed at a reduced level (Nguyen et al., 2016; Tsuboyama et al., 2016).

Taken together, the combination of *in vitro* reconstitutions with *in vivo* experiments revealed that the Atg8 conjugation system is an important regulator of phagophore expansion, but not essential for autophagy. Which membranes promote expansion in response to different environmental conditions, how their transport is coordinated and how incoming membranes merge with the phagophore remain to be answered.

Fusion or fission? – maturation and sealing of the phagophore

Electron tomography of autophagic structures in human cells showed that the fully expanded phagophore adopts a spherical shape (Biazik et al., 2017). Sealing of the structure requires that the

membrane rims of the remaining spherical pore come into very close proximity to eventually merge. How this process is coordinated and which proteins promote sealing of the phagophore remained unknown. In principle, the process should be similar to the fusion of two small vesicles of high membrane curvature or to the scission of a membrane neck that projects away from the cytoplasm into the lumen of organelles or the extracellular space. Research over the past decades tried to clarify which of the two diametrically opposed mechanisms serve this purpose.

Initial efforts focused on the fusion model in yeast and human cells. Many fusion reactions in cells are carried out by SNARE proteins, which merge membranes of vesicles with those of organelles or the plasma membrane. To overcome the high energy barrier for membrane fusion, SNAREs from two opposite membranes form a four-strand helical bundle that brings the opposite membranes in such close proximity that fusion takes place (Poirier et al., 1998). However, a recent study reported that although priming of SNARE proteins is required for maturation, they are not involved in the formation of autophagosomes (Abada et al., 2017; Reggiori et al., 2004).

The lack of experimental evidence for a SNARE-mediated sealing mechanism has shifted the focus towards Atg proteins with potentially fusogenic properties. First indications for the involvement of yeast Atg8 in this process came from reconstitutions of the lipidation reaction on LUVs. Conjugation of Atg8 to LUVs resulted in massive clustering of the vesicles, which was macroscopically visible by an increase in the turbidity of the suspension (Nakatogawa et al., 2007). Further characterization of this process revealed that Atg8 molecules on opposite membranes interact with each other to promote tethering. Atg8-mediated fusion of theses membranes was, however, only observed if membranes with unphysiologically high levels of unsaturated phospholipids were used (Nair et al., 2011). This does not necessarily exclude the participation of Atg8 during phagophore closure since packaging defects of lipids lead to a destabilization of the membrane at the phagophore rim. However, additional experiments are needed to confirm the contribution of Atg8 in sealing of phagophores *in vivo* (Nair et al., 2011). Reconstitution reactions of human ATG8 homologs on model membranes revealed another potential mechanism by which ATG8 can promote fusion. The two ATG8 family members light chain 3B (LC3B) and gamma-aminobutyric acid receptor-associated protein L2 (GABARAPL2) possess highly basic N-terminal helices that are required and sufficient to tether and fuse membranes *in vitro* by binding negative charges of opposing phospholipid bilayers (Weidberg et al., 2011). Since lipidation of ATG8 might be restricted to the phagophore rim (Nath et al., 2014), these ATG8 proteins would be positioned

at the right place to promote fusion (**Fig. 2D**). However, their small size would allow them to bridge only distances that are smaller than ~5 nm. Spontaneous membrane fusion occurs already if membranes are as close as 2 nm (Kozlovsky and Kozlov, 2003), suggesting that ATG8 proteins can only catalyze the very last step of sealing at which the remaining phagophore-pore is sufficiently constricted. Furthermore, other proteins such as Atg16 have been found to tether membranes in *in vitro* reconstituted systems. As for ATG8s, tethering of model membranes was based on interactions of positively charged amino acids with acidic phospholipids (Romanov et al., 2012). Further studies thus need to clarify how these different tethering events contribute to autophagosome formation.

Recent progress in identifying biophysical principles of membrane scission reactions indicates that sealing of the autophagosome does not necessarily require fusion, but scission of membranes (Knorr et al., 2015; Renard et al., 2018). Although intuitively less obvious, the topology of the phagophore pore is very similar to that of a membrane neck in membrane abscission processes during multivesicular body biogenesis or viral budding. Both scission reactions are carried out by the endosomal sorting complex required for transport (ESCRT) complex III (Wenzel et al., 2018; Wollert et al., 2009). *In vitro* reconstitutions of this process on giant unilamellar vesicles (GUVs) (**see Box 2**) using purified ESCRT components demonstrated that the correct membrane topology is required and sufficient for cutting of the membrane by ESCRT-III (Wollert and Hurley, 2010). *In vivo,* ESCRT-III appears to cooperate with the AAA-ATPase VPS4 in order to sever not only membrane necks in multivesicular body biogenesis, but also during nuclear envelope assembly, midbody abscission during cell division, as well as for repair of damaged plasma or lysosomal membranes (Adell et al., 2017; Christ et al., 2017; Mierzwa et al., 2017). Owing to the diversity of cellular processes that rely on ESCRT-mediated membrane scission and that share a similar topology of the membrane neck that needs to be cut, a potential role of ESCRTs in autophagy was proposed. However, convincing experimental evidence was missing until a recent study reported that the ESCRT-III subunit charged multivesicular body protein 2A (CHMP2A) is required for sealing of the phagophore membrane (Takahashi et al., 2018). In related reactions, ESCRT-III cooperates with upstream factors to generate membrane necks that are narrow enough to be cleaved by ESCRT-III. This suggests that - as discussed for a potential ATG8-mediated fusion reaction - the phagophore rim needs to be sufficiently constricted to become a substrate for ESCRT-III. Whether phagophore expansion inherently leads to a sufficiently constricted pore remains a

challenging question. Furthermore, a collaboration of Atg8-mediated fusion and ESCRT-III mediated scission mechanisms remain a possibility. Innovative experiments using physiologically relevant templates such as cup-shaped membranes might be able to answer this question in the future.

