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J Mol Biol. Author manuscript; available in PMC 2021 June 01.

Published in final edited form as:
J Mol Biol. 2016 December 4; 428(24 Pt A): 4819–4827. doi:10.1016/j.jmb.2016.10.029.

Phospholipids in Autophagosome Formation and Fusion

Sascha Martens[1,*], **Shuhei Nakamura**[2], **Tamotsu Yoshimori**[2,*]

[1]Max F. Perutz Laboratories, University of Vienna, Dr Bohr-Gasse 9/3, 1030 Vienna, Austria

[2]Department of Genetics, Graduate School of Medicine, Osaka University, Yamadaoka 2-2, Suita, Osaka, 565-0871, Japan

Abstract

Autophagosomes are double membrane organelles that are formed during a process referred to as macroautophagy. They serve to deliver cytoplasmic material into the lysosome for degradation. Autophagosomes are formed in a *de novo* manner and are the result of substantial membrane remodeling processes involving numerous protein-lipid interactions. While most studies focus on the proteins involved in autophagosome formation it is obvious that lipids including phospholipids, sphingolipids and sterols play an equally important role. Here we summarize the current knowledge about the role of lipids, especially focusing on phospholipids and their interplay with the autophagic protein machinery during autophagosome formation and fusion.

Keywords

Autophagy; lysosome; conjugation; phosphatidylethanolamine; phosphatidylinositol 3-phosphate; lipid phosphatase; lipid kinase

Introduction

The term autophagy describes processes wherein cytoplasmic material is delivered into the endo-lysosomal compartment for degradation. During macroautophagy (hereafter autophagy) a portion of the cells' cytoplasm is sequestered within a double membrane organelle referred to as autophagosome. After induction of autophagy by extrinsic or intrinsic stimuli, autophagosomes form in a *de novo* manner. Initially, small membrane structures called isolation membranes (or phagophores) are assembled. These gradually expand around a portion of the cytoplasm referred to as cargo and eventually close to isolate the cargo from the rest of the cytoplasm. Subsequently, the outer autophagosomal membrane fuses with lysosomes wherein the inner membrane and the cargo are degraded (Figure 1) [1, 2].

Autophagy can be massively induced by stress conditions such as starvation but also by cell intrinsic stimuli such as the accumulation of damaged mitochondria, protein aggregates, the presence of damaged endosomes and lysosomes and some cytosolic pathogens [3]. Autophagy thereby serves ensure cellular homeostasis and quality control. Consequently,

*to whom correspondence should be addressed: S.M sascha.martens@univie.ac.at, T.Y tamyoshi@fbs.osaka-u.ac.jp.

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dysfunctions of autophagy have been implicated in various pathological conditions including neurodegeneration, cancer and uncontrolled infections [4].

Essentially, autophagosome formation is a membrane sculpting process during which cells form a new membrane-bound organelle and as such lipids play a central role. While the lipid composition of autophagosomes is surprisingly still unknown, specific lipids have been shown to be important for the induction and expansion of isolation membranes as well as for their fusion with lysosomes. Here we summarize the recent knowledge of the lipid requirements for autophagosome formation.

Autophagosome induction

The membrane donor for autophagosomes is still under debate and various sources including the endoplasmic reticulum (ER), the Golgi, mitochondria, the plasma membrane, lipid droplets and endosomes have been suggested to supply lipids to the growing isolation membrane [5–18]. It is possible that multiple sources are able to act as lipid donors, that various cell types or distinct types of autophagy employ different membrane sources or that certain organelles contribute only indirectly to autophagosome formation. However, in mammalian cells it seems well established that when triggered by starvation, isolation membrane formation occurs close to, or at a specialized domain of the ER [5, 6, 9, 10, 16, 19]. Electron microscopy pictures have shown that isolation membranes are connected to the ER [10, 19]. These sites are likely to be identical to structures identified by light microscopy referred to as omegasomes [5]. In yeast, current evidence suggests that isolation membrane growth is driven at least in part by vesicle fusion [20, 21]. Connections of the isolation membranes with the ER as observed in mammalian cells have not yet been reported although ER-derived structured have been seen close to the autophagosome formation site [7, 22]. The distance between the two membranes of the isolation membranes is just a few nanometers and therefore much smaller than for the ER [10, 19]. For this reason, isolation membranes do not simply represent an area of deformed ER.

