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Autophagosome-lysosome fusion

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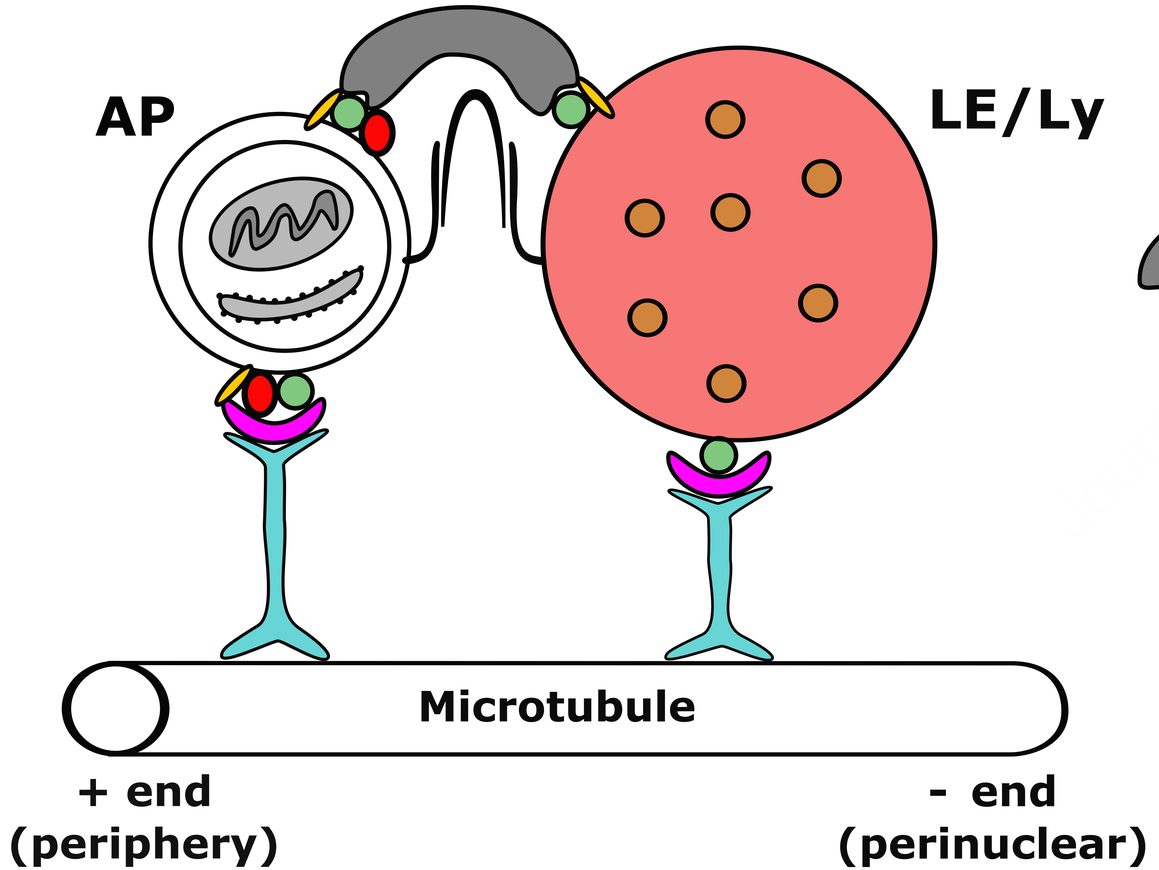

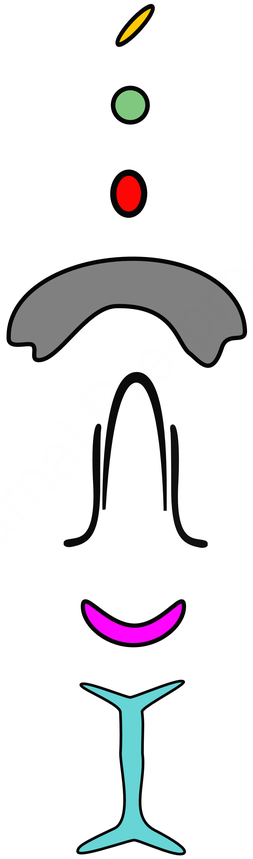

Phosphoinositides: PI(3)P; PI(3,5)P_2; PI(4)P

Small GTPases: RAB7A; RAB2A; ARL8A/B

Atg8 family proteins: LC3s and GABARAPs

Tethers, adaptors: HOPS; EPG-5; ATG14L; TECPR1; GRASP55; BRUCE; RUFY4

SNARE complexes: STX17-SNAP29-VAMP7
STX17-SNAP29-VAMP8
YKT6-SNAP29-STX7

Motor adaptors: BORC; FYCO1; PLEKHM1; PLEKHM2/SKIP; RILP; ORP1L

Motors: **+ end motors** (kinesins)
- end motors (dynein-dynactin)

Autophagosome-lysosome fusion

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autophagy; autophagosome; lysosome; fusion; vesicle tethering

Abstract

Macroautophagy is a conserved catabolic process observed in all eukaryotic cells, during which selected cellular components are transported to and broken down within lysosomes. The process starts with the capture of unnecessary material into autophagosomes, which is followed by autophagosome-lysosome fusion to generate autolysosomes that degrade the cargo. In the past quarter-century, our knowledge about autophagosome formation almost exponentially increased, while the later steps were much less studied. This fortunately changed in the past few years, with more and more publications focusing on the fate of the completed autophagosome. In this review we aspire to summarize the current knowledge about the molecular mechanisms of autophagosome-lysosome fusion.

A brief overview of autophagy

Autophagy (cellular “self-eating”) is one of the two main degradation pathways in eukaryotes besides the proteasome system. Since proteasomes are limited to degrading individual ubiquitinated proteins, autophagy is utilized for en mass degradation of parts of the cytoplasm including whole organelles and large amounts of proteins, lipids and glycogen. It is important to note that autophagic degradation is not simply a catabolic process, as breakdown is followed by recycling of resulting monomers in biosynthetic and energy production processes [1, 2].

Several autophagic routes have been described and all of them have one thing in common: their ultimate goal is to transport cellular cargo into the lysosomal lumen. How a cell achieves this serves as the basis for the classification of different autophagic processes. The most common form of autophagy is macroautophagy, in which a membrane cistern called phagophore or isolation membrane grows around a certain portion of cytoplasm, thereby isolating it into a double-membrane autophagosome. This organelle transfers the

sequestered material to the lysosomal compartment via fusion of its outer membrane with the limiting membrane of the lysosome. The result is an autolysosome, which will degrade the autophagic cargo as well as the inner autophagosomal membrane. An autophagosome may also fuse with late endosomes to generate an amphisome, but the fate of the cargo remains the same, as amphisomes subsequently also fuse with lysosomes [1, 2]. Late-endosome-autophagosome fusion was even suggested to be an obligatory step that must precede lysosomal fusion in metazoans [3, 4]. The lysosome itself is capable of taking up smaller amounts of cytoplasmic cargo via lysosomal membrane invaginations and engulfment, in a topologically similar manner to multivesicular body formation during endosome maturation. This form of autophagy is termed microautophagy. Finally, lysosomal membranes in mammalian, bird and possibly all vertebrate cells contain Lysosome-associated membrane protein 2A (Lamp-2A), which can form a homohexameric channel that directly transports proteins into the lysosomal lumen with the assistance of Hsc70 during chaperone-mediated autophagy [1, 2, 5]. Since these latter two processes do not involve lysosomal fusion steps, in our review we refer to macroautophagy simply as autophagy.

The principle and key players of membrane fusion

The eukaryotic cell is limited by a plasma membrane and its biochemical processes are compartmentalized into membrane bound organelles. This compartmentalization allows the cell to precisely control separate functions and transport routes between organelles, which are mainly achieved via membrane fusion. With some exceptions such as the fusion of mitochondria, most membrane fusions rely on very similar components as follows [6].

All cellular membranes are characterized by specific lipid and protein composition, with selected components controlling the movement and fusion of the given organelle. These include proteins of the Rab (Ras-associated binding) and Arf (ADP-ribosylation factor) small GTPase families [7-9]. These proteins can be thought of as molecular identifiers: they are able to recruit effector proteins in their GTP bound (active) state, which will then determine the subsequent fate of the given organelle. Among these are tethering factors, SNAREs (SNAP Receptor, SNAP: Soluble NSF attachment protein), SM (Sec1/Munc-18) proteins and motors as key players in membrane fusions [6]. The main fusion factors involved in autophagosome-lysosome fusion will be discussed in detail below (see also **Figure 1**).

