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COMMENTARY

New insights into autophagosome–lysosome fusion

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ABSTRACT

Macroautophagy (autophagy) is a highly conserved intracellular degradation system that is essential for homeostasis in eukaryotic cells. Due to the wide variety of the cytoplasmic targets of autophagy, its dysregulation is associated with many diseases in humans, such as neurodegenerative diseases, heart disease and cancer. During autophagy, cytoplasmic materials are sequestered by the autophagosome – a double-membraned structure – and transported to the lysosome for digestion. The specific stages of autophagy are induction, formation of the isolation membrane (phagophore), formation and maturation of the autophagosome and, finally, fusion with a late endosome or lysosome. Although there are significant insights into each of these steps, the mechanisms of autophagosome–lysosome fusion are least understood, although there have been several recent advances. In this Commentary, we will summarize the current knowledge regarding autophagosome–lysosome fusion, focusing on mammals, and discuss the remaining questions and future directions of the field.

KEY WORDS: Phosphoinositides, Autophagy, Fusion, Lysosome

Introduction

Macroautophagy, hereafter referred to as autophagy, is a catabolic process that targets a wide variety of cellular components including proteins, lipids, damaged organelles and pathogens. Autophagy normally occurs at a basal level, but it is accelerated by a variety of stresses such as starvation, accumulation of abnormal proteins, organelle damage and pathogen infection. Autophagy was originally considered to be a bulk, non-selective degradation system; however, it is now known that autophagy selectively degrades targets and contributes to intracellular homeostasis (Kawabata and Yoshimori, 2016). During autophagy, a small cisterna, called the isolation membrane (phagophore), elongates and surrounds a part of the cytoplasm to form a double-membraned structure, called the autophagosome. Autophagosomes either fuse with late endosomes to form amphisomes, which then fuse with lysosomes, or they fuse directly with lysosomes (Berg et al., 1998; Fader et al., 2008). After fusion with the lysosome, they are called autolysosomes and the sequestered contents are digested (Fig. 1).

Since the identification of autophagy-related genes (ATGs) in yeast (Tsukada and Ohsumi, 1993), the functions of their homologs have been identified and extensively studied, especially in mice and mammalian cells (Mizushima et al., 2011). Briefly, activation of the unc-51-like kinase 1 (ULK1; Atg1 in yeast) complex is crucial for the initiation of autophagy. Then, activation of the class III phosphatidylinositol 3-kinase complex, which comprises PI3K (Vps34 in yeast), beclin 1, VPS15 (PIK3R4) and ATG14L (ATG14), triggers vesicle nucleation. The subsequent elongation and closure of the isolation membrane are mediated by two ubiquitin-like ATG conjugation pathways, ATG5-ATG12 and LC3/Atg8. In mammals, there are seven Atg8 orthologues; MAP1LC3A/B/C, GABARAP and GABARAPL1/2/3 (all of which are hereafter referred to as LC3). LC3 is widely used as a marker for the microscopic detection of isolation membranes and autophagosomes. After synthesis, LC3 is processed at its C terminus by Atg4 and becomes LC3-I, which has a glycine residue at the C-terminal end. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II by a ubiquitination-like enzymatic reaction. In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome. PE-conjugated LC3 (LC3-II) and unconjugated LC3 (LC3-I) can be detected separately by immunoblot analysis, and the amount of LC3-II is also widely used for the quantification of autophagic activity (Kabeya et al., 2000).

Although the extensive characterization of ATG genes has yielded insights into the mechanisms of autophagy activation and autophagosome formation, how the fusion of autophagosomes with endosomes and/or lysosomes is controlled remains poorly understood. Nevertheless, recent studies have started to uncover the molecular mechanisms that regulate the fusion steps. Several experimental approaches have contributed to identifying the conditions that are necessary for the autophagosome–lysosome fusion step to occur. For instance, the V-ATPase inhibitor bafilomycin A1 (BafA1), a macrolide antibiotic derived from *Streptomyces griseus*, which blocks degradation in autolysosomes and/or autophagosome–lysosome fusion (Klionsky et al., 2008; Yamamoto et al., 1998; Yoshimori et al., 1991), and LC3 tandem tagged with RFP and GFP (RFP-GFP–LC3), which loses its GFP fluorescence after fusion with the lysosome, have facilitated to detect the blockage of autophagosomal fusion (Kimura et al., 2007). In this Commentary, we survey recent findings with regard to the molecular mechanisms underlying the autophagosome–lysosome fusion step, with a focus on mammalian studies, and also discuss future perspectives for the field.

Completion of autophagosomes

The timing of autophagosome–lysosome fusion is very important and only the closed autophagosomes can fuse with lysosomes. This raises the question how the closure of autophagosomes is regulated. In mammals, a defect in the ATG-conjugation system results in accumulation of unclosed autophagosomes (Fujita et al., 2008a; Kishi-Itakura et al., 2014; Mizushima et al., 2001; Sou et al., 2008), implying that it is likely to function in elongation and closure of autophagosomes, and is important for transition of the isolation membrane into the autophagosome. In addition to its role in autophagosome maturation, the ATG conjugation system (consisting of ATG3, ATG5 and ATG7) has been recently shown

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Received 3 January 2017; Accepted 9 February 2017