Born to die – fusion of autophagosomes with lysosomes

The final step in autophagy requires the outer membrane of the autophagosome to fuse with the limiting membrane of the lysosome. Before this, autophagosomes need to be transported to lysosomes and both compartments need to be tethered to each other. Yeast autophagosomes form in immediate vicinity to the vacuole, allowing their completion to be coordinated with fusion independently of microtubule-based transport mechanisms (Kirisako et al., 1999). In human cells, however, autophagosomes are formed at various locations (Jahreiss et al., 2008) and therefore need to be transported to the perinuclear region where lysosomes are located (Korolchuk et al., 2011). This process depends on microtubules, the minus-end directed motor dynein, which mediates retrograde transport of cargo to lysosomes, but also the plus-end directed kinesin motor KIF5B, which is important for autophagosome positioning and fusion with lysosomes (Cardoso et al., 2009; Kimura et al., 2008; Kochl et al., 2006). Advanced *in vitro* approaches were based on fusion of single dynein or kinesin motors to fluorescent beads and their incubation with microtubule-coated coverslips (Svoboda and Block, 1994). Optical traps allowed movement of single motor proteins on microtubules to be recorded and forces in the pico-Newton range to be measured (Simmons, 1996): Dynein, which drives the anterograde transport of autophagosomes, moves with a speed of ~0.7 µm per sec and can exert a force up to 5 pN (King and Schroer, 2000; Toba et al., 2006). Neurons are particularly well-suited to study transport processes *in vivo*. Most autophagosomes are produced at the distal axon of neurons and thus need to be transported to the soma, where lysosomes are located, over distances of 500–1000 µm (Maday and Holzbaur, 2014). Based on the measured velocity of dynein, it takes 12–24 min for an autophagosome to be transported from the distal axon to the soma. Compared to myosin-based transport on actin filaments, dynein-mediated trafficking is rather slow (Schott et al., 2002).

Having reached perinuclear lysosomes, autophagosomes need to be recognized as fusion partners. This requires selective markers on their surface to provide identity and one such marker, the

SNARE protein Syntaxin17 (STX17), is recruited to autophagosomes after their completion (Itakura et al., 2012; Tsuboyama et al., 2016).

The activity of SNARE proteins is tightly regulated; before fusion occurs, organelles need to be brought in close vicinity. This tethering is mediated by the lysosomal HOPS complex, which binds autophagosomes and lysosomes through its interaction with the small GTPases such as ADP-ribosylation factor-like protein 8 (ARL8) and ras-related protein rab-2 (RAB2) in humans and Ypt7 (RAB7 homolog) in yeast (Reggiori and Ungermann, 2017). The molecular architecture of the yeast HOPS complex was revealed by single-particle electron microscopy (Bröcker et al., 2012). The complex spans 30 nm, possesses Rab-binding sites (in Vsp41 and Vps39) on both ends and its headpiece contains the SNARE-interacting subunit Vps33. *In vitro* reconstitution experiments with LUVs revealed that the HOPS complex tethers membranes based on its interaction with Rab GTPases (Ho and Stroupe, 2015), but also through a curvature-dependent direct interaction with membrane vesicles (Ho and Stroupe, 2016). Moreover, the cooperation of HOPS, SNAREs, Ypt7 and the SNARE chaperones Sec17 and Sec18 is required to drive robust membrane fusion (Stroupe et al., 2009). The HOPS complex thus not only tethers autophagosomes to lysosomes, it also promotes SNARE-assembly (**Fig. 3A**). In autophagy, the HOPS complex promotes fusion by cooperating with STX17 (Jiang et al., 2014) as well as with the SNAREs synaptosomal-associated protein 29 (SNAP29) and vesicle-associated membrane protein 8 (VAMP8) (**Fig. 3A**) (Itakura et al., 2012). Mechanistic details on how SNAREs drive membrane fusion have been revealed using SNARE-containing LUVs. Examples include the specificity of SNARE proteins (Paumet et al., 2004), the process of zippering into a four-strand helical bundle (Jakhanwal et al., 2017; Stein et al., 2009), as well as the process of membrane hemi-fusion and fusion (Hernandez et al., 2012).

Even in the presence of tethering factors, the spontaneous assembly of SNAREs is inefficient and controlled by Sec1/Munc18-like (SM)-proteins (Gerber et al., 2008). Insights into the mechanism by which SM-proteins promote assembly of SNAREs came from *in vitro* reconstitutions and structural characterizations: the SM-protein Vps33 helps to assemble the t-SNARE motifs of SNAREs within the target membrane such that they are in register for interaction with the v-SNARE within the vesicle membrane (Baker et al., 2015). A similar assisted assembly of SNAREs was found in autophagy. Unexpectedly, however, ATG14, the component of the PtdIns(3)-kinase complex that initiates autophagy, promotes this step (**Fig. 3A**). The structure of the SNARE-

ATG14 complex revealed that the coiled-coil domain of ATG14 stabilizes the interaction of STX17 and SNAP29 to prime both SNAREs for zippering with the corresponding partner VAMP8 (Baker et al., 2015). Consequently, reconstitutions on LUVs showed that SNARE-mediated membrane fusion was strongly enhanced in the presence of ATG14 (Diao et al., 2015). Taken together, ATG14 appears to have different functions in autophagy: As component of the PtdIns(3)-kinase complex, it promotes induction of autophagy at the ER, whereas its function in autophagosome-lysosome fusion is independent of the other PtdIns(3)-kinase complex subunits **(Fig. 3A)**.