Autophagosome closure and the acidification of the lysosome may not be required for fusion

Proteins mediating autophagosome formation were first identified in *Saccharomyces cerevisiae* and are called Atg (Autophagy-related) proteins [10]. Most of these have orthologues in higher metazoans and kept their original functions [11]. Atg proteins regulate the initiation and elongation of the phagophore and the formation of the autophagosome [12, 13]. Autophagosome formation utilizes two ubiquitin-like conjugating systems: the Atg12 and Atg8 conjugating systems. Atg8 and Atg12 are ubiquitin-like proteins, and the ultimate purpose of these enzyme reactions is to lipidate and thus anchor Atg8 family proteins to the growing phagophore membrane. [14]. In mammals, Atg8 family proteins belong to two subgroups: the LC3 (light chain 3) subfamily that includes LC3A, LC3B, LC3C and the GABARAP (γ-aminobutyric acid receptor-associated protein) subfamily that includes GABARAP, GABARAPL1, and GABARAPL2. All 6 of these appear to promote autophagosome biogenesis in various ways: Atg8/LC3s are important at the earlier stages of

this process by mediating the elongation of the phagophore and GABARAPs are likely required for later steps [15]. Accordingly, defects in the conjugating systems or in Atg2-mediated lipid transport to the growing phagophore results in the accumulation of abnormal isolation membranes, LC3 positive pre-autophagosomal vesicles and incomplete, non-sealed autophagosomes in both mammalian, fruit fly and worm cells [16-25].

Since the inner membrane of an autophagosome is not resistant to lysosomal hydrolases and is degraded within the autolysosomal/vacuolar lumen, an incomplete/unclosed autophagosome is expected to avoid premature fusion with lysosomes. Interestingly, apparently this is not always the case:, probably unclosed autophagosomes forming in ATG conjugation–deficient MEF cells were found to fuse with lysosomes, although at a slower rate [26]. The newly formed autolysosomes could degrade cargo without leaking lysosomal enzymes to the cytosol. A potential explanation is that the closure pore (which is likely tiny as it could not be directly visualized) can provide a substantial barrier and is sufficient to seal the internal content. However, the degradation of the inner membrane was significantly slower compared in conjugation-deficient cells compared to controls [26]. Importantly, these incomplete, unsealed autophagosomes could still acquire the autophagosomal SNARE Syntaxin17/STX17 (discussed later) [26]. As STX17 promotes fusion, this suggests that Atg8 lipid conjugation is required for autophagosome completion but it is dispensable for fusion and STX17 targeting. In contrast, another study reported that Atg8 family members are important for autophagosome–lysosome fusion by recruiting PLEKHM1 (see later) to autophagosomes but are dispensable for autophagosome formation [27]. Finally, GABARAP proteins were also suggested to mediate autophagosome-lysosome fusion by regulating the lipid composition of autophagosomes [28].

Atg8 proteins may have fusogenic abilities by tethering pre-autophagosomal membranes to the expanding phagophores [29, 30]. In addition, the *C. elegans* homolog of LC3 (but not GABARAP) has been shown to interact with the HOPS tethering complex to promote fusion of autophagosomes with lysosomes [31]. Interestingly, Atg8 is recycled and cleaved from the external membrane of autophagosomes by the cysteine protease Atg4 [32, 33]. Accordingly, spatial restriction of Atg4 impedes autophagosome-vacuole fusion in yeast, suggesting that Atg8 must be removed from autophagosomes prior to successful fusion [34]. This suggests that Atg8 proteins have dual roles during autophagosome-lysosome fusion: they can contribute to the recruitment of tethers and other proteins, but thereafter they must be completely removed to allow subsequent fusion.

Besides Atg8, other Atg proteins have also been found to promote vesicle fusion. For example, the Atg17-Atg31-Atg29 complex and Atg11 recruits the SNARE protein Vam7 to autophagosomes in yeast [35] and Atg14 is suggested to act as a tethering factor in mammalian cells (both discussed later) [36].

The closure of the phagophore - which is topologically equivalent to multivesicular body formation - was suspected to rely on the ESCRT (Endosomal sorting complexes required for transport) machinery [37]. Indeed, it has been shown in both yeast and mammalian cells that ESCRT promotes the sealing of the autophagosome, which might also involve Vps21/Rab5 in yeast [38-41]. Importantly unclosed autophagosomes accumulated and successfully acquired STX17 in ESCRT depleted U2OS cells [40]. Therefore, in spite of occurring at a slower rate, these autophagosomes were still able to fuse with lysosomes. As the degradation of the inner membrane was also impaired, the phenotype of ESCRT loss is remarkably similar to that of ATG conjugation–deficiency [26, 40]: the inner membrane of the unsealed autophagosome acquired Lamp1 (Lysosome-associated membrane protein 1) after lysosomal fusion, which normally cannot happen in the case of a completely closed autophagosome [40]. The intraluminal part of Lamp proteins is heavily glycosylated, which is

thought to protect lysosomal membranes from lysosomal hydrolases that normally digest membrane components [42-44]. This suggests that the degradation delay of the autophagosomal inner membrane in ESCRT or ATG conjugation deficient cells is most likely the consequence of acquiring lysosomal membrane proteins and/or lipids. This could explain the autophagosome accumulation seen in ESCRT depleted cells [3, 4, 45, 46].

Bafilomycin A1 is a potent inhibitor of Vacuolar ATPase (V-ATPase), thereby it inhibits acidification and degradation in lysosomes [47]. Bafilomycin also inhibits autophagosome-lysosome fusion [48], which raised the possibility that lysosomal acidification is required for fusion events. However, it was later shown that non-degrading autolysosomes accumulate in V-ATPase depleted cells, indicating that autophagosome-lysosome fusion occurs independently of lysosomal pH and thus, lysosomal acidification and fusion are two separate events [49]. Unlike pH, the ion composition in the lysosomal lumen seems to regulate autophagosome-lysosome fusion. Lysosomal voltage-gated calcium channel and Ca^{2+} efflux from lysosomes promote fusion, as the loss of this channel causes accumulation of autophagosomal-lysosomal fusion intermediates both in fly and mouse cells [50]. Importantly, elevated levels of Ca^{2+} can also be inhibitory as heavy metal induced ER stress increases intracellular Ca^{2+} levels above physiological, which in this case inhibits autophagic flux [51, 52].

Summary and future directions. *Based on the aforementioned data, we can conclude that autophagosome completion, Atg8 conjugation proteins and lysosomal acidification are not absolutely essential for STX17 recruitment and executing the fusion with lysosomes. In addition to promoting phagophore assembly and sealing of its edges, Atg8 proteins may be required for the timing of autophagosome-lysosome fusion, as premature fusion of unsealed autophagosomes also decreases autophagic flux due to the delayed degradation of the inner membrane of the autophagosome. The lysosomal content flows into the space between the external and the internal membranes of the autophagosomes, which is normally followed by breakdown of the internal membrane. If the closure pore remains open as is likely the case in Atg8 conjugation and ESCRT deficient cells, respectively, the inner membrane can also acquire lysosomal membrane proteins that probably limits its access by lysosomal hydrolases. It is thus a very interesting question how the cargo can still be degraded without autolysosomal "leaking". Perhaps the closure pore closes spontaneously?*

Multiple small GTPases promote autophagosome-lysosome fusion

Rab GTPases are essential regulators of membrane traffic in eukaryotic cells. These proteins, as their name suggests, are able to bind GTP and hydrolyze it to GDP, thereby acting as molecular switches. What makes them of particular interest is that these proteins are prenylated and recruited to target membranes upon GTP binding, providing molecular identity to the organelle membrane [7, 9]. The cycle between GTP-bound (membrane-associated) and GDP-bound (cytosolic) states is mediated by Guanosine exchange factors (GEFs) and GTPase-activating proteins (also known as GTPase-accelerating proteins – GAPs). GEFs trigger the release of GDP from the Rab protein to allow GTP binding, thus prompting their active state. GAPs antagonize this by stimulating the GTPase activity of the Rab, thereby "switching them off". The GDP loaded, inactive Rab is then released from the membrane by chaperone-like molecules called GDP dissociation inhibitors (GDIs) [53]. Besides Rabs, other small GTPases belonging to the Ras superfamily that function in a

similar manner have been shown to regulate membrane trafficking events, with Arf and Arf-like (Arl) small GTPases being most significant [8].