Journal of Cell Science (2017) 0, 1-8 doi:10.1242/jcs.196352

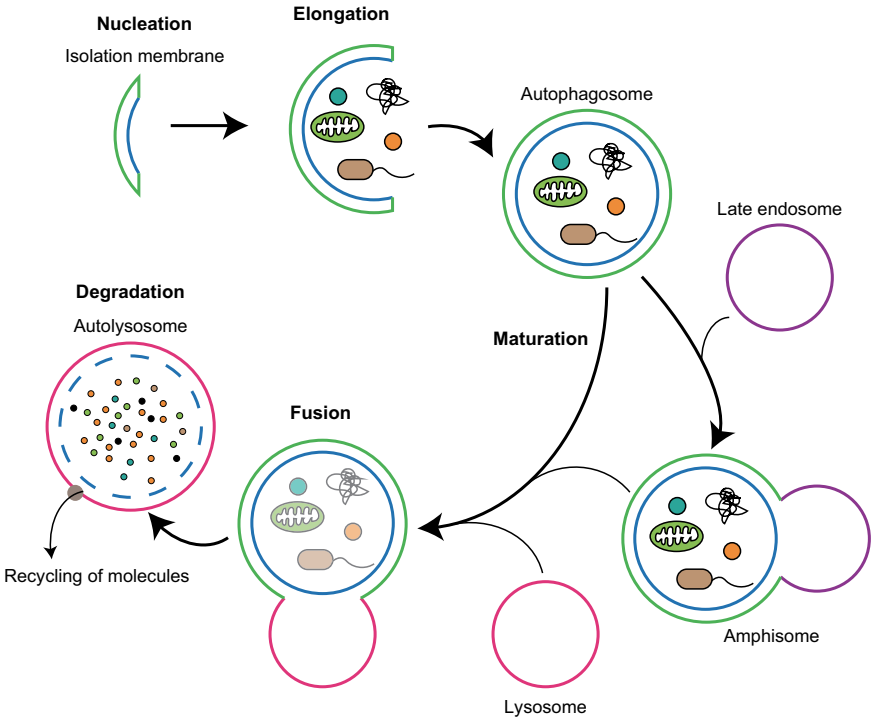

Fig. 1. Overview of autophagy. Upon induction of autophagy by stress, cytoplasmic materials are sequestered by a double-membraned structure, called autophagosome. These autophagosomes fuse with late endosomes (termed amphisomes) or lysosomes to become autolysosomes, in which the sequestered cargos are degraded and recycled for the maintenance of cellular homeostasis. Autophagy can be divided into several steps: formation of the isolation membrane (nucleation), elongation of the isolation membrane (elongation), completion and transport of the autophagosome (maturation), docking and fusion between autophagosome and lysosome (fusion), and degradation of the cargos inside the autolysosome (degradation).

to be required for efficient degradation of the inner autophagic membrane; however, it is not required for autophagosome–lysosome fusion, although the rate of autophagosome formation is reduced to ∼30% in ATG conjugation-deficient cells (Tsuboyama et al., 2016). By contrast, another study that was using cell lines in which the entire ATG8 protein family had been knocked out revealed that LC3 and GABARAP proteins are not required for autophagosome formation, but are crucial for autophagosome–lysosome fusion (Nguyen et al., 2016). The lack of fusion is probably due to the impaired recruitment of the adaptor protein PLEKHM1 (McEwan et al., 2015) (see also below) to autophagosomes. The inconsistency between these two studies might reflect the non-overlapping function of the ATG conjugation system and LC3 and GABARAP. Why only the closed autophagosomes are recognized by several fusion factors (discussed below) is currently unclear.

Movement of autophagosomes and lysosomes

The cytoskeleton has many functions, including the structural maintenance of cells, cell division and movement. Although microtubules are dispensable for autophagy in yeast (Kirisako et al., 1999), they are essential for the fusion step in mammals (Aplin et al., 1992; Kochl et al., 2006; Monastyrska et al., 2009). Autophagosomes are thought to form randomly throughout the cytoplasm, whereas late endosomes and lysosomes are predominantly found in the perinuclear region. Therefore, once complete and closed autophagosomes have been generated, they need to be delivered to the perinuclear region. The minus-end-directed dynein–dynactin motor complex moves cargo to the perinuclear region, whereas most kinesins are plus-end-directed motor proteins that drive their cargo towards the cell periphery (Gross et al., 2007). Given that lysosomes localize to the perinuclear regions, the minus-end-directed transport of autophagosomes appears reasonable and, indeed, live-imaging shows that mature autophagosomes move along microtubule tracks towards the lysosomes (Kimura et al., 2008). The efficient movement of autophagosomes is inhibited by microinjection of antibodies against LC3, suggesting a role for LC3 during this process (Kimura et al., 2008). In addition, kinesin-dependent plus-end-directed transport is essential for the correct positioning of autophagosomes because the depletion of the kinesin KIF5B blocks autophagy and results in perinuclear clustering of autophagosomes (Cardoso et al., 2009). Similarly to mammals, in *Drosophila*, the PX-domain-containing kinesin Klp98A controls the formation, fusion and intracellular positioning of autophagic vesicles (Mauvezin et al., 2016).