Before fusion of autophagosomes and lysosomes occurs, Atg/ATG proteins - including the cytoplasmic accessible pool of Atg8–PE or its human homologs - are released from the autophagosome. Recycling of Atg8 is achieved through proteolytic cleavage of the amide-bond between the C-terminal glycine of Atg8 and the amine function of PE by Atg4 (Kirisako et al., 2000). The recruitment and activity of Atg4 needs to be tightly regulated to prevent premature cleavage of Atg8 (Abreu et al., 2017). This is achieved by Atg1/Ulk1-mediated phosphorylation of Atg4 or its human homologs at a conserved serine residue near its catalytic site (Pengo et al., 2017; Sanchez-Wandelmer et al., 2017). The importance of this serine residue was confirmed by reconstituting the deconjugation reactions using SUVs with enzymatically conjugated Atg8 and incubating these with Atg4 or related serine mutants (Sanchez-Wandelmer et al., 2017). The observation that Atg1/ULK1 is present on phagophore membranes during their expansion and released shortly after autophagosome completion strongly imply that deconjugation of Atg8 proteins and fusion of autophagosomes with lysosomes are interdependent and precisely regulated processes (**Fig. 1D, E**) (Cebollero et al., 2012; Yu et al., 2012). Spatiotemporal insights into Atg4-mediated deconjugation were obtained using fluorescently labeled Atg8 conjugated to GUVs (Kaufmann et al., 2014). Atg4 efficiently recycles Atg8 from model membranes *in vitro,* even if Atg8 is engaged in a membrane scaffold with Atg12–Atg5-Atg16. Consequently, scaffold formation that was predicted to regulate phagophore expansion and recruitment of Atg4 by Atg8 are mutually exclusive (**Fig. 1D**) (Kaufmann et al., 2014).

The deconjugation of human ATG8 proteins is more complex, given that six ATG8 homologs are conjugated to autophagosomes and four ATG4 homologs are catalyzing priming and recycling reactions (Kabeya et al., 2004). The human ATG4B protein is the most potent isoform to prime human ATG8s and efficiently cuts LC3, GABARAP and GABARAP-L2 (Li et al., 2011; Sou et

al., 2006). Although the other ATG4 homologs are considerably slower in priming of human ATG8s, they possess similar catalytic activities as ATG4B in deconjugating ATG8s *in vitro* (Kauffman et al., 2018). The precise timing of the recycling reaction as well as the physiological functions of the individual ATG4 homologs in human cells remain to be characterized. Interestingly, depletion of Atg4 in yeast or ATG4 homologs in humans leads to the formation of considerably smaller autophagosomes and impact on the sealing of the phagophore (Fujita et al., 2008; Hirata et al., 2017; Nair et al., 2012). This suggests that deconjugation of Atg8/ATG8 not only takes place shortly before, but also during phagophore-expansion and sealing.

After autophagosomes and lysosomes have fused and autophagic cargo has been degraded, lysosomes need to be regenerated. The involved process has been termed autophagic lysosome reformation (ALR) and involves the formation of membrane extrusions from autolysosomes. Proto-lysosomes bud from theses tubes and their maturation restores the population of functional lysosomes (Chen and Yu, 2017).

Insights into the inside – cargo selection in autophagy

The fusion of the outer membrane of autophagosomes with lysosomes delivers autophagic cargo to the lysosomal lumen where it is degraded. Much attention has thus been devoted to reveal how specific cargo is selected and targeted to phagophores. This involves a family of conserved autophagy receptors, which bind cargo on the one hand and the phagophore on the other (Khaminets et al., 2016). Tethering of cargo to the phagophore depends on a LC3-interacting region (LIR), or Atg8-family interacting motif (AIM)-mediated interaction of autophagy receptors with Atg8 family proteins (Lamark et al., 2017) **(Fig. 3B)**. The classical LIR motif is characterized by two bulky hydrophobic residues that are separated by two random residues and flanked by acidic amino acids. More recently, a number of motifs that distinguish between different ATG8 family members have been identified, suggesting that binding of cargo receptors is more complex in higher eukaryotes (Muhlinen et al., 2012; Rogov et al., 2017; Stolz et al., 2017). The interaction of purified yeast and human Atg8 proteins with peptides that contain the various LIR(AIM) motifs has been investigated and the underlying structural, and to a certain extend regulatory principles, are well understood (Kim et al., 2016; Wild et al., 2013). Despite the important contributions of these *in vitro* binding studies, they have some limitations. The LIR motif of tectonin beta-propeller repeat-containing protein 2 (TECPR2), for example, binds all human ATG8 proteins with similar

affinity *in vitro*, but TECPR2 selectively interacts with LC3C *in vivo* (Stadel et al., 2015). Furthermore, increasing evidence suggests that lipidated and non-lipidated Atg8 proteins adapt distinct structural conformations which impact on their affinity to LIR motifs (Coyle et al., 2002; Ichimura et al., 2004; Kumeta et al., 2010; Nakatogawa et al., 2007), which should be systematically investigated in the future.

Another interesting insight into selectivity of autophagy was that multiple Atg8-binding sites on Atg19 are critical for a tight tethering of the cargo ApeI to membranes in order to avoid uptake of other material (Sawa-Makarska et al., 2014). A related mechanism to achieve selective and exclusive uptake of distinct cargo was revealed by recapitulation of aggrephagy: the receptor p62 oligomerizes upon binding to protein aggregates, which facilitates efficient tethering of such aggregates to LC3-conjugated membranes *in vitro* (Wurzer et al., 2015). However, cargo such as yeast ApeI does not only activate its corresponding cargo-receptor Atg19, it also appears to initiate lipidation of Atg8 by recruiting the E3-ligase complex (**Fig. 3B**). This couples receptor-mediated tethering of cargo to Atg8-conjugation, providing a possible mechanism to spatiotemporally coordinate cargo tethering with expansion of the isolation membrane in a selective and exclusive manner (Fracchiolla et al., 2016).

Taken together, the combination of *in vitro* and *in vivo* studies greatly advanced our understanding of how cargo is tethered to membranes during selective autophagy. One of the most important questions that remain to be investigated concerns the two faces of autophagy – how can the cell transform a highly selective pathway that degrades defined cargo in an exclusive manner into a non-selective pathway that degrades bulk cytoplasm?