Generally both GTP-loaded Rab and Arl proteins recruit effectors to the target membranes, thus determine the fate of the given organelle. Rab proteins were mainly shown to bind effectors required to drive fusion (such as motors, adaptors and tethers), while Arf and Arl proteins were mainly shown to bind coat proteins, thus driving vesicle budding and organelle positioning. This is not a strict rule, though: several exceptions (such as Arl8, discussed below) are known [8, 54].

At least 60 Rab protein isoforms are present in mammalian cells [55], some of which participate in autophagic processes including autophagosome formation and maturation [54, 56]. Prior to autophagy, the endocytic role of selected Rab proteins were already extensively studied. Among them, Rab5 and Rab7 can be considered as key players: early stages (ending with ESCRT-dependent MVB formation) are regulated by early endosomal Rab5, while late stages - including late endosome/MVB-lysosome fusion – are regulated by late endosomal Rab7 [57]. Importantly, Rab7, along with its GEF the Mon1-Ccz1 (Monensin sensitivity protein 1 - Caffeine, calcium, and zinc 1) complex was shown to be required for autophagosome-lysosome fusion in yeast, fly and mammalian cells and it has been therefore considered as a central regulator of autophagosome clearance [32, 58-65]. Rab7 localizes to autophagosomes and autolysosomes in yeast, fly and mammalian cells [59, 63, 66-68] and GTP-bound Rab7 is able to recruit various effectors including motor proteins and tethering factors to target membranes, thus it is important both for the movement and the subsequent fusion of autophagosomes (discussed later).

It has long remained unclear how autophagosomes acquire Rab7. In the case of endosomes, GTP loaded, membrane associated Rab5 likely recruits Mon1-Ccz1, which binds to Rab5 and endosomal PI(3)P (Phosphatidylinositol 3-phosphate). Mon1-Ccz1 subsequently recruits and activates Rab7, this way Rab5 is sequentially replaced by Rab7 [69-71]. In contrast to endosomes, Rab5 has never been found on phagophores or autophagosomes. In line with this, Rab5 is dispensable for autophagosome formation and fusion in *Drosophila* and Rab5 contributes to autophagosome closure but not fusion in yeast [39, 59]. This implies that Rab5 is not required for autophagosome-lysosome fusion and Mon1-CCz1 is recruited to autophagosomes in a Rab5-independent manner. Similar to endosomes, autophagosomal membranes also contain PI(3)P [72], but it is a question whether this is enough to recruit Mon1-Ccz1. Yeast CCZ1 contains two LIRs (LC3-interacting motif) at its C-terminal part that can bind to Atg8. The disruption of these motifs prevents autophagosome-vacuole fusion while endosomal transport remains intact [66]. Since these motifs were found to be conserved [66], it is reasonable to assume that similar to yeast, Atg8/LC3 proteins also recruit Mon1-Ccz1 to autophagosomes in animal cells, also bringing Rab7 to autophagosomal membranes this way. However, as discussed above, unsealed autophagosomes in ATG conjugation mutants could still fuse with lysosomes, thus most likely autophagosomal PI(3)P and Atg8/LC3 cooperate in Rab7 recruitment and one of these mechanisms may be still sufficient to drive fusions at a lower rate.

Interestingly, a recent paper showed that autolysosomes accumulate in starved Rab7 knockout MCDK-II cells and these autolysosomes are quickly cleared in a Rab7 independent manner when only glutamine was absent from the medium [73]. This suggests the possibility that in contrast to insects or yeast that have only one Rab7 gene, mammalian paralogs of Rab7 (Rab7B (also called Rab42) and Rab7L (also called Rab29)) may compensate for Rab7 loss, or perhaps other Rab proteins or small GTPases are involved in autophagosome-lysosome fusion. Indeed, Rab2, a mainly Golgi associated Rab protein, was shown to be required for autophagosome clearance [74-76]. Rab2 is also essential for endolysosome

formation in flies similar to Rab7 [75, 76] and the constitutively active form of Rab2 relocates to (auto)lysosomes and increases their size in a Rab7 dependent manner. Interestingly, small vesicles positive for GTP-locked Rab2 may fuse with large autolysosomes [75]. Since Rab2 is known to promote vesicle transport from the Golgi, this observation raises the possibility that autophagic vesicles are capable of fusing with Rab2-positive, Golgi-derived carrier vesicles as well besides lysosomes or endosomes, and the role of active Rab2 on (auto)lysosomes may be to continuously maintain incoming fusions. Interestingly, the GTP-locked form of Rab7 could not increase autolysosome size, suggesting that the amount of active Rab2 is rate-limiting for lysosomal fusions, perhaps because it has a short dwelling time on lysosomes [75]. The GAP of Rab7 is the TBC/RabGAP protein Armus (TBC1D2A) [77] and by binding to LC3, it can inactivate Rab7 on autophagosomes [78]. Although Rab7 is required for autophagosome-lysosome fusion, the rapid cycling and removal of Rab7 seems to be essential for completion of fusion as the depletion of Armus delayed autophagosome clearance [78].

Interestingly, silencing of Rab2A in MDA-MB-231 human breast cancer cells led to the accumulation of immature amphisomes or autolysosomes but not bona fide autophagosomes [75], similar to starved Rab7 knockout MCDK-II cells [73], altogether supporting the idea that these Rab proteins act redundantly during autophagosome-lysosome fusion (note that there is no homolog of Rab2 in the yeast genome).

Interestingly, Rab2 and Rab7 are not the only small GTPases involved in autophagosome-lysosome fusion: Arl8 is mostly known to mediate lysosomal positioning and motility, and it is recruited to lysosomes by the BORC (BLOC-1–related complex, BLOC: Biogenesis of lysosome-related organelles complex 1) complex [79, 80]. Arl8 also binds to the HOPS tethering complex similar to Rab7 and Rab2 (discussed later) and thus promotes autophagosome-lysosome fusion [81, 82]. Rab21, an endosomal Rab, along with its GEF Sbf/MTMR13, was also shown to modulate fusion by regulating the trafficking of the SNARE Vamp8 (discussed later) to lysosomes. Depletion of Sbf/MTMR13 or Rab21 blocked endolysosomal trafficking of Vamp8, leading to impaired autophagosome-lysosome fusion [83]. Rab24 is another Golgi Rab that can relocate to autophagosomes and autolysosomes upon starvation and regulate endocytic cargo degradation by interacting with Rab7-RILP [84, 85]. More recently, Rab24 was found to promote the clearance of autophagic compartments in full culture medium condition but not under starvation [86], thus its involvement and role during autophagosome-lysosome fusion is under debate. Of course different autophagic settings such as basal or stress induced autophagy may rely on different fusion machineries.

Yet another Golgi small GTPase, Rab33B and its GAP (OATL1) has been shown to regulate autophagosome maturation in a particularly interesting manner [87]. Rab33B was previously found to bind Atg16 and thus promote phagophore elongation [88]. Interestingly, overexpression of both wild-type or constitutively active Rab33B inhibited autophagosome-lysosome fusion in a similar manner to OATL1 [87]. At first this seems to be contradictory: opposing phenotypes could be expected because OATL1 is the inactivator of Rab33B, so thus its overexpression should mimic the loss of its target. Interestingly, the same group earlier identified OATL1 as the GAP of Rab2A [89] and later found that its GAP activity towards Rab33B is higher than to Rab2A [88]. We suggest that these results could be resolved if OATL1 has GAP activity towards both Rab proteins. Based on the overexpression experiments, Rab33B seems to be a negative regulator of autophagosome maturation/autolysosome formation, in contrast to Rab2A which seems to be a positive one. OATL1 is recruited to LC3 positive vesicles and localizes to the outer membrane of autophagosomes [90]. Thus it can possibly inactivate Rab33B on autophagosomes, but autophagosomes were still forming in the absence of Rab33B [87]. These suggest that

Rab33B promotes but it is not essential for autophagosome formation and may delay its maturation. Since activated Rab2 relocates to and increases the size of (auto)lysosomes but not that of autophagosomes [75], it is possible that Rab33B inhibits premature autolysosome fusion and it is inactivated by OATL1 after maturation is finished. Next, OATL1 may remain on the newly formed autolysosomal membrane to inactivate Rab2, preventing excessive fusions this way.