Interestingly, the localization of lysosomes determines the rate of autophagosomal fusion. Increasing the perinuclear localization of lysosomes by depletion of kinesins KIF1B-β and KIF2A leads to increased autophagosomal fusion, whereas dispersion of lysosomes to the periphery by overexpressing the motors reduces fusion rates (Korolchuk et al., 2011). Thus, the coordinated transport of both autophagosomes and lysosomes is essential for fusion; but how are autophagosomes and lysosomes connected to microtubules? The small GTPase Rab7, which acts as a molecular switch and, presumably, is recruited to late autophagosomes (Gutierrez et al., 2004), links autophagosomes to microtubule motors through FYCO1 (FYVE and coiled-coil domain-containing 1), thereby mediating kinesin-driven movement towards the cell periphery (see below) (Pankiv et al., 2010). Rab7 also works in the reverse direction by interacting with Rab-interacting lysosomal protein (RILP), the cholesterol sensor ORP1L (also known as OSBPL1A) and dynein, in order to facilitate transport of autophagosomes, autolysosomes and lysosomes to the perinuclear region (Jordens et al., 2001; Wijdeven et al., 2016) (Fig. 2).

Similarly to microtubules, actin filaments form tracks to move various intracellular cargos by using the myosin family of motor proteins. Several pieces of evidence suggest that actin is involved in autophagosome–lysosome fusion. For instance, histone deacetylase 6 (HDAC6) recruits the cortactin-dependent actin remodeling machinery, which in turn assembles the actin network that stimulates autophagosome–lysosome fusion (Lee et al., 2010). Interestingly, HDAC6 and actin assembly are dispensable for

Journal of Cell Science (2017) 0, 1-8 doi:10.1242/jcs.196352

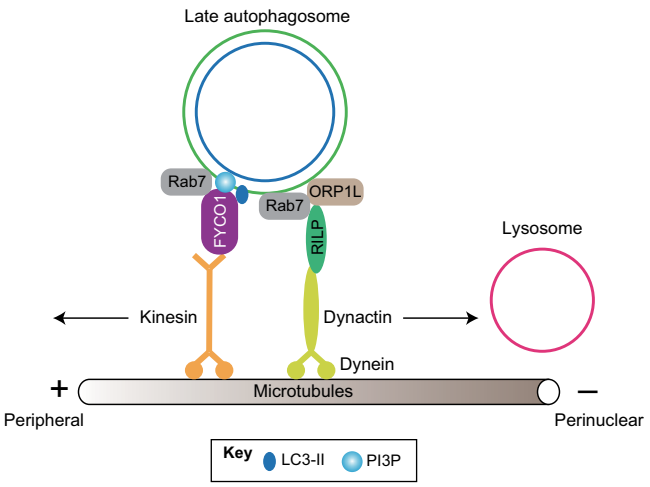

Fig. 2. Transport of autophagosomes. Rab7 GTPase links autophagosomes to a microtubule motor through FYCO1 to facilitate kinesin-driven movement towards the cell periphery. FYCO1 has been identified to bind to LC3, but it also binds to the phospholipid PI3P, a component of the autophagosome membrane. Rab7 also binds to RILP and ORP1L in order to mediate dynein and/or dynactin-driven movement towards the perinuclear region under normal cholesterol conditions. When levels of cholesterol are low, ORP1L forms a contact site with VAP-A, which prevents dynactin recruitment and blocks minus-end transport (not shown in this figure). Lysosomal positioning also determines the rate of autophagosome–lysosome fusion.

starvation-induced autophagy, but are required for the selective degradation of aggregated proteins (Lee et al., 2010). Moreover, the actin motor myosin VI and Tom1, a component of the ESCRT machinery and binding partner of myosin V1 on endosomes, are involved in fusion, as the loss of both factors reduces autophagosomal delivery of endocytic cargo and blocks autophagosome–lysosome fusion (Tumbarello et al., 2012).

Fusion of autophagosome and lysosome – Rabs, SNAREs and tethering factors

After autophagosomes arrive at their destination, they fuse with the endocytic system. However, it is difficult to distinguish the movement from the actual fusion, most likely because these two processes seem to occur almost simultaneously. Currently, our knowledge of the machinery involved in this process is based on the general understanding of intracellular membrane trafficking, particularly with regard to three sets of protein families: Rab GTPases, membrane-tethering complexes and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). Rab proteins localize to specific membranes and recruit tethering complexes that act as bridges to bring the compartments intended for fusion together. These tethering complexes, in turn, help SNARE proteins to physically drive the fusion of opposing lipid bilayers.

Rab proteins

The small GTPases of the Ras-related protein in brain (Rab) family are evolutionally conserved, crucial regulators of membrane trafficking in eukaryotic cells. They recruit specific effector proteins, such as cargo adaptors to form transport vesicles, motor proteins to move the vesicle to its target membrane, as well as tethering proteins to aid the fusion machinery when the vesicular cargo reaches its destination (Stenmark, 2009; Zhen and Stenmark, 2015). Each Rab protein localizes to a distinct membrane compartment and, by doing so, Rabs are thought to provide specificity to membrane trafficking. Membrane-associated Rabs are activated by specific guanine nucleotide exchange factors (GEFs) that drive GTP binding. Upon binding GTP, Rabs conformationally change to interact with their effector proteins. Subsequently, Rabs are inactivated by specific GTPase-activating proteins (GAPs) that hydrolyze the bound GTP to GDP, causing loss of effector binding and extraction from the membranes.