Conclusions and perspectives

In vitro reconstitutions of biological processes have proven to be powerful tools to reveal unprecedented insights into fundamental mechanisms in biology. The combination of different model membranes such as vesicles and supported lipid bilayers **(see Box 2)** with structural, fluorescent-based, biophysical and biochemical methods **(see Box 3)** dissected molecular principles that govern autophagy induction, phagophore expansion and maturation, as well as fusion with lysosomes. The advantage of *in vitro* systems to provide a chemically well-defined environment is, however, at the same time its strongest limitation. Often, biophysical properties of isolated components *in vitro* simply cannot be correlated one-to-one with their functions *in vivo*.

This might reflect fundamental differences of artificial membranes, which are composed of pure phospholipids (see **Box 2**), and biological membranes which are much more complex in terms of their lipid composition. Biological membranes contain also a substantial amount of membrane proteins which strongly impact on physical properties, spatial organization and shape of theses membranes. Moreover, many experiments with reconstituted systems are 'out of equilibrium', relying on physical separation of components to analyze or quantify them independently (see **Box 3**). Examples include a vast number of binding studies with liposomes and recombinant proteins. *In vivo* systems are, however, mostly in chemical equilibrium and fine-tuned by variations in expression levels of proteins or physical compartmentalization of membranes into microdomains. A successful strategy to overcome these limitations is a combinatorial approach in which *in vitro* experiments are complemented by *in vivo* approaches. Quite often, these combinations create positive feedback loops in which one method complements limitations of the other. The resulting synergy is well suited to tackle the most challenging remaining questions in autophagy in the future. Combined with new biophysical approaches, even more complicated aspects of autophagy can hopefully be reconstituted *in vitro*. The final goal in 'reconstructing destruction' remains to build an autophagosome from purified components in the test tube.

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Box 1: Autophagy in human cells.

In contrast to autophagy initiation in yeast that occurs at a single PAS (Cheong et al., 2008; Suzuki et al., 2001), many autophagosomes are formed simultaneously in human cells. Moreover, the endoplasmic reticulum (ER) has been shown to serve as a platform for nucleation of phagophores whereas the yeast PAS is in close proximity to the vacuole with the phagophore edges contacting the ER (Axe et al., 2008; Biazik et al., 2015; Hayashi-Nishino et al., 2009). The ER-domain at which autophagosomes are nucleated, termed omegasome, adopts a characteristic omega-like shape and is enriched in PtdIns(3)P (Hayashi-Nishino et al., 2009). Similar to yeast, the recruitment of the human ULK1-kinase complex (Atg1-kinase complex homolog) is the earliest

event in autophagy and requires ATG13 to be dephosphorylated (Hosokawa et al., 2009; Kim et al., 2011; Puente et al., 2016). After translocation to the ER, the ULK1 complex recruits the PtdIns(3)-kinase complex III (Park et al., 2016; Park et al., 2018), which consists of the enzyme VPS34 that converts PtdIns into PtdIns(3)P, VPS15, its autophagy specific subunit ATG14 and the adaptor protein Beclin 1. The enrichment of the omegasome in PtdIns(3)P leads to the recruitment of double FYVE-containing protein 1 (DFCP1) and the WD repeat domain phosphoinositide-interacting (WIPI) proteins (Proikas-Cezanne et al., 2004). WIPI1 is involved in the nucleation of the phagophore membrane at the omegasome (Axe et al., 2008; Proikas-Cezanne et al., 2015). The expansion of the phagophore is regulated by WIPI2, which recruits ATG16L1 to initiate the conjugation of ATG8 homologs to the phagophore (Dooley et al., 2014). Although the Ub-like conjugation system in yeast and humans is highly conserved, human cells express six ATG8 homologs with non-redundant functions (Weidberg et al., 2010). According to their sequence homology, two ATG8-subfamilies with the members light chain 3A (LC3A), LC3B and LC3C as well as gamma-aminobutyric acid receptor-associated protein (GABARAP), GABARAPL1 and GABARAPL2/ Golgi-associated ATPase enhancer of 16 kDa (GATE-16) can be distinguished (Schaaf et al., 2016). GABARAPs have been shown to play essential functions during both phagophore initiation and closure (Joachim et al., 2017; Wang et al., 2015; Weidberg et al., 2011). Deletion of LC3 homologs leads to the formation of smaller autophagosomes, suggesting that LC3s coordinate the expansion of autophagosomes (Nguyen et al., 2016; Weidberg et al., 2010), a process that also requires cooperation with the ATG9 compartment (Karanasios et al., 2016). Furthermore, all ATG8 homologs target specific cargo to the phagophore based on their interaction with a linear peptide motif, LC3-interacting region (LIR), that is present in most autophagy receptors (Rogov et al., 2014). After cargo has been confined by the expanded phagophore, the membrane is sealed giving rise to the double-membrane surrounded autophagosome that fuses with lysosomes (Itakura et al., 2012; Jiang et al., 2014).

Box 2: Membrane model systems in synthetic biology.

Model membranes are composed of synthetic phospholipids with defined compositions or lipid extracts with complex lipid-mixtures. A widely applied phospholipid-extract is derived from brain tissues based on the Folch extraction method (FOLCH et al., 1957). The lipids are, depending on their hydrophobicity, dissolved in chloroform or chloroform:methanole(:water) mixtures.

Liposomes of various sizes can be generated by producing a thin film of dried lipids on glass surfaces. Multilamellar vesicles of various sizes are spontaneously formed upon hydration of lipid films with buffer solutions. Unilamellar vesicles can be generated by sonication (small unilamellear vesicles, SUVs) or extrusion through filters with defined pore sizes (large unilamellar vesicles, LUVs) (Mui and Hope, 2009). Giant unilamellar vesicles (GUVs) can be formed by electroformation (Angelova and Dimitrov, 1986). SUVs and LUVs are widely used membrane structures to investigate protein-lipid interactions or to incorporate transmembrane proteins. GUVs are often used to investigate protein binding and protein mobility based on fluorescence microscopy using fluorescent-labeled, recombinant proteins (Wollert, 2012). The dynamics of proteins or lipids within membranes or at the surface of membranes can also be investigated using fluorescence correlation spectroscopic techniques (Ries and Schwille, 2008). In contrast to liposomes, which are 'free-standing membranes', supported lipid bilayers (SLBs) are formed on a solid support (for example glass or mica). SLBs are produced from SUVs that are spontaneously fusing upon contact with the surface of the support (Hardy et al., 2013). The advantage of SLBs is that high-resolution techniques such as atomic force microscopy or total internal reflection microscopy can be applied. However, the diffusion rates of lipids and membrane proteins are significantly lower in SLBs compared to free-standing membranes.