Summary and future directions. *A subset of small GTPase proteins are required for autophagosome lysosome fusion, of which Rab7 (Ypt7 in yeast), Rab2A (Rab2 in flies), and Drosophila Arl8 are best characterized so far. These proteins are well known players during endolysosome formation, during which they mediate the maturation of Rab7 positive late endosomes into (endo)lysosomes. They function in a very similar fashion during autophagy. Autophagosomes acquire Rab7 during autophagosome maturation and so they become competent to fuse with Rab7, Rab2 or Arl8 positive organelles such as late endosomes, lysosomes or Golgi-derived vesicles. How autophagosomes acquire Rab7 is still not entirely clear. It seems that similar to endosomes, autophagosomes become enriched with Rab7 in a Mon1-Ccz1 dependent manner. However, as opposed to endosome maturation, this is independent of the early endosomal Rab5. Future studies are required to identify the upstream events leading to Rab7 recruitment during autophagy. Three independent papers showed that Rab2 mediates autophagosome-lysosome fusion in flies, while the roles of Rab2A (and Rab2B) are not that clear in mammalian cells. In mammals, autolysosomes can form in Rab7 KO cells unlike in invertebrates or fungi, indicating increasing gene redundancy. Thus, future studies are needed to elucidate the exact roles of these small GTPases in autolysosome formation and to define the extent of redundancy between them.*

Motor proteins ensure that autophagosomes encounter lysosomes

Autophagosomes are thought to be generated at random positions throughout the cytoplasm and are then transported towards the center of the cell [91, 92]. In contrast, late endosomes and lysosomes usually exhibit perinuclear distribution in most cell types. It is a pre-requisite of successful fusions that autophagosomes encounter lysosomes or late endosomes. Effector proteins of the previously discussed Rab proteins and Arl8 include motor protein binding adaptors, which are able to drive the transport of autophagosomes and late endosomes/lysosomes towards each other along the cytoskeleton [93].

Vesicular transport mainly relies on the microtubular network and associated motor proteins. The minus-end directed dynein−dynactin motor complex moves vesicles (including autophagosomes) to the perinuclear region, and the plus-end-directed motor proteins kinesins drive vesicles to the periphery [92, 93]. Accordingly, the cytoskeleton has multiple roles in metazoan cells in autophagy from autophagosome formation to fusion (reviewed in [94] and discussed below).

Among the first papers dealing with the molecular mechanisms of autophagosome-lysosome fusion was the study of dynein mutations that cause motoneuron disease [95]. Data from both cell culture and mice revealed the accumulation of autophagosomal LC3 staining upon loss of dynein function, which was attributed to a fusion defect [95]. Importantly, mutation of dynein light chain 1 also caused autophagic clearance defects and decreased larval motility in *Drosophila* [96].

There are several adaptor proteins among the effectors of small GTPases that can link a vesicle to motor proteins. For example, Rab7 binds to FYCO1 (FYVE and coiled-coil

domain-containing 1), ORP1L (Oxysterol-binding protein-related protein 1L, also known as Oxysterol binding protein like 1A (OSBPL1A)) and RILP (Rab-interacting lysosomal protein) and so it can mediate both plus-end and minus-end transport of vesicles, as discussed below [97-103].

In addition to its interaction with Rab7, FYCO1 also binds LC3 and PI(3)P and its depletion leads to autophagosome accumulation at the perinuclear region of cells, suggesting that FYCO1 is a kinesin adaptor [98, 104, 105]. Accordingly, FYCO1 was shown to directly bind to KLC2, the light chain of kinesin 1 on Rab7 positive late endosomes [106]. Moreover, depletion of the heavy chain (KIF5B) of kinesin-1 induces the perinuclear accumulation of autophagosomes, similar to FYCO1 loss [107]. Kinesin-3 and kinesin-13 were also suggested to mediate autophagosomal-lysosomal transport, as depletion of these kinesins resulted in the localization of autophagosomes mostly at the center of the cell. This also increased fusion rates, while the overexpression of kinesin-3 redistributed autophagosomes to the periphery and the rate of their fusion with lysosomes greatly decreased [108]. These results suggest that lysosome positioning is able to determine the rate of autophagosome–lysosome fusion. Another kinesin-3 family member Klp98A (the orthologue of human KIF16B) has been demonstrated to carry autophagic vesicles in *Drosophila*, but in this case the kinesin can directly bind to Atg8a. Interestingly, it can also capture endosomes by binding the endolysosomal Rab14, thus possibly directly mediating the fusion between autophagosomes and endosomes [109].

Rab7 also binds PLEKHM1 (Pleckstrin Homology And RUN Domain Containing M1) and this is facilitated by DEF8 (Differentially Expressed In FDCP 8 Homolog). Binding of FAM98A (Family with sequence similarity 98 member A) and NDEL1 (Nuclear distribution protein nudE-like 1) to PLEKHM1 was shown to connect lysosomes to microtubules and regulate their movement. Accordingly, the loss of these proteins attenuates the peripheral distribution of lysosomes in osteoclasts [110]. This suggests that PLEKHM1 and its interactors regulate the kinesin-mediated plus-end transport of Rab7 positive organelles.

In contrast to FYCO1, ORP1L and RILP interact with the dynein-dynactin complex to mediate inward transport [99-101]. ORP1L also functions as a cholesterol sensor and it interacts with the ER resident VAP-A protein at a low cholesterol level, thereby releasing $p150^{Glued}$ from the dynein-dynactin complex and thus inhibiting vesicle movement. This way, ORP1L ensures that autophagosome-endosome/lysosome fusion takes place at normal cholesterol levels [97, 102]. Moreover, VAP-A bound ORP1L inhibits HOPS recruitment by PLEKHM1, thus it can directly inhibit fusion [97].

Arl8 also binds adaptors such as the BORC complex and regulates the minus-end transport of lysosomes. Arl8 associates with lysosomes in a BORC-dependent manner and it recruits the kinesin-1/KIF5 and kinesin-3/KIF1 motors to promote the movement of lysosomal to the periphery. In line with this, depletion of BORC or Arl8 causes lysosomes to accumulate at the pericentral region of cells, reducing the possibility of encounters between autophagosomes and lysosomes [103, 111-113]. The interaction of Arl8 with BORC is inhibited by Ragulator, a protein complex important for lysosome-controlled growth signaling. This way, Ragulator inhibits lysosomal movement and thereby it may reduce fusion rates [112, 114]. Arl8 also binds to both the PLEKHM1 and PLEKHM2 (also known as SKIP/SKI-interacting protein) adaptor proteins. These proteins compete for Arl8 binding and so have opposing roles: PLEKHM2/SKIP interacts with kinesin 1 and mediates lysosomal movement towards the periphery, while PLEKHM1 promotes juxtanuclear clustering of lysosomes [115, 116].

GTP-locked *Drosophila* Rab2 and human RAB2A have also been found to bind the dynein adaptor Bicaudal D, both in fly and mammalian cells [117, 118]. This suggests that Rab2 may contribute to the recruitment of minus-end directed motors to lysosomes or Golgi derived vesicles, but this has not been demonstrated yet.

Of note, not just small GTPases but also Atg8/LC3 itself has been shown to directly bind a motor adaptor: Jip1, to promote dynein mediated autophagosome transport along the axon in neurons [119].

Less is known about the involvement of actin and its associated motors in autophagosome-lysosome fusion, but there are some evidences that the actin network and myosins may also regulate this process. Actin and associated motors have been suggested to contribute to autophagy from autophagosome formation to maturation (reviewed in: [120]), but very few factors have been studied. HDAC6 (histone deacetylase 6) was suggested to recruit an F-actin network to facilitate autophagosome–lysosome fusion during selective but not starvation induced autophagy [121]. Lysosome associated cortactin and stabilized actin filaments were also speculated to promote autophagosome-lysosome fusion [122].

Currently two myosins, MYO6 and MYO1C have been shown to promote autophagosome-endosome/lysosome fusion. These myosins interact with autophagy receptors (such as OPTN/Optineurin, NDP52/Nuclear dot protein 52 and TAX1BP1/Tax1 binding protein 1) on forming autophagosomes and Tom1 (ESCRT-0 component) on endosomes, thereby facilitating their movement and subsequent fusion [123-125].