It has been suggested that some members of the Rab family regulate autophagy. Rab7, which is localized on late endosomes and lysosomes, and is essential for subsequent endocytic membrane trafficking from late endosome to lysosome, is also important for autophagosome–lysosome fusion and the subsequent degradation of autophagosomal contents (Gutierrez et al., 2004; Jager et al., 2004; Kirisako et al., 1999). Rab7 might be also recruited to late autophagosomes. Gutierrez et al. showed that, upon induction of autophagy, an increase in the labeling intensity of Rab7 is observed on the autophagic vacuole (notice that the term autophagic vacuole refers to nascent autophagosomes and autophagosomes that have fused with late endosomes and lysosomes) because Rab7 staining on late autophagic vacuole is stronger than that on early autophagic vacuoles (based on by immunofluorescence microscopy and immunoelectron microscopy) (Gutierrez et al., 2004). The group also claimed that Rab7 delivery to autophagosomes is detected before fusion with a LAMP-1-positive compartment. Knockdown of Rab7 causes accumulation of late autophagic vacuoles, indicating that Rab7 function is only needed for the final maturation of late autophagic vacuoles, probably the fusion with lysosomes (Gutierrez et al., 2004; Jager et al., 2004). Interestingly, Rab7 is involved in the formation of GAS-containing autophagosome-like structures (GcAVs) that sequester invading group A streptococcus, (Yamaguchi et al., 2009). Rab7 also functions in isolation membrane expansion during mitophagy (Yamano et al., 2014). These results indicate that Rab7 functions during the early phase of selective types of autophagy. Owing to such multiple and overlapping roles, it has proven difficult to determine the specific role of Rabs during autophagosome–lysosome fusion. Nevertheless, thapsigargin, an ER stressor widely used to induce autophagy, blocks the recruitment of Rab7 to mature autophagosomes and inhibits their fusion with endocytic vesicles without affecting endocytosis, highlighting the vital role of Rab7 in the fusion step (Ganley et al., 2011).

In mammals, a GEF that activates Rab7 for fusion has not been identified. In *Drosophila* fat cells, the guanosine exchange complex of Ccz1 and Mon1 (Ccz1−Mon1) recruits Rab7 to PI3P-positive autophagosomes (Hegedus et al., 2016), and loss of the Ccz1–Mon1–Rab7 complex impairs autophagosome–lysosome fusion. Here, Rab5 recruits Ccz1−Mon1 to endosomes in order to activate Rab7, which facilitates endosome maturation and fusion with the lysosome. However, Rab5-null mutants exhibit normal autophagosome–lysosome fusion, and Rab5 is dispensable for Ccz1–Mon1-dependent recruitment of Rab7 (Hegedus et al., 2016).

Two components of the PI3K complex, UV radiation resistance-associated (UVRAG) and Rubicon (RUBCN) are involved in endocytic transport, autophagosome maturation and/or autophagosome–lysosome fusion through Rab7 (Liang et al., 2008; Matsunaga et al., 2009; Tabata et al., 2010; Zhong et al., 2009), although they have opposite effects. UVRAG promotes autophagosome–lysosome fusion, whereas Rubicon inhibits it. UVRAG, which localizes to the endoplasmic reticulum and endosomes, binds to VPS16, a subunit of the homotypic fusion and protein sorting (HOPS) complex (Liang et al., 2008) to stimulate Rab7 GTPase activity and autophagosome–lysosome fusion, whereas Rubicon binds to UVRAG and negatively regulates

VPS34 activity (Sun et al., 2011). Under nutrient-rich conditions, UVRAG is phosphorylated by mechanistic target of rapamcycin complex 1 (mTORC1) (Kim et al., 2015), which enhances the interaction with Rubicon and impairs VPS34 kinase activity, as well as the interaction between UVRAG and the HOPS complex, thus affecting autophagosome maturation. Prevention of UVRAG phosphorylation increases the rate of autophagosome maturation and lysosomal degradation, indicating that mTORC1 not only regulates the induction of autophagy but also facilitates fusion through UVRAG. However, the function of UVRAG during fusion is controversial because another study found that UVRAG neither interacts with HOPS nor regulates autophagosome–lysosome fusion (Jiang et al., 2014). Similarly, UVRAG is dispensable for autophagosome–lysosome fusion in *Drosophila* (Takats et al., 2014). Further studies are needed to clarify the function of UVRAG during the fusion.

An active, GTP-bound Rab protein binds to various effectors that usually regulate vesicle motility and fusion with the correct membrane compartment. Recent findings suggest that PLEKHM1, which has originally been identified as a Rubicon homolog, functions as a Rab7 effector and is involved in autophagosome–lysosome fusion through Rab7, the HOPS complex, and LC3 and/or GABARAP (McEwan et al., 2015; Tabata et al., 2010) (Fig. 3). Several other Rab7 effectors have been characterized, such as RILP and FYCO1, both of which function in autophagosome transport (see above and below).

In addition to Rab7, Rab33b is known to regulate the fusion step. Rab33b is a Rab protein localized at the Golgi complex that plays a role in autophagosome formation through interaction with ATG16 (Itoh et al., 2011). Its GAP ornithine aminotransferase-like 1 (OATL1, also known as TBC1D25) is recruited onto autophagosomes through direct interaction with ATG8, and OATL1 overexpression has been shown to inhibit autophagosome–lysosome fusion (Itoh et al., 2011). It has been recently shown that during T-tubule remodeling in *Drosophila*, Rab2 localizes to completed autophagosomes and interacts with the HOPS complex to promote autophagosome–lysosome fusion (Fujita et al., 2017). These results suggest that, although Rab7 plays a pivotal role during autophagosome–lysosome fusion, further small GTPases are involved in this process and other – so-far unappreciated – functions of members of this protein family might be discovered in future.