Box 3: Experimental approaches involving artificial membranes.

Vesicles are particularly useful to study protein-lipid interactions as well as interactions of membrane proteins with soluble factors. It is based on the physical separation of membrane vesicles from unbound proteins in order to quantify bound and unbound protein fractions. This can be achieved by sedimentation assays, in which both fractions are separated by ultracentrifugation, which sediments vesicles much faster than soluble proteins (Julkowska et al., 2013). The disadvantage of this approach is that protein aggregates co-sediment, leading to contamination of the vesicles fraction with unbound proteins. This problem can be circumvented by applying floatation assays, which are based on centrifugation of the sample in a density gradient. Membranes have a much lower density as proteins, allowing them to 'float' to the lightest top fraction of a density gradient. Bound proteins are co-floating together with membrane vesicles, whereas unbound proteins remain in the dense fraction and protein aggregates sediment. This allows all species to be separated (Busse et al., 2016). The drawback of the physical separation of

unbound and bound fractions is the perturbation of the biophysical equilibrium and the dissociation of weak interactions. In order to study protein-lipid interactions under equilibrated conditions, fluorescence-based methods can be applied. One such method utilizes GUVs with diameters of 10 to 100 µm and the incorporation of fluorescent lipids. In combination with fluorescent-labeled proteins, binding can be observed in real time by imaging GUVs upon addition of proteins (Sezgin and Schwille, 2012). Moreover, fluorescent recovery after photobleaching of the membrane-bound protein fraction reveals insights into the mobility of proteins on the membrane as well as dynamics of the exchange of bound and unbound proteins (Rayan et al., 2010). Fluorescence correlation and cross-correlation spectroscopy can be used to determine the two-dimensional diffusion coefficients of membrane bound proteins and fluorescent lipids independently from each other (Bacia et al., 2014). The interaction of fluorescent proteins with membranes can be studied at a single-molecule level by combining reconstitutions on supported lipid bilayers with total internal reflection microscopy (Kiessling et al., 2017). Furthermore, new, ultrafast atomic force microscopes allow assembly of macromolecular machines as well as their dynamics to be structurally characterized with unmatched spatial resolution of height profiles in real time (Chiaruttini et al., 2015). Electron microscopy, electron tomography and subtomogram averaging are, in turn, powerful methods to reveal static high-resolution structures of proteins on membranes. Structural characterization of large membrane proteins by EM is achieved through nano-discs, which are lipid bilayer islands that are surrounded by amphipathic proteins such as apolioprotein A1 (Thonghin et al., 2018).

Figure legends

Fig. 1: Schematic representation of autophagy in yeast.

(A) The nucleation of phagophores occurs at the phagophore assembly site and requires activation and assembly of the Atg1-kinase complex as well as recruitment and tethering of Atg9 vesicles. (B) The phosphatidylinositol(3)-kinase (PI3K) complex generates PtdIns(3)P at the early phagophore, which is required to recruit the Atg8 conjugation machinery (shown here: E2-enzyme Atg3). Before Atg8 is conjugated to the phagophore, its C-terminus is cleaved by Atg4 (priming). (C) The concave (inner membrane) pool of Atg8 tethers cargo to the phagophore, whereas the convex pool forms, together with Atg12–Atg5-Atg16, a two-dimensional protein network that stabilizes the membrane. Expansion of the phagophore requires incorporation of membranes,

probably by fusion of vesicles of different origins with the rim of the phagophore. (D) Sealing of the phagophore gives rise to the double-membrane surrounded autophagosome. The kinase activity of Atg1 promotes release of most Atg proteins on the cytoplasmic face of autophagosomes into the cytoplasm. Recycling of Atg8 requires proteolytic activity of Atg4. (E) The fusion of autophagosomes with the yeast vacuole is coordinated by the HOPS complex, which tethers both compartments to each other, and by SNARE proteins. Zippering of the lysosomal SNARE with autophagic SNAREs drive membrane fusion. Proteolytic enzymes within the lysosome degrade the inner autophagosome membrane, cargo and Atg proteins (e.g. Atg8).

Fig. 2: Expansion of autophagosomes requires spatiotemporal coordination of many processes.

(A) The human WIPI2 protein binds PtdIns(3)P at the outer face of the phagophore and recruits the ATG12–ATG5-ATG16L1 complex, which facilitates the transfer of ATG8 proteins from ATG3 to phosphatidylethanolamine of the phagophore membrane. (B) The yeast Atg1-kinase complex strictly localizes to the phagophore rim, whereas Atg1 is also recruited to the phagophore membrane (by binding Atg8). The Atg1-kinase complex might be involved in coordinating fusion of vesicles with the phagophore rim. Expansion of the membrane requires transport of membranes from different sources by vesicular carriers such as Atg9 or COPII vesicles. (C) The amphipathic helix of ATG14 targets the human phosphatidylinositol(3)-kinase (PI3K) complex to the phagophore rim where it converts PtdIns (PI) into PtdIns(3)-phosphate (PI3P). It is unknown how the asymmetric distribution of PI3P, which is only found in the outer membrane of the phagophore, is achieved and maintained. (D) Human ATG3 possesses an ALPS motif which might target a subpopulation of ATG3 to the phagophore rim. This localization allows ATG8 to reach the convex (outer) and concave (inner) face of the phagophore after conjugation. Insertion of the ALPS motif of ATG3 or the amphipathic helix of ATG14 into the membrane is facilitated by lipid packaging defects that are caused by strong bending of the membrane at the rim of phagophores.