Summary and future directions. *Rab7 and Arl8 are key players in autophagosome and lysosome positioning because they bind several motor adaptor proteins including FYCO1, ORP1L, RILP, PLEKHM1/2, and BORC. These adaptors link autophagosomes and lysosomes to cytoskeleton-associated motor proteins. Autophagosomes and lysosomes mostly travel along the microtubules. This is driven by the minus end directed dynein−dynactin motor complex that moves these vesicles to the perinuclear region, while plus end directed kinesins transport lysosomes (and other vesicles) towards the periphery of the cells. The transport of autophagosomes and lysosomes towards each other is essential for successful encounters that result in fusions. Depletion of either type of motors will result in the accumulation of autophagic vesicles, either in the cell periphery (dynein loss) or at the cell center (kinesin loss). Very little is known about the involvement of the actomyosin network in autophagosome-lysosome fusion, pointing to the more prominent role of the microtubule system in this process, at least for now.*

Tethers and adaptors anchor autophagosomes to endosomes or lysosomes

Vesicles moving along cytoskeletal elements eventually reach their destination and need to be tethered to their partner organelle. Tethering is usually carried out by large coiled-coil proteins or multisubunit tethering complexes, both of which bind to GTP-loaded small GTPases and/or lipids on the surface of interacting vesicles. Next, SM (Sec1/Munc-18) family proteins arrive and facilitate SNARE complex assembly and zippering [6].

The best known tether in autophagosome-lysosome (vacuole in yeast) fusion is the multisubunit complex HOPS (Homotypic fusion and vacuole protein sorting). This complex was first identified in yeast endocytic maturation studies and consists of six subunits: the 4-subunit class C core and the two Ypt7 (Rab7 in metazoans) binding subunits: Vps41 and Vps39 on its opposing ends. This way, HOPS is able to tether Ypt7/Rab7 positive vesicles

(autophagosomes and late endosomes) to the vacuole, the equivalent of lysosomes in yeast cells [62, 126-133]. HOPS is conserved in metazoan organisms but it does not bind directly to Rab7, unlike in yeast [75, 82, 118, 134]. Instead, HOPS binds the previously mentioned Rab7 adaptors Plekhm1 and RILP [58, 75, 99, 118, 135, 136]. Interestingly, HOPS directly binds GTP-loaded Rab2 in *Drosophila* or RAB2A in human breast cancer cells [75, 117, 137], although this has not been confirmed in HeLa cells, suggesting the necessity of adaptors in some cell types [118]. HOPS is also able to bind PI(3)P [138], the autophagosomal SNARE Syntaxin 17 [139, 140], the lysosomal small GTPase Arl8 [79, 82, 141] and its GEF the Mon1-Ccz1 complex [69].

Based on the number of its interactions with small GTPases and other proteins in metazoan cells, it is not surprising that HOPS is the key tether of lysosomal fusions. HOPS is essential for almost all lysosome-related fusion events including late endosome-lysosome fusion, lysosome related organelle (LRO) biogenesis, secretory granule-lysosome fusion during crinophagy and autophagosome-lysosome fusion (**Figure 2**) [58, 74, 75, 98, 99, 133, 137, 139, 142-154]. These data suggest that HOPS can be targeted to different membranes via several different interactions: for example, HOPS may be recruited to autophagosomes via STX17 [139, 140] or Rab2 [74] and to lysosomes via Arl8 and the BORC complex [82, 113]. In contrast, late endosomal targeting of HOPS depends on Rab7 and Rab2 but not Arl8 in fruit fly nephrocytes [155]. The regulatory subunit of a PI3K complex, UVRAG (ultraviolet radiation resistance-associated gene protein), was suggested to interact with and recruit HOPS to autophagosomes to facilitate their subsequent fusion with lysosomes [156]. However, it was shown later that UVRAG is dispensable for autophagosome-lysosome fusion in both *Drosophila* and mammalian cells, while it is required for endolysosomal degradation and lysosomal function [139, 140, 157], so it may affect autophagy indirectly.

HOPS is not only a tethering factor, as one of its central subunits, Vps33 (Vps33A in metazoa) also functions as an SM protein. This way, HOPS facilitates SNARE complex assembly and zippering besides its tethering function [158-164]. Moreover, yeast HOPS protects properly assembled trans-SNARE complexes from disassembly by αSNAP [165].

HOPS shares 4 or 3 central subunits with the related CORVET (in yeast and mammals) or *Drosophila* miniCORVET complexes, respectively, which are tethers of Rab5 positive early endosomes [127, 131, 147, 166, 167]. Although yeast Vps21/Rab5 and CORVET has been implicated in autophagosome clearance [38, 168], miniCORVET is dispensable for autophagy in flies [155], whilemammalian CORVET's involvement in autophagy is unknown. Interestingly, the overexpression of Vps8 (the defining subunit of miniCORVET in Drosophila) inhibits autophagosome-lysosome fusion and other HOPS-dependent processes because Vps8 outcompetes Vps41 from shared subunits [155].This suggests that the fine balance between the intracellular amount of CORVET and HOPS is essential for normal trafficking to lysosomes.

Additional autophagic tethering factors or adaptors have also been found. EPG-5 (Ectopic P-granules 5) was first identified in *Caenorhabditis elegans* and the name refers to its loss of function phenotype: *epg-5* mutants accumulate ectopic germline protein granules due to defective autophagic flux and failed autolysosome formation [169]. Similar to HOPS, EPG-5 is required for the degradation of endocytic cargo [170]. Importantly, EPG-5 binds and is recruited to lysosomal and autophagosomal SNAREs along with Rab7 and LC3, and facilitates SNARE assembly and promotes the fusion of autophagosomes with late endosomes/lysosomes [144]. Enlarged vesicles containing both autophagic and endocytic markers accumulate in EPG-5 depleted cells, suggesting that autophagosomes are mistargeted to other endocytic vesicles instead of late endosomes/lysosomes in these cells [144], which has never been reported in HOPS deficient cells.

The adaptor protein Tectonin Beta-Propeller Repeat Containing 1 (TECPR1) was first shown to be required for *Shigella* targeting for selective pathogen autophagy [171] as another possible tethering protein. TECPR1 binds to PI(3)P and the Atg12-Atg5 conjugate. The authors suggested that lysosome-localized TECPR1 binds and tethers autophagosomes, because its loss prevents autophagosome-lysosome fusion in U2OS cells [172]. The tethering ability of TECPR1 may be vertebrate specific, as the loss of its *C. elegans* homolog did not cause an autophagy defect [144].

Additional potential tethers include Golgi reassembly stacking protein 55 (GRASP55), a protein crosslinking Golgi cisternae, which relocates to autophagosomes and lysosomes upon starvation and interacts with LC3B (via a LIR motif) and LAMP2 [173]. Baculovirus IAP repeat-containing ubiquitin conjugating enzyme (BRUCE) was also shown to interact with the Atg8 family proteins GABARAP and GABARAPL1 as well as with STX17 and SNAP29. BRUCE localizes to lysosomes to promote autophagosome lysosome fusion, and its depletion caused STX17 to accumulate on autophagic membranes [174]. Interestingly, the core autophagy protein ATG14 has also been suggested to harbor tethering abilities in addition to its role in autophagosome biogenesis as a PI3K complex subunit: it binds to and stabilizes the STX17-SNAP29-VAMP8 SNARE complex and it successfully tethered reconstituted proteoliposomes in a PI3K independent manner *in vitro*. [36]. Finally, RUN and FYVE domain–containing protein 4 (RUFY4) has also been suggested to promote the tethering of autophagosomes to lysosomes in an Interleukin-4 dependent manner [175].

Summary and future directions. *Tethering factors cross-link the autophagosomal membranes to late endosomal/lysosomal membranes. This is usually carried out by binding GTP-loaded small GTPases on the opposing surfaces. Additionally, some tethers also bind Atg8/LC3 family proteins and autophagosomal PI3P. The most well characterized tethering factor is the heterohexameric HOPS complex, which is able to bind - sometimes via adaptors - Rab7, Rab2 and Arl8. The Vps33A subunit of HOPS also functions as an SM protein, this way HOPS promotes SNARE complex assembly and zippering and prevents it from disassembly. HOPS is conserved from yeast to man, but interestingly, additional proteins have been found to promote the tethering of autophagosomes with lysosomes. These include EPG-5, TECPR1, ATG14, GRASP55, BRUCE and RUFY4. With the exception of EPG-5, these were identified in certain cultured human cell lines and it is not entirely clear yet whether these can really function as autophagosome-lysosome tethers in all cell types or in vivo. Further studies are required to address this.*

SNARE proteins execute the fusion of autophagosomes with lysosomes

About 40 Soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins are critical components of intracellular membrane fusions in mammalian cells. Based on the identity of the amino acid located at the center of their 60 amino acid long eponymous domain (often referred to as a zero layer in the assembled SNARE complex), they are grouped into Q-SNARE-s (aka target/t-SNAREs) and R-SNAREs (aka vesicular/v-SNAREs). Q-SNAREs can be further classified into Qa-, Qb-, and Qc-SNARE-s [176], with some of them containing both Qb- and Qc-SNARE motifs: the Qbc-SNAREs [177, 178]. During vesicle fusion, an R-SNARE located on the membrane of one vesicle and three different Q-SNAREs on the other form a trans-SNARE complex, which contains all four different SNARE domains: Qa, Qb, Qc and R. The zippering of these domains fuses the membranes, after which all of the SNAREs will be located on the same membrane. This cis-

SNARE complex is then recognized and disassembled by NSF (N-ethylmaleimide sensitive fusion protein) and α-SNAP (alpha soluble NSF-attachment protein) proteins for recycling [176, 178].