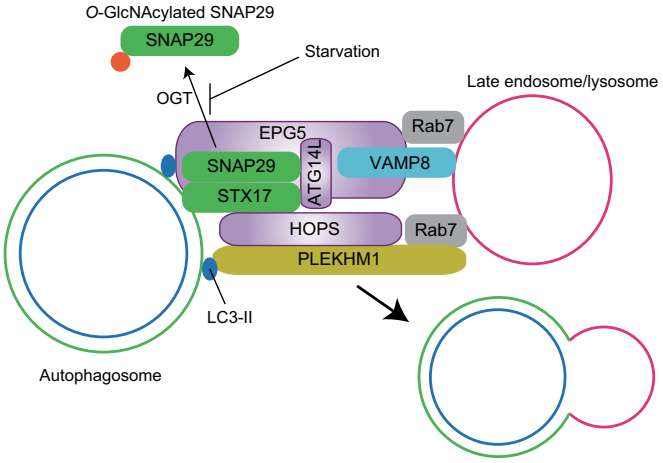

Fig. 3. Role of SNAREs in mediating autophagosome–lysosome fusion. EPG5 is recruited to late endosomes/lysosomes together with Rab7 and VAMP-8, where it tethers late endosomes/lysosomes by binding to LC3 and STX17–SNAP29; this facilitates the assembly of the trans-SNARE complex for fusion. In contrast to EPG5, ATG14L binds to STX17 and STX17–SNAP29 Qabc intermediate SNARE complexes on autophagosomes, but not to STX17–SNAP29-VAMP8 RQabc SNARE complexes, suggesting that ATG14L acts earlier than EPG5. PLEKHM1 is an adaptor protein that interacts with Rab7, HOPS–SNARE complexes and LC3 and/or GABARAP proteins to facilitate autophagosome–lysosome fusion. EPG5, ATG14L and the HOPS complex function as tethering factors, but their exact relationship still needs to be clarified. *O*-GlcNAcylated SNAP-29 which is generated by O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT), has a reduced binding affinity for its partner SNAREs. This modification is suppressed by starvation, and a reduce in the levels of *O*-GlcNAcylated SNAP-29 acts as a signal for the assembly of SNAP-29-containing trans-SNARE complexes, thus stimulating autophagy.

SNAREs

More than 60 SNAREs determine membrane fusion specificity and drive the fusion processes of mammalian cells. Functionally, SNAREs are grouped into v-SNAREs on donor vesicles and t-SNAREs on target vesicles. Structurally, SNAREs are divided into Q-SNAREs (which have a Q amino acid residue) and R-SNAREs (which have an R amino acid residue). Q-SNAREs are further divided into Qa-, Qb- and Qc-SNAREs based on the amino acid sequence of the SNARE domain. These SNAREs form a parallel four-helix bundle composed of Qa-, Qb-, Qc- and R-SNAREs to bridge the two fusing membranes.

Upon starvation in mammals, the Qa-SNARE syntaxin 17 (STX17) is recruited, presumably from the cytosol to closed autophagosomes, and mediates autophagosome–lysosome fusion by binding to its partners, the Qbc-SNARE SNAP29 and the lysosomal R-SNARE VAMP8 (Itakura et al., 2012) (Fig. 3). Accordingly, depletion of STX17 causes accumulation of autophagosomes. Similar machineries and mechanisms are also used for autophagosome–lysosome fusion in *Drosophila* (Takats et al., 2013). Furthermore, the *O*-linked *N*-acetylglucosamine (O-GlcNAc) modification of SNAP29 negatively regulates SNARE-dependent fusion between autophagosomes and lysosomes (Guo et al., 2014). Consequently, knockdown of O-GlcNAc transferase or mutating SNAP29 O-GlcNAc sites promotes formation of the SNAP29-containing SNARE complex and increases fusion between autophagosomes and lysosomes (Fig. 3).

It has recently been shown that the isolation membrane forms at the ER-mitochondria contact site and STX17 is also involved in this process (Hamasaki et al., 2013). STX17 localizes to the ER under feeding conditions but, upon starvation, it relocalizes to ER-mitochondria contact sites (Hamasaki et al., 2013). Here, STX17 binds to, and so recruits, ATG14L to ER-mitochondria contacts to initiate formation of the isolation membrane. By contrast, STX17, which is involved in autophagosome–lysosome fusion, is presumably recruited from the cytosol to closed autophagosomes (Itakura et al., 2012). Further studies are required to understand how cells utilize different pools of STX17 depending on the context (ER or cytoplasm). In addition to STX17, ATG14L binds to a binary complex formed between STX17 and SNAP29 (STX17–SNAP29) and facilitates its interaction with VAMP8 to promote autophagosome–lysosome fusion (Diao et al., 2015) (Fig. 3), indicating the interaction between STX17 and ATG14L both in the early and late steps of autophagy.