Fig. 3: Autophagosome-lysosome fusion and cargo capture by Atg8.

(A) Fusion of autophagosomes and lysosomes is driven by the membrane-tethering HOPS complex and SNAREs. RAB7 is a small GTPase that is recruited to autophagosomes and lysosomes. Direct or RAB effector-mediated interactions with RAB7 allow the HOPS complex to

bind and tether both membranes. The two cytoplasmic SNAREs, Syntaxin 17 (STX17) and SNAP29, are recruited to the phagophore after phagophore completion. ATG14 facilitates assembly and priming of the autophagic SNAREs. The HOPS complex promotes zippering of lysosomal and autophagic SNAREs into a four-strand helical bundle that drives membrane fusion. (B) The multiple functions of Atg8 on phagophores. The concave pool of Atg8 binds autophagy receptors to capture cargo. Depicted here is the interaction of the Ape1 complex (a cargo in the yeast cytoplasm-to-vacuole targeting pathway) with its receptor Atg19 and Atg8. Atg19 binds Atg16 *in vitro*, which might promote recruitment of the Atg12–Atg5-Atg16 complex to the phagophore and position it close to the phagophore rim, where it could stabilize Atg3. The amphipathic helix of Atg3 contributes to the rim localization of Atg3. As a result, conjugation of Atg8 to lipids at the rim of the phagophore is promoted, which would allow Atg8 to reach the inner (concave) and outer (convex) face of the phagophore. At the convex membrane, a scaffold composed of the Atg12–Atg5-Atg16 complex and Atg8 might promote and guide phagophore expansion.