In 1992, Wada and colleagues identified vacuole morphology (vam) mutants in yeast, in which vacuolar fusions were impaired [179]. Seven of them later turned out to encode the 6 subunits of HOPS and Ypt7 (Rab7 in yeast) [126, 180]. Two others of the affected genes encode the Qa-SNARE Vam3 and Qc-SNARE Vam7, which, along with the later identified Qb-SNARE Vti1 (Vps10 Interacting) and R-SNARE Ykt6, have all been suggested to participate in the fusion of autophagosomes with the vacuole [181-185]. Accordingly, HOPS was shown to interact with Ykt6, the H_{abc} domain of Vam3 and the PX (Phox) domain of Vam7 to facilitate SNARE assembly [160, 186]. Two independent groups recently showed that Ykt6 is recruited to autophagosomes in yeast and it is able to form a trans-SNARE complex with vacuolar Vam3, Vam7 and Vti1 to facilitate the fusion of autophagosomes into the vacuole [187, 188]. Importantly, Vam3 and Vam7 have no clear homologues in metazoans, indicating that different SNARE complexes regulate autophagosome-lysosome fusion in these organisms.

Initially, the mammalian R-SNAREs Vamp8, Vamp7 (vesicle-associated membrane protein, not related to yeast Vam-s) and Qb-SNARE Vti1b were suggested to be involved in autophagosome-lysosome fusion [189, 190]. Another R-SNARE, Vamp3 (not related to yeast Vam3) was suggested to be required for multivesicular-body autophagosome fusion, hence for amphisome formation [189].

Later studies in mammalian and *Drosophila* cells made it clear, however, that the R-SNAREs Vamp8 and Vamp7 (*Drosophila* only has a single homologue of these that is called Vamp7), the autophagosomal Qa-SNARE Syntaxin17 (STX17/Syx17 in *Drosophila*) and the Qbc-SNARE SNAP-29 (aka. Ubisnap in *Drosophila*) form the SNARE complex that directly mediates autophagosome-lysosome fusion [164, 191, 192]. STX17 localizes to autophagosomes, Vamp7 or Vamp8 localize to lysosomes/late endosomes and by recruiting the cytosolic, two SNARE-domain containing SNAP-29, these SNARE proteins assemble into trans-SNARE complexes [164, 191, 192]. Besides their role in autophagosome-lysosome fusion, these proteins may also have roles in earlier stages of autophagy. Mammalian STX17 has been recently shown to be phosphorylated at Ser202 by TBK1 (TANK-binding kinase 1) to control formation of PAS [193]. STX17 has also been suggested to interact with Atg14 at ER-mitochondria contact sites where autophagosomes may form [194]. Additionally, VAMP7 has been suggested to interact with Syntaxin16 and SNAP-47 to fuse Atg9a-positive vesicles in order to promote autophagosome formation [195].

STX17 normally localizes to the ER and it rapidly relocates to LC3 positive autophagosomes upon starvation, which is mediated by its two transmembrane domains [192] This does not require the Atg8 conjugation system or the HOPS tethering complex, as depletion of neither could prevent the recruitment of STX17 to autophagic membranes [26, 139, 140]. However, STX17 association to autophagic vesicles may also be facilitated by Atg8 proteins, the small guanosine triphosphatase IRGM (immunity-related GTPase M) and Lamp2 [196, 197]. Importantly, the recruitment of HOPS appears to be promoted by STX17, which then facilitates trans-SNARE assembly and fusion [139, 140, 164]. In line with this, the interaction of both STX17 and Vamp7 with SNAP-29 decreases in Vps33A (the SM subunit of HOPS) depleted cells, based on FLIM-FRET experiments [164].

The N-terminal domain of STX17 seems to be an important factor for the assembly and function of the SNARE complex, as the overexpression of N-terminally tagged full length GFP-STX17 exhibits a dominant negative effect and causes accumulation of

autophagosomes [198]. The N-terminal peptide of STX17 contains a string of four negatively charged amino acid residues flanked by a phosphoserine site, and the expression of the phosphomimetic mutant form abolished the interaction of STX17 with the two other SNAREs and inhibited autophagosome clearance. In contrast, a non-phosphorylatable mutant had no effect on SNARE assembly [164]. The phosphomimetic mutant associated with VPS33A significantly more than the non-phosphorylatable or wild-type forms of STX17 [164]. Thus, VPS33A may associate with the phosphorylated STX17 N-peptide at the prefusion site, followed by dephosphorylation to enable SNARE bundle formation [164]. Interestingly, the phosphorylation status of this serine had no effect on the interaction of Atg14 with STX17 [164].

SNAP-29 can also be post-translationally modified by the addition of O-linked β-N-acetylglucosamine (O-GlcNAc) that is catalyzed by O-GlcNAc transferase (OGT). This modification decreases the interaction of SNAP-29 with STX17 and VAMP8. Accordingly, depletion of OGT promotes autophagic activity and starvation reduces SNAP-29 O-GlcNAcylation levels [199]. The rate of fusion can also be modulated by altering the protein levels of these SNAREs: the amounts of intracellular STX17 and SNAP-29 appear to be regulated by the 20S proteasome in an ubiquitin independent manner [200].

In human cells, Vamp7 has several isoforms and interestingly, the SNARE domain of Vamp7B is not functional. Thus, Vamp7B acts as an inhibitory protein by competing with Vamp7A for the SNARE domain of STX17. Lysosome localized DIPK2A (divergent protein kinase domain 2A) interacts with both VAMP7B and STX17 and it is able to promote autophagosome-lysosome fusion by decreasing the amount of free VAMP7B, thus increasing the ratio of VAMP7A-containing fusogenic complexes [201]. The subcellular localization of Vamp7 is regulated by the atypical SNARE SNAP-47 [202] and accordingly, autophagic flux is inhibited in SNAP-47 siRNA cells [203].

YKT6 is also involved in autophagosome-lysosome fusion in metazoan cells. In *Drosophila*, Ykt6 is suggested to act as a regulatory SNARE by forming a fusion incompetent prefusion SNARE complex together with Syx17 and Snap-29, later to be replaced by Vamp7 to form a fusion competent complex [204]. In mammals, YKT6 localizes to autophagosomes and forms a fusion competent complex with SNAP-29 and the Qa-SNARE STX7, which may act in parallel to the STX17-SNAP-29-VAMP7/VAMP8 complexes. YKT6 is also essential for Golgi function but its recruitment to autophagosomes depends on its N-terminal longin domain rather than the C-terminal palmitoylation and farnesylation sites that are essential for its Golgi localization [205]. It is not clear why mammals have two autophagic SNARE complexes, but the phenotype of the loss of the autophagic SNAREs YKT6 or STX17 seems to be additive [205]. While this raises the possibility that two populations of autophagosomes (STX17+ vs YKT6+) might be found in mammalian cells, this seems rather unlikely as STX17 and YKT6 colocalize [205]. However, given that yeast autophagosomes are smaller than their mammalian counterparts [206], Kriegenburg and colleagues point out that the lipid anchor of YKT6 could be better suited for the smaller autophagosomes (possibly transporting selective cargo), unlike the two transmembrane domain containing STX17, which might preferably integrate into larger, bulk autophagosomes [207]. This contradiction may be resolved if both SNAREs are targeted to all forming autophagosomes, but on smaller ones YKT6 may be more important than STX17, which on the other hand may fuse larger autophagosomes and lysosomes more effectively. Besides the above-mentioned possibilities, Yong and Tang suggest in their recent review that Ykt6 could mediate fusion on its own by engaging SNAP29 and STX7, albeit at a low basal level and when autophagy is induced for example by starvation, STX17 could take over as the main SNARE that drives

fusion [208]. Alternatively, YKT6 and STX17 may provide the selectivity for fusion with either lysosomes or late endosomes [207]. Further studies are needed to address these questions.