Tethering factors

Membrane tethers are thought to provide another level of specificity, and to facilitate docking and fusion by bridging opposing membranes and by stimulating SNARE complex formation. In addition to its well-known role in the endocytic pathway, the HOPS complex functions as a tethering factor for autophagosomal fusion (Jiang et al., 2014) (Fig. 3). STX17 interacts with the HOPS complex, including VPS33A, VPS16, VPS11,

VPS18, VPS39 and VPS41. Consistent with this, these HOPS subunits are recruited to STX17-positive autophagosomes upon starvation. Importantly, knockdown of VPS33A, VPS16 or VPS39 blocks the autophagic flux and causes accumulation of STX17- and LC3-positive autophagosomes, suggesting that HOPS promotes autophagosome–lysosome fusion with STX17 (Jiang et al., 2014). Most of these findings are also observed in *Drosophila* (Takats et al., 2014). A recent structural study of Vps33 in the thermophilic fungus *Chaetomium thermophilum* suggest that Vps33 promotes SNARE assembly by precisely positioning and aligning SNAREs (Baker et al., 2015). This finding provides us with a novel insight how the HOPS complex promotes SNARE assembly.

How are HOPS complexes recruited to autophagosomes and lysosomes? On late endosomes and lysosomes, Rab7 recruits its effectors PLEKHM1 and RILP that bind to the HOPS components VPS39 and VPS41, respectively (Wijdeven et al., 2016). These effectors, thus, jointly recruit the HOPS complex for fusion. This is similar in yeast, where the Rab7 homologue Ypt7 acquires HOPS by binding to both Vps39 and Vps41 (Ostrowicz et al., 2010; Plemel et al., 2011). In addition to PLEKHM1 and RILP, Rab7 recruits another effector, the cholesterol sensor ORP1L, that binds to Rab7 in the presence of RILP and negatively regulates fusion (Johansson et al., 2007; Wijdeven et al., 2016). At low levels of cholesterol, ORP1L on late endosomes or lysosomes interacts with the ER protein VAP-A to form the ER-autophagosome contact site. This contact site prevents the recruitment of PLEKHM1 to Rab7, and consequently, that of the HOPS complex, which results in a defect in autophagosome-late endosome/lysosome fusion. Interestingly, ORP1L and the presence of this contact site prevent the recruitment of dynein and/or dynactin by RILP and, thus, minus-end-directed transport of late autophagosomes (Wijdeven et al., 2016). These observations suggest that the fusion of autophagosomes to late endosomes and lysosomes and the transport of late autophagosomes are regulated by cholesterol. However, the *in vivo* function of cholesterol during the fusion is still unclear and needs to be addressed in future studies.

Ectopic P granules protein 5 (EPG5) was originally identified in a *C. elegans* genetic screen and is another Rab7 effector and tethering factor that determines the fusion specificity of autophagosomes with endosomes/lysosomes (Tian et al., 2010; Wang et al., 2016) (Fig. 3). EPG5 is recruited to late endosomes/lysosomes through direct interaction with Rab7 and the late endosomal/lysosomal R-SNARE VAMP7/VAMP8. EPG5 also binds to LC3/LGG-1 (LGG-1 is the *C. elegans* Atg8 homolog) through its LC3-interacting region (LIR) motif and to assembled STX17–SNAP29 complexes (Qabc-SNARE) on autophagosomes. EPG5 stabilizes and facilitates the assembly of STX17–SNAP29–VAMP8 trans-SNARE complexes and, thus, is most likely to promote the fusion between autophagosomes and lysosomes. Loss of EPG5 causes abnormal fusion of autophagosomes with various endocytic vesicles, partly due to increased assembly of the STX17–SNAP25–VAMP8 complex. Consistent with this, SNAP25 knockdown partially suppresses the effect on autophagy because of EPG5 depletion (Wang et al., 2016). These findings have, thus, begun to answer the question as to why autophagosomes specifically fuse with late endosomes and lysosomes. In addition to HOPS and EPG5, as mentioned previously, ATG14L directly binds to the STX17–SNAP29 binary complex on autophagosomes and promotes STX17–SNAP29–VAMP8-mediated autophagosome fusion with lysosomes, thus functioning as a tethering factor (Diao et al., 2015) (Fig. 3). However the precise relationship between HOPS, EPG5 and ATG14L is still unclear, and needs to be clarified in future studies. Tectonin beta-propeller repeat-containing 1 (TECPR1) also functions as a tethering factor and we discuss its role further on in the text (Chen et al., 2012).

Phosphoinositides in autophagosome–lysosome fusion

Phosphoinositides (PIs) function in intracellular membrane trafficking. Phosphorylation of the third, fourth, and fifth position of the PI inositol ring produces different variants, in particular PI3P [phosphatidylinositol (3)-phosphate] that has a well-characterized role in autophagosome formation. Although most studies focus on the proteins that are involved in the autophagy process, several recent studies have revealed roles for PIs during the late stage of autophagy, including the transport of autophagosomes and the autophagosomal fusion with the lysosome.