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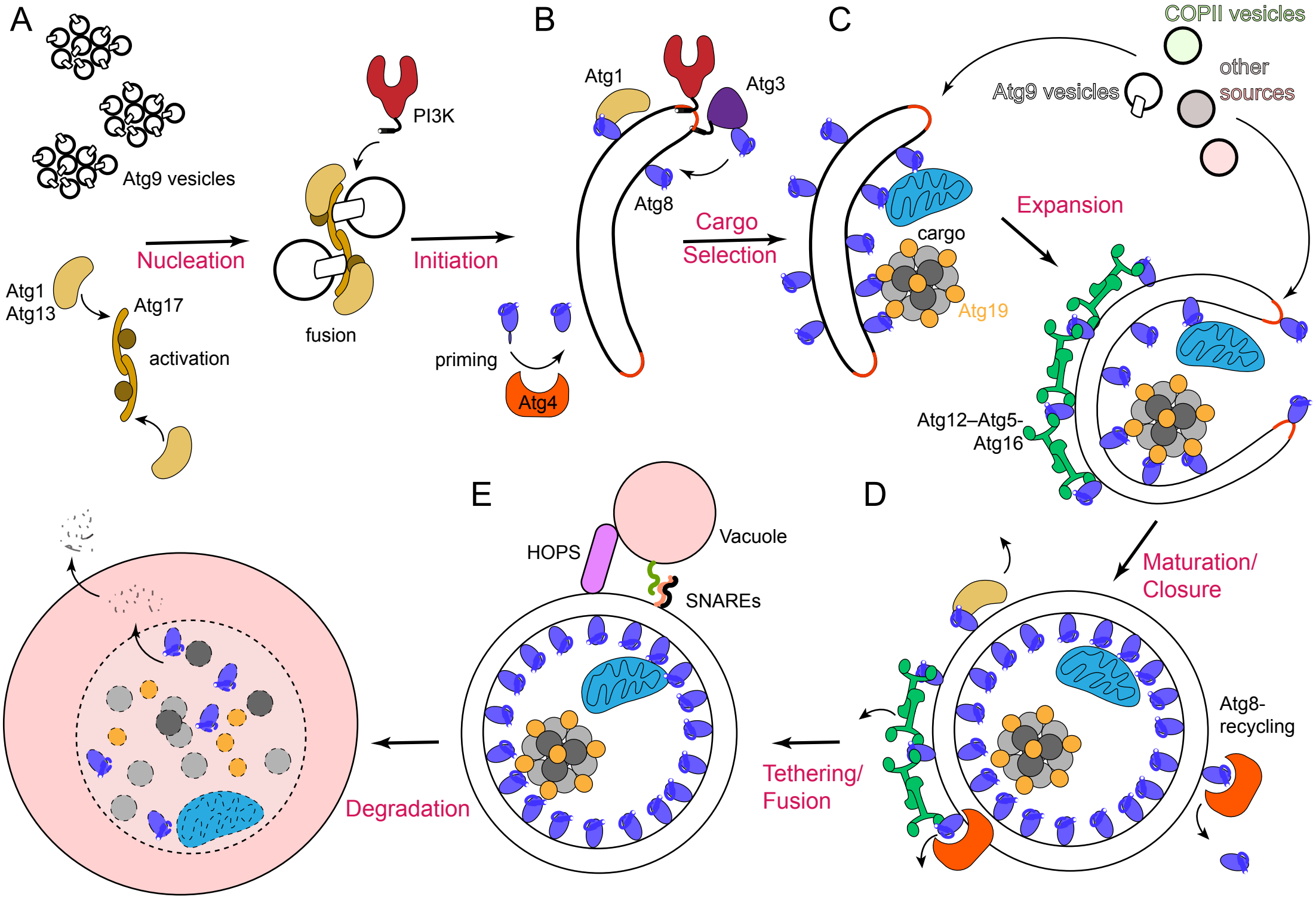
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activation
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fusion
Initiation
B
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priming
Atg4
Cargo
Selection
C
COPII vesicles
other
sources
Atg9 vesicles
cargo
Atg19
Expansion
Atg12–Atg5-
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Tethering/
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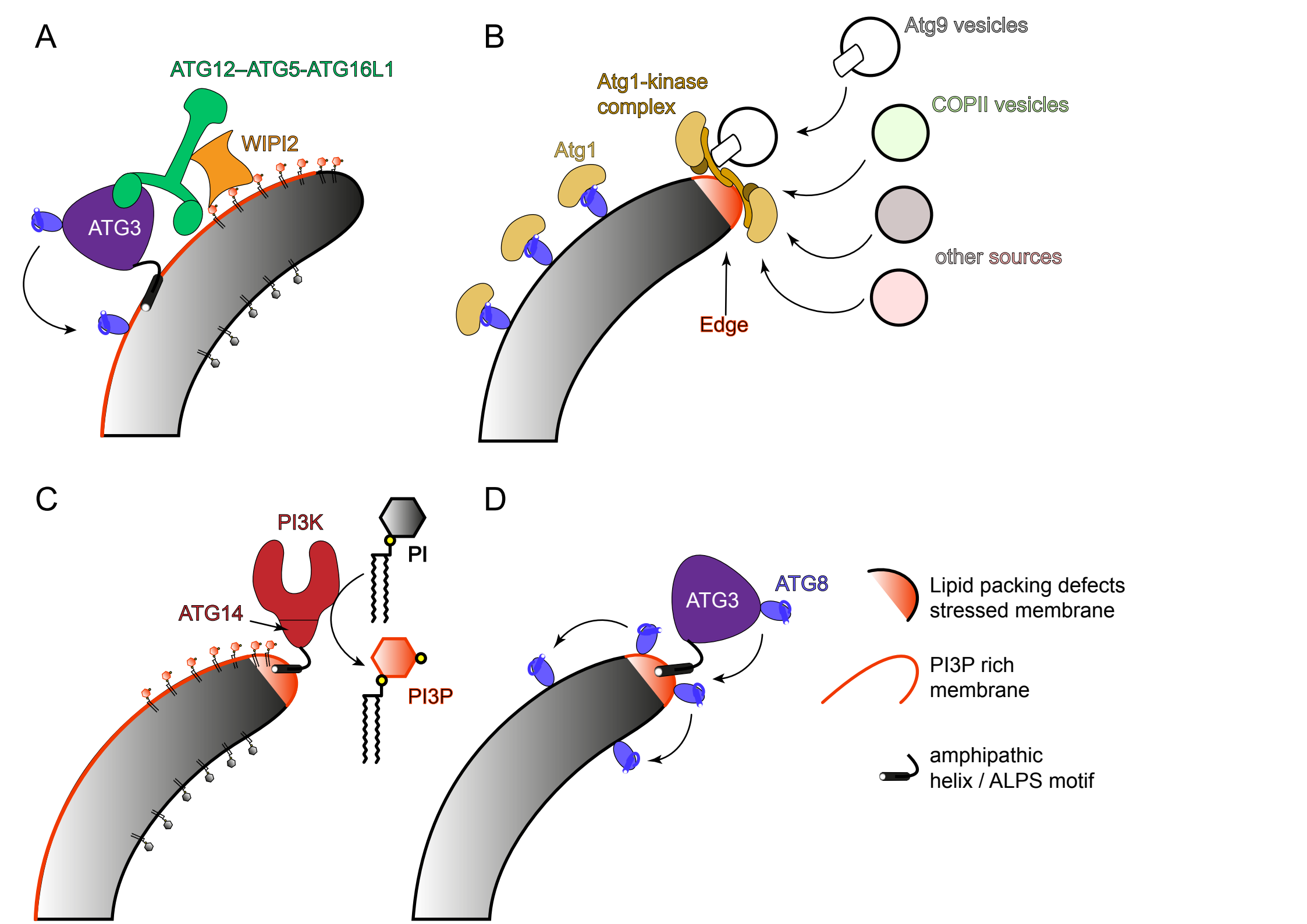