In addition to these, the STX6-VTI1B-VAMP3 complex facilitates the fusion between Group A Streptococcus (GAS) containing autophagosome-like vacuoles and recycling endosomes, which is required for the subsequent clearance of intracellular GAS by autophagy [209]

The disassembly of cis-SNARE complexes and the recycling of the SNAREs are also crucial for fusion, as depletion of α-SNAP or NSF proteins or the expression of an NSF dominant negative mutant version lead to decreased autophagic flux, even though the formation of autophagosomes remains largely unaffected [190, 210]. It is important to note that α-SNAP (aka Sec17 in yeast) appears to promote vesicle fusion directly, as its N-terminal hydrophobic loop can penetrate into the membrane to facilitate the fusion reaction [211, 212]. Trans-SNARE complexes are also recognized by α-SNAP and are subject of disassembly. Importantly, this is inhibited by HOPS, which prevents the disassembly of properly assembled trans-SNARE complexes during membrane fusion in yeast [165]. This way, HOPS may have proofreading activity by allowing the disassembly of improper SNARE complexes [213].

Besides these, there are other autophagy related fusion events mediated by SNAREs. For example, STX17-containing mitochondrial-derived vesicles are able to directly fuse with lysosomes in a STX17-SNAP-29-VAMP7 and HOPS dependent manner [214]. Crinophagy is another special form of autophagy, in which excess secretory granules directly fuse with lysosomes. The developmentally programmed crinophagy of glue granules in the *Drosophila* salivary gland is mainly Atg gene-independent and mostly relies on the same small GTPases and tethering proteins discussed above, with one important exception: instead of a Syx17 containing SNARE complex, crinophagy is executed by a Syx13-Snap-29-Vamp7 complex [81, 143]. As Syx13 has no clear homologue in mammals, it will be interesting to discover which SNARE is crinophagy specific in mammals.

Summary and future directions. *SNARE complexes execute autophagosome-lysosome* fusion *from yeast to human cells*. *However, these SNARE complexes are not conserved. Ykt6 is recruited to autophagosomes in yeast to form a trans-SNARE complex with vacuolar Vam3, Vam7 and Vti1. In fruit flies, autophagosomal Syx17 binds the two SNARE domain containing, thus cytosolic Snap-29, which also binds to Vamp7 located on late endosomes or lysosomes. Ykt6 is also required for autophagosome-lysosome fusion here, possibly by facilitating the formation of the former complex. In contrast, at least three different SNARE complexes have been identified to promote autophagosome-lysosome fusion in mammalian cells. STX17 may form two similar complexes, both containing Snap-29 and either Vamp7 or Vamp8. An alternative complex also exist that contains Snap-29, Ykt6 (on autophagosomes) and STX7 (on lysosomes/endosomes), which likely act in parallel to the STX17 containing ones. An important question of the field is how the autophagosomal SNARE STX17/Syx17 is recruited to this organelle, as its two transmembrane domains are sufficient to mediate its translocation to the autophagosome from the cytosol [192]. Also, it is not clear why multiple SNARE complexes act in parallel in mammals, as in invertebrates one combination of SNAREs is necessary and sufficient to fuse autophagosomes with lysosomes.*

The lipid composition of the autophagosome regulates autophagosome-lysosome fusion.

It is clear that the global lipid content of the cell affects autophagosome-lysosome fusion as we discussed about the cholesterol sensor ORP1L [97, 99, 102]. In addition, too much lipid intake can be disadvantageous, as high fat diet usually leads to inhibited autophagic flux *in vivo* [215, 216].

A series of events take place in a well-defined order during autophagosome maturation and so the protein and lipid composition of the autophagosome dynamically changes during its lifetime. One of the most typical lipids involved in autophagosome formation is PI(3)P, which is generated by the Atg14 containing PI3K complex both in yeast and metazoan organisms [72, 217-221]. Several proteins harboring FYVE (for example ESCRT), PX (for example Vam7) or PROPPIN (for example Atg18) domains can bind PI(3)P to drive the maturation of PI(3)P positive autophagic vesicles or endosomes (in this latter case, endosomal PI(3)P is generated by the UVRAG containing PI3K complex) [72, 222-226]. The Mon1-Ccz1 complex also binds both PI(3)P and Atg8 and it is required for autophagosome-lysosome fusion by recruiting Rab7 to autophagosomes [59, 66, 70, 71]. This way, PI(3)P is critical not only for autophagosome formation but also for subsequent fusion. After these events PI(3)P must be removed from the autophagosomal membranes, which is facilitated by the sole yeast PI(3)P phosphatase Ymr1 (Yeast Myotubularin Related 1) to allow autophagosome-lysosome fusion [227]. In higher organisms, there are several PI(3)P phosphatases called Myotubularins (MTMRs), but most of them antagonize the PI(3)P production of PI3K(III) and thus block autophagosome formation rather than modulating its maturation [228-230]. However, MTM-3, the closest homolog of Ymr1 in *C. elegans* (and of MTMR4 in mammals) promotes autophagosome maturation as autophagosomes fail to fuse with lysosomes in *mtm-3* mutants [231]. It would be interesting to know whether the function of this phosphatase is conserved or worm specific.

$PI(3,5)P_2$ is a lysosomal membrane phospholipid required for autophagosome clearance. The PIKfyve/Fab1 PI(3)P 5-kinase complex is responsible for the synthesis of $PI(3,5)P_2$ from PI(3)P and while it is dispensable for autophagy in yeast [232], its depletion inhibits autophagic flux in mammalian cells [233, 234] and leads to the accumulation of Atg8/LC3 positive autophagosomes in fly and worm cells [45, 235]. The amount of $PI(3,5)_2$ is negatively regulated by Inositol polyphosphate-5 phosphatase E (INPP5E), which converts $PI(3,5)P_2$ back to PI(3)P and its activity is also required for successful autophagosome-lysosome fusion [122]. This is perhaps due to the increase of PI(3)P and the decrease of $PI(3,5)P_2$ and INPP5E on lysosomes, leading to the phosphorylation and activation of cortactin, which then binds and stabilizes lysosome associated actin filaments. These filaments may then facilitate autophagosome-lysosome fusion [122]. Taken together, these results suggest that the correct amount and ratio of PI(3)P and $PI(3,5)P_2$ in autophagosomal and lysosomal membranes, respectively, are essential for autolysosome formation.

Autophagosomal membranes also become positive for PI(4)P via the action of phosphatidylinositol 4-kinase IIα (PI4KIIα or PI4K2A), which was also shown to be required for autophagosome-lysosome fusion [28]. It is the GABARAP proteins that recruit palmitoylated PI4KIIα to autophagosomes upon autophagy induction to produce PI(4)P. Accordingly, PI4K2A depletion or the overexpression its kinase-dead, dominant negative form leads to the buildup of autophagosomes, inhibits autophagic flux and impairs autophagosome-lysosome fusion [28]. RAB7 positive late endosomes/lysosomes are also enriched with PI(4)P by PI4K2A. Phosphatidylinositol 4-Phosphate-5 kinase γ (PI4P5Kγ or PIP5Kγ) then converts PI(4)P to $PI(4,5)P_2$, leading to RAB7 inactivation and release from the membranes along with its effector PLEKHM1. Accordingly, simultaneous depletion of both PI4K2A and PIP5Kγ leads to a RAB7 cycling defect and impaired autophagosome-lysosome fusion [236].

Summary and future directions. *While a lot of (perhaps most) proteins involved in autophagosome formation and fusion are known, we know much less about how autophagosomal or lysosomal membrane lipids regulate autophagosome maturation. Of all the membrane lipids, the phosphoinositides PI(3)P, PI(3,5)P_2, PI(4)P and PI(4,5)P_2 have been found to be involved in autophagosome-lysosome fusion. It is important to emphasize that very little is known about the overall lipid composition of autophagosomal membranes, so this should be explored in future studies.*

Impaired autophagosome clearance causes neurodegeneration and other disorders.