Studies in yeast provide hints on how autophagosomes become competent to fuse with lysosomes. Dephosphorylation and clearance of PI3P by the PI3P phosphatase Ymr1 on the completed autophagosome are essential for fusion of autophagosome and vacuole (Cebollero et al., 2012). Clearance of PI3P triggers the dissociation of the ATG machinery from the outer autophagosomal membrane, which makes the autophagosome competent for fusion. By contrast, mammalian forms of PI3P phosphatases, such as myotubularin-related protein 3 (MTMR3) and MTMR14 (also known as Jumpy), appear to have different functions during autophagy (Taguchi-Atarashi et al., 2010; Vergne et al., 2009). Knockdown of MTMR3 and MTMR14 increases autophagosome formation, indicating that these phosphatases are negative regulators of autophagy and that they function during the early stages of autophagosome formation. It is, thus, worthwhile to further investigate whether other PI phosphatases exert similar functions in mammals.

As mentioned above, FYCO1 binds to LC3, PI3P and Rab7, and is involved in the movement of autophagosomes (Fig. 2). Endogenous FYCO1 localizes on perinuclear cytosolic vesicles but, upon starvation, it is also distributed in the cell periphery in a microtubule-dependent manner (Pankiv et al., 2010). FYCO1 functions as an adaptor protein between autophagosomes and microtubule plus-end-directed molecular motors – as evidenced by FYCO1-depleted cells that accumulate autophagosomes in perinuclear clusters (Pankiv et al., 2010). Recently it has been shown that FYCO1 on endosomes interacts directly with the KLC2 light chain of kinesin1 during the translocation of endosomes to the cell periphery (Raiborg et al., 2015). Thus, it seems worthwhile to examine whether the same kinesins are used during the transport of autophagosomes.

In addition to the HOPS complex, the PI3P-binding proteinTECPR1 has been suggested to function as a tethering factor in autophagosomal fusion (Chen et al., 2012) (Fig. 4). TECPR1-depleted cells have impaired autophagic flux, and accumulate autophagic vacuoles and substrates, including p62 (officially known as SQSTM1) and lipidated LC3 (LC3-II) (Chen et al., 2012). TECPR1 was originally identified through its interaction with ATG5 (Behrends et al., 2010). Whereas phagophore (isolation membrane) localization of the Atg12–Atg5–Atg16 complex is dependent on the presence of PI3P, Chen et al. show that TECPR1 and ATG16 form mutually exclusive complexes with the ATG12–ATG5 conjugate, and TECPR1 binds PI3P upon association with the Atg12–Atg5 conjugate (Chen et al., 2012; Fujita et al., 2008b). Furthermore, TECPR1 localizes to lysosomes/autolysosomes and recruits the ATG12–ATG5 conjugate, which enables its binding to PI3P, thereby possibly facilitating autophagosome maturation and autophagosome–lysosome fusion. However, another study found that TECPR1 also functions in phagophore biogenesis and maturation during selective autophagy (Ogawa et al., 2011). These discrepancies might either reflect dual roles of TECPR1 or distinct functions in

Journal of Cell Science (2017) 0, 1-8 doi:10.1242/jcs.196352

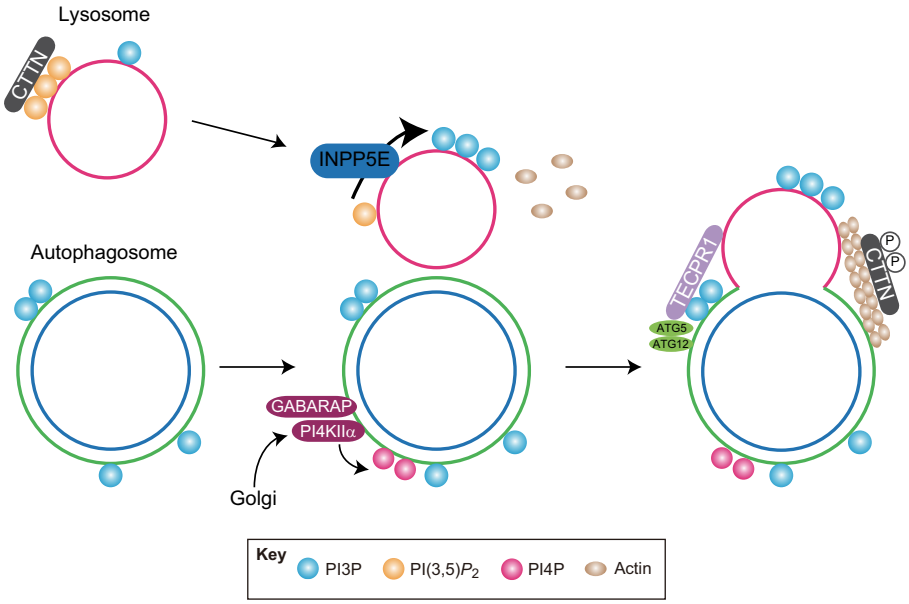

Fig. 4. Roles of phospholipids during autophagosome–lysosome fusion. A certain amount of cellular INPP5E localizes to lysosomes where it converts PI(3,5)P2 to PI3P, which then leads to the phosphorylation and activation of cortactin (CTTN). Activated CTTN is essential for the stabilization of actin filaments and thus for autophagosome–lysosome fusion. TECPR1 on lysosomes functions as a tethering factor that interacts with ATG12–ATG5 and PI3P on autophagosomes. GABARAP recruits palmitoylated PI4KIIα from the trans-Golgi network to autophagosomes, PI4P that is produced by PI4KIIα is essential for fusion, although the its exact function is still unknown.

different biological contexts. Moreover, the functional relationship among the several above-mentioned tethering factors is still unclear and needs to be addressed.