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complex
COPII vesicles
Atg1
other sources
Edge
C
PI3K
PI
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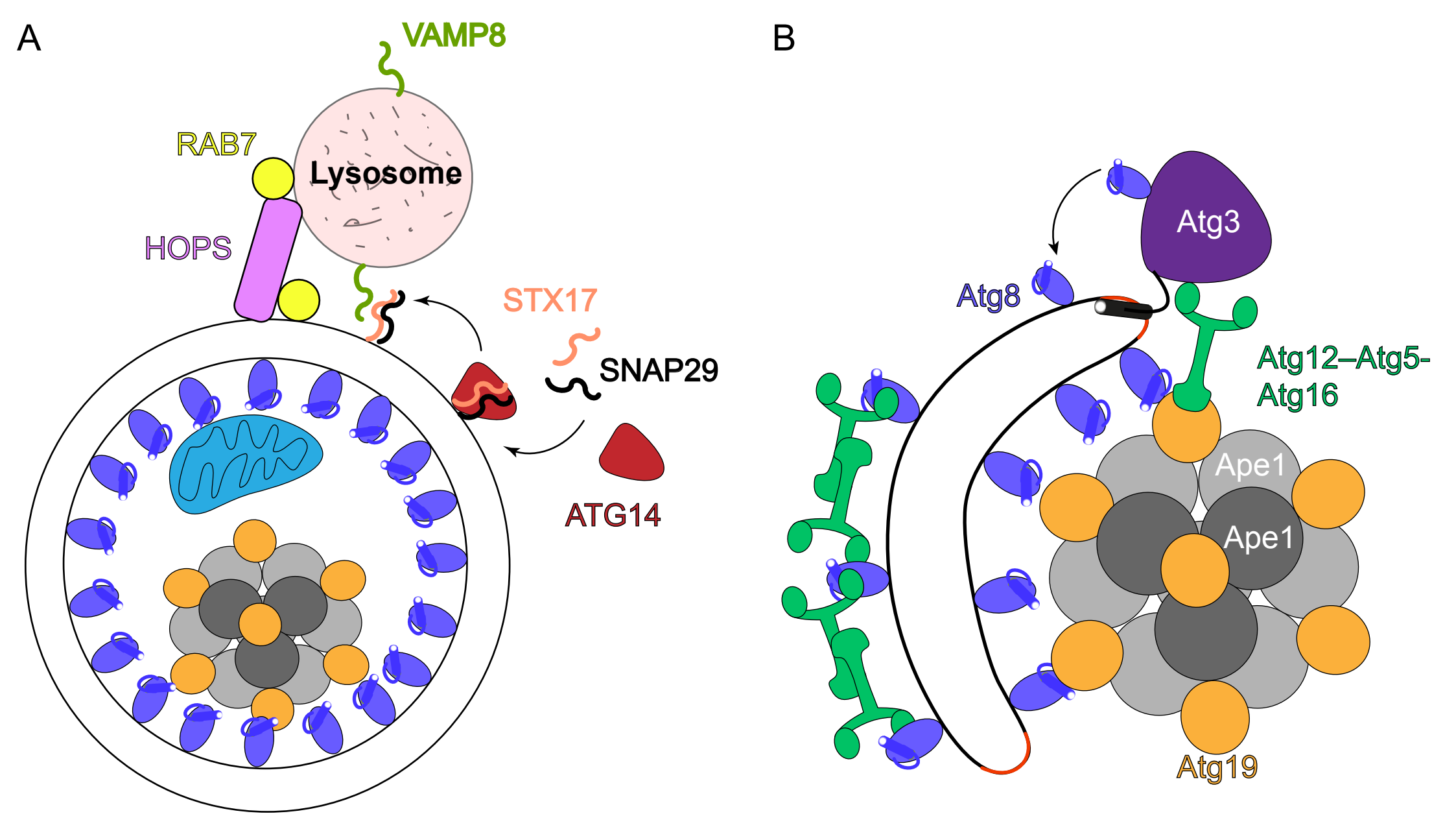
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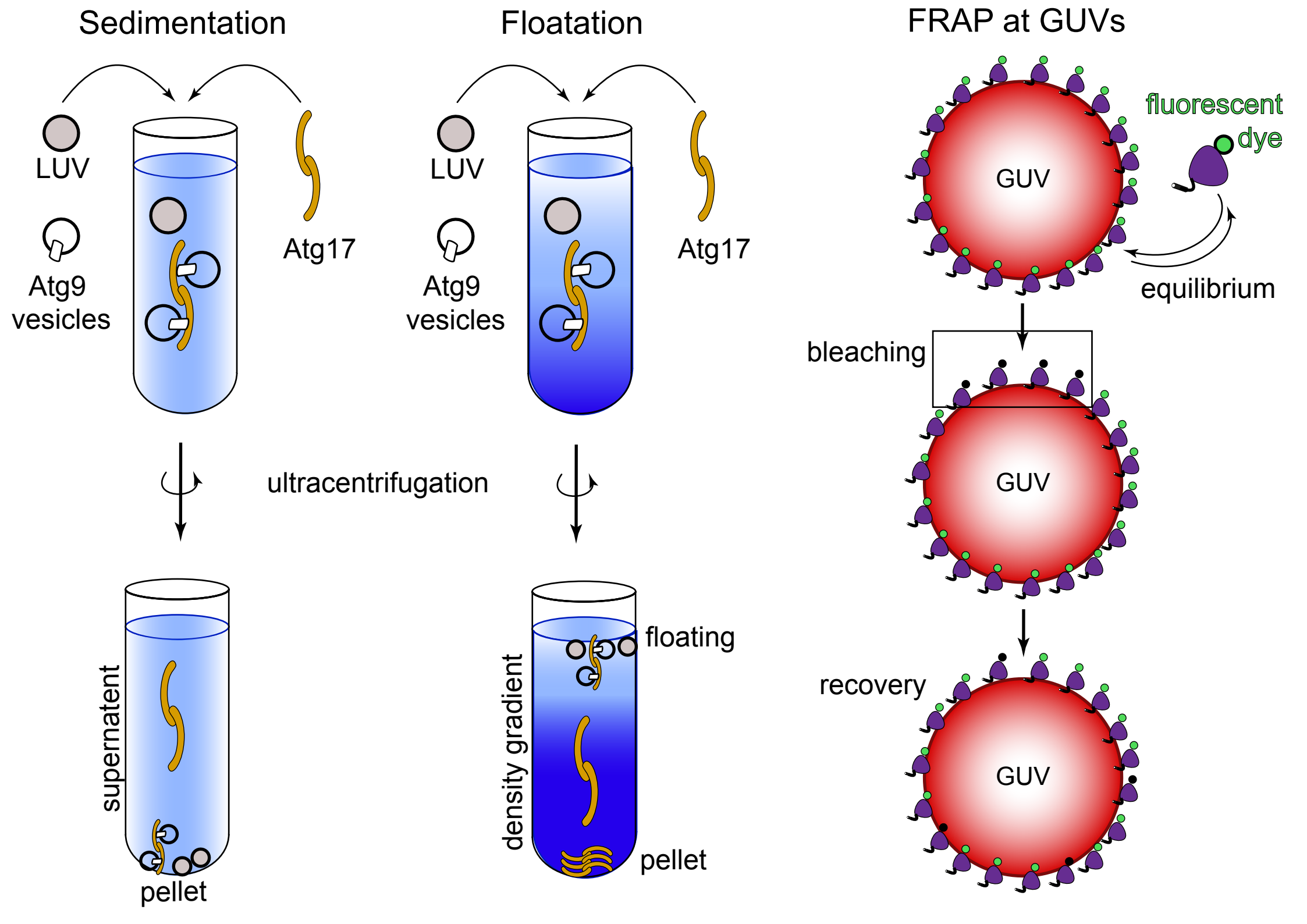
Sedimentation
Floatation
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GUV
fluorescent
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equilibrium
bleaching
recovery