As autophagy is essential for cellular homeostasis, impaired autophagy can lead to multiple diseases [237, 238]. Accordingly, the loss of several genes encoding proteins with a role in autophagosome-lysosome fusion have been linked to various diseases. Mutations affecting the gene encoding the tethering factor/adaptor EPG-5 leads to Vici syndrome, characterized by agenesis of the corpus callosum, cataracts, retinitis pigmentosa, cardiomyopathy and immunodeficiency [239-241]. Mutations affecting Rab7 lead to Charcot-Marie-Tooth type 2B neuropathy [242]. Mutations in Rab7 interactors can also cause diseases, for example PLEKHM1 mutations lead to osteopetrosis [243]. Mutations of the HOPS subunit VPS11 induce autosomal recessive leukoencephalopathy in humans and neuroaxonal dystrophy in dogs [244, 245] and VPS33A mutations cause a lysosomal storage disorder: mucopolysaccharidosis-plus syndrome [246, 247]. One base pair deletions or insertions in SNAP-29 leading to premature termination cause the very severe CEDNIK (cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma) syndrome [248, 249]. Finally, PIKfyve mutations can cause François-Neetens mouchetée fleck corneal dystrophy [250].

As the gene products listed above all participate in endosomal-lysosomal processes other than autophagy, possibly lysosomal dysfunction or impaired endosomal traffic is also important in these diseases. However, Syx17 is not required for endocytic degradation in *Drosophila* and mutant flies still displayed clear locomotion defects and neurodegeneration [191]. Therefore it is most likely that combined effects in autophagy and endocytic traffic/degradation are involved in the development of these syndromes.

Nonetheless, one clear exception is known. Mutations affecting INPP5E lead to Joubert syndrome 1, which is a brain disorder characterized by several neurodevelopmental defects [251]. INPP5E is not required for lysosomal integrity or fusion between endosomes and lysosomes. When the Joubert syndrome 1 mutant form of this protein was expressed in INPP5E knock-down neurons, the protein could not rescue the autophagy defect, suggesting that impaired autophagic flux may be indeed the cause of the disease [122].

With next-generation sequencing becoming relatively cheap and straightforward to do, large-scale analyses of rare genetic diseases and cancer subtypes will likely identify additional patients whose symptoms are at least in part due to a defect in autophagosome-lysosome fusion or in lysosomal degradation.

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Figure legends

Figure 1. Main factors involved in autophagosome-lysosome fusion in human cells.

The fusion of the autophagosome (AP) with a late endosome (LE) or lysosome (Ly) is carried out by specific SNARE proteins, small GTPases and their effectors including tethers, adaptors and motor proteins, with the help of additional factors shown in this model. See main text for details.

Figure 2. Autophagosomes accumulate upon inhibition of autophagosome-lysosome fusion.

Knockdown of the HOPS subunit Vps39 leads to accumulation of Atg8a puncta (representing autophagosomes) in GFP-positive cells in the fat tissue of a starved mosaic *Drosophila* larva. GFP negative neighboring cells serve as built-in controls. Right panel shows the anti-Atg8a (red) channel separately.

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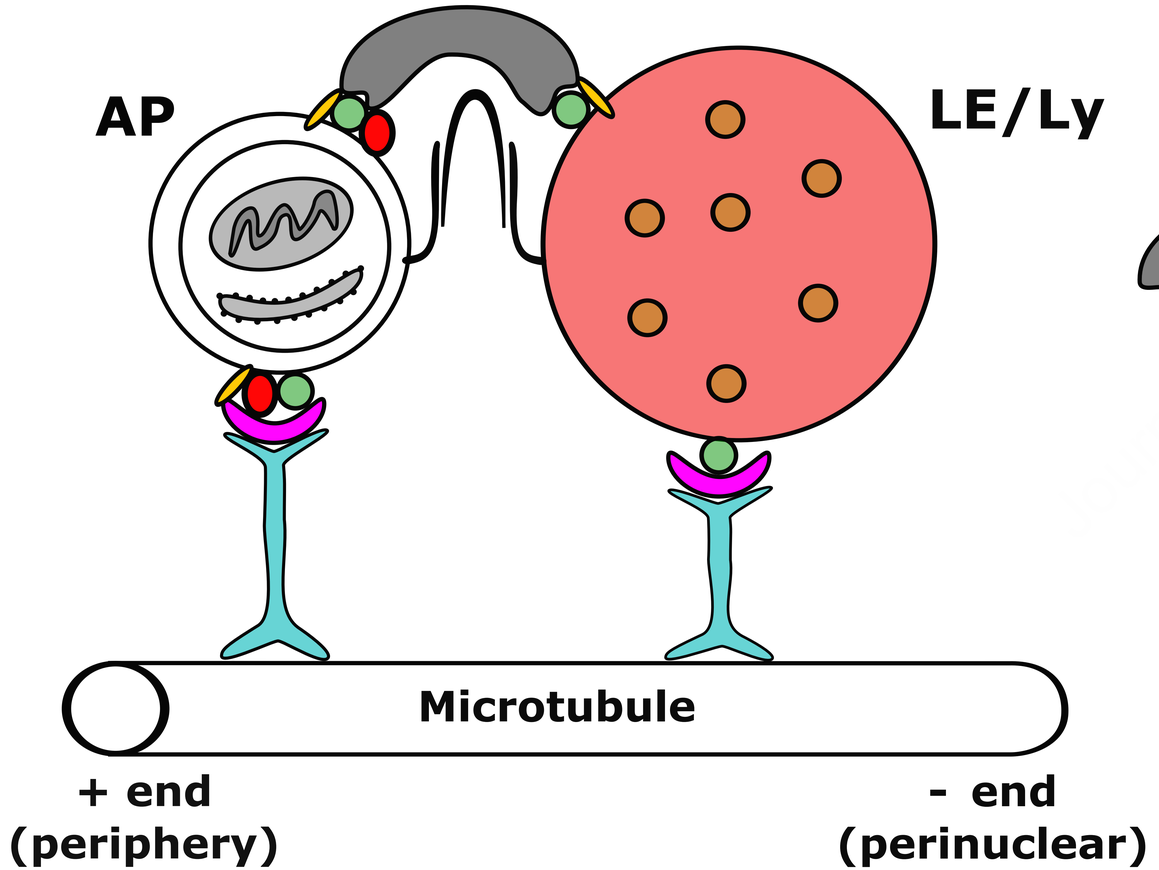
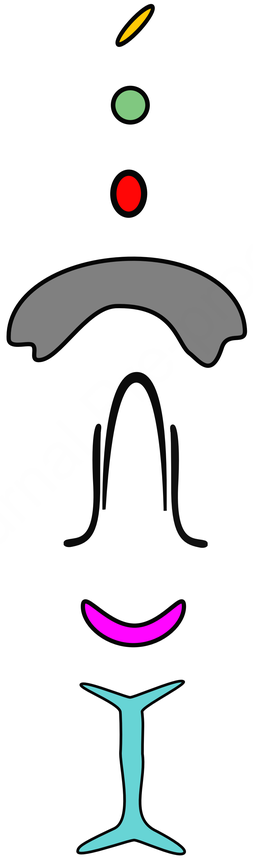




Phosphoinositides: PI(3)P; PI(3,5)P_2; PI(4)P

Small GTPases: RAB7A; RAB2A; ARL8A/B

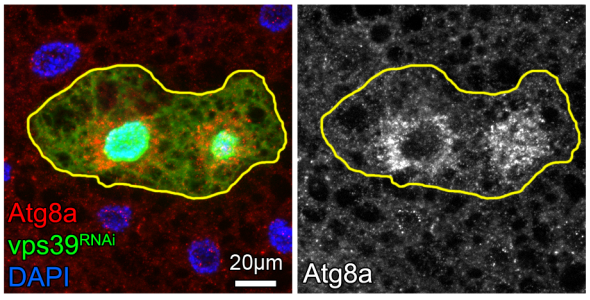
Atg8 family proteins: LC3s and GABARAPs

Tethers, adaptors: HOPS; EPG-5; ATG14L; TECPR1; GRASP55; BRUCE; RUFY4

SNARE complexes: STX17-SNAP29-VAMP7
STX17-SNAP29-VAMP8
YKT6-SNAP29-STX7

Motor adaptors: BORC; FYCO1; PLEKHM1; PLEKHM2/SKIP; RILP; ORP1L

Motors: **+ end motors** (kinesins)
- end motors (dynein-dynactin)



Highlights:

- Autophagosome closure and lysosome acidification may not be required for fusion
- Multiple small GTPases promote autophagosome-lysosome fusion
- Motor proteins ensure that autophagosomes encounter lysosomes
- Tethers and adaptors anchor autophagosomes to endosomes or lysosomes
- SNARE proteins execute the fusion of autophagosomes with lysosomes
- The lipid composition of the autophagosome regulates autophagosome-lysosome fusion