The generation of PI4P [phosphatidylinositol (4)-phosphate] on autophagosomes is crucial for autophagosome–lysosome fusion in mammals (Wang et al., 2015) (Fig. 4). Phosphatidylinositol 4-kinase type 2-alpha (PI4KIIα) normally localizes to the perinuclear region and the trans-Golgi network (TGN). Upon starvation, PI4KIIα exits the TGN and disperses into the cytoplasm, with some PI4KIIα localizing to autophagosomes in a palmitoylation-dependent manner with a similar redistribution of PI4P (Wang et al., 2015). Consistent with this, depletion of PI4KIIα reduces the concentration of PI4P in autophagosomes. Interestingly, PI4KIIα interacts with the Atg8 homologs GABARAP and GABARAPL2, but not with LC3. Depletion of GABARAP inhibits translocation of PI4KIIα to autophagosomes, whereas depletion of PI4KIIα does not affect GABARAP distribution, indicating that GABARAP functions upstream of PI4KIIα in this context (Wang et al., 2015). Knockdown of either GABARAP or PI4KIIα creates defective, large autophagosomes and impairs the degradation of LC3 and p62, owing to defective autophagosomal fusion with the lysosome. Importantly, this fusion defect is rescued by introduction of PI4P, but not of phosphatidylinositol (4,5)-bisphosphate, suggesting that the generation of PI4P specifically – and not its downstream metabolites – is essential for autophagosome–lysosome fusion (Wang et al., 2015). However, the exact role of PI4P in the fusion process needs to be clarified in future studies. In line with the function of GABARAP during autophagosome–lysosome fusion, a recent systematic functional analysis of the Atg8 family in mammals revealed that the GABARAP subfamily promotes recruitment of PLEKHM1 and governs autophagosome–lysosome fusion, whereas the LC3 subfamily has a less prominent role in these particular processes (Nguyen et al., 2016).

In contrast to the clear roles of phospholipids in autophagosomal membranes, it was unclear whether the specific composition of phospholipids in the lysosomal membrane is important for autophagosome–lysosome fusion. But, recently, we identified the phosphoinositide phosphatase inositol polyphosphate-5 phosphatase E (INPP5E) as a so-far-unknown regulator of autophagy that promotes the fusion step (Hasegawa et al., 2016; Nakamura et al., 2016) (Fig. 4). Inhibition of INPP5E causes the accumulation of autophagosomes, as defined by LC3 puncta, by impairing autophagosome–lysosome fusion. Importantly, knockdown of INPP5E does not affect the endocytic pathway or the integrity of lysosomes, further highlighting a specific role of INPP5E during the autophagosome–lysosome fusion step. Notably, some of the INPP5E protein localizes to lysosome membranes, where it mediates the conversion of phosphatidylinositol (3,5)-bisphosphate [PI(3,5)P2] to PI3P, which is crucial for fusion with autophagosomes. Knockdown of 1-phosphatidylinositol 3-phosphate 5- kinase (PIKFYVE) decreases the levels of PI(3,5)P2 on lysosomes and causes severe autophagy defects due to the loss of lysosomal function (de Lartigue et al., 2009). Therefore, correct levels of PI(3,5)P2 in the lysosomal membrane appear to be required for lysosome function and for their fusion with autophagosomes during autophagy. INPP5E activity on lysosomes has shown to be ultimately required for phosphorylation of cortactin (CTTN), which leads to actin polymerization followed by autophagosome–lysosome fusion (Hasegawa et al., 2016) (Fig. 4). Mutations in INPP5E are linked to Joubert syndrome, a rare brain disorder (Bielas et al., 2009). Interestingly, these mutant forms of INPP5E do not rescue the autophagy defects that are caused by INPP5E knockdown in neuronal cells, suggesting that the impairment of autophagy causes this disease (Hasegawa et al., 2016). Because INPP5E is predominantly expressed in neuronal cells, future studies are needed to clarify whether a similar mechanism is involved in autophagosome–lysosome fusion in other cell types.

Although several PIs, both in the autophagosome and lysosome, participate in the autophagosome–lysosome fusion, future studies should investigate the exact role of each PI, the timeline of PI function, and how different PIs function cooperatively during the fusion process.

Conclusions and future directions

In this Commentary, we have summarized recent findings regarding autophagosome–lysosome fusion. Although several molecular mechanisms have so-far been uncovered, there are still remaining questions with regard to the autophagosome–lysosome fusion step. We still do not know how different fusion machineries are recruited to mature autophagosomes, endosomes and lysosomes at the right time. It is also unclear whether the fusion process is actively

regulated. As has been shown for ATG14L and syntaxin 17, some factors are involved in early as well as late stages of autophagy; also, we might have overlooked some of their roles owing to the severe defects caused by their functional ablation during early steps of the fusion process. The tissue and cell specificity of the fusion mechanism must also be addressed in future studies. Answers to these questions will provide the opportunity to develop new therapeutic strategies in order to control activity of autophagy.

Acknowledgements
We thank all members of our laboratory for the critical reading of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Funding
S.N. is supported by the Japan Society for the Promotion of Science (JSPS) Kakenhi, the MSD Life Science Foundation and a Kishimoto Foundation Research Grant. Y.T. is supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT)/JSPS Kakenhi and Japan Agency for Medical Research and Development (AMED).

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