The molecular mechanisms underlying autophagosome formation are largely unclear. The ULK1 kinase complex (Atg1 in yeast) and the class III PI3K complex 1 are essential for autophagosome formation and act together with the transmembrane protein ATG9 during the induction and nucleation of isolation membranes [23]. The ULK1 kinase complex consists of the ULK1 (Atg1) kinase subunit, FIP200 (Atg17/Atg11 in yeast), ATG13 (Atg13 in yeast) and ATG101 [24–27]. The mechanism of ULK1 recruitment to the autophagosome formation site is not well understood, in particular during starvation induced autophagy but the membrane composition and/or shape of the autophagosome formation initiation site may play an important role. The Atg1 kinase subunit has been shown to contain an amphipathic region termed EAT domain that binds preferentially to highly curved membranes [28]. Amphipathic regions in proteins recognize the curvature as regions with membrane defects as these aid insertion of the protein into the hydrophobic core of membrane and, analogously, lipids with small headgroups will have a similar effect [29, 30].

The class III phosphatidylinositol 3-phosphate kinase (PI3K) complex 1 consists of the VPS34 lipid kinase subunit as well as VPS15, Beclin/ATG6 and ATG14 [31, 32]. VPS34

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phosphorylates phosphatidylinositol (PI) to generate phosphatidylinositol 3-phosphate (PI3P) [33]. The enzymatic activity of this complex is the major source of PI3P for autophagosome formation. In addition, other PI3K complexes such as class II PI3K could contribute to PI3P generation [34]. The class III PI3K complex 1 may be targeted to the autophagosome formation site by the ULK1/Atg1 complex and interactions with ER-resident proteins such as syntaxin-17 [9, 35]. In addition, several subunits of the class III PI3K complex 1 have been shown to directly interact with membranes [31, 32]. The VP34 subunit contains a membrane inserting amphipathic helix [36], VPS15 is anchored to the membrane by a N-terminal myristoylation [37], ATG14 contains a C-terminal amphipathic helix [38] and Beclin1 interacts with the membrane via aromatic residues located in its C-terminal evolutionary conserved domain [39]. All these interactions are facilitated on membranes with high degree of defects and thus when they are highly curved and/or contain lipids with small headgroups such as phosphatidylethanolamine (PE). The PI3P generated by the class III PI3K complex 1 subsequently recruits further factors (see below) that mediate the expansion and subsequent closure of the isolation membrane. Downregulation of PI3P levels by the PI3P phosphatase MTMR3 and Jumpy/MTMR14 was shown negatively regulate autophagosome formation [40–42].

Isolation membrane expansion and closure

After their nucleation, isolation membranes expand and eventually close. While the lipid composition of isolation membranes and autophagosomes is still unknown, individual lipids are likely to play critical roles. One of the hallmarks of isolation membrane expansion is the attachment of ATG8 proteins to the membrane [43–45]. In yeast there is one Atg8 protein while there are several ATG8 isoforms in mammalian cells, which are subdivided into the LC3s and GABARAPs [46]. ATG8 proteins are ubiquitin-like proteins that become covalently linked to the headgroup of PE via their C-terminal glycine residue (Figure 2)[43–45]. ATG8 proteins are synthesized as precursors with short C-terminal extensions and the C-terminal glycine residues become exposed after proteolytic cleavage by cysteine proteases of the ATG4 family [43]. ATG8 proteins are subsequently activated under consumption of ATP by the E1-like enzyme ATG7. From ATG7, ATG8 is transferred to the E2-like ATG3 and from there to the headgroup of PE [44, 47]. This last step is vastly accelerated by a protein complex composed of the ATG12–ATG5 conjugate and ATG16 [48]. The ATG12–ATG5-ATG16 complex acts analogous to the E3-like enzymes during ubiquitination and is itself a product of a protein conjugation reaction during which the ubiquitin-like ATG12 proteins becomes conjugated via its C-terminal glycine residue to a lysine residue in ATG5 [49]. The localization of the ATG12–ATG5-ATG16 complex is a critical determinant for the localization of ATG8–PE formation [50]. The regulation of selective autophagy and the transport of autophagosomes to lysosomes by ATG8 proteins via interaction with cargo receptors and adaptors is well established [51]. In contrast, the role of ATG8 proteins during the membrane sculpting processes is less well understood. Current evidence points to a contribution of these proteins to isolation membrane expansion and closure [52–55].

Since PE is the main target for ATG8 conjugation *in vivo* it is likely that this lipid is present on the isolation membrane. In addition, PE is one of the most abundant lipids in cells and may therefore have a significantly contribution to the lipid mass of the autophagosomal

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membrane [56]. High levels of PE have been shown to stimulate Atg8-PE conjugation and to facilitate Atg8-mediated hemifusion of small vesicles [47, 48, 53, 54, 57, 58]. In vitro, phosphatidylserine (PS) can also serve as acceptor for ATG8 protein conjugation and it is possible that a small fraction of ATG8 is conjugate to this lipid [59]. Most of the ATG8 proteins are removed from the outer autophagosomal membrane after autophagosome completion by ATG4. The fact that ATG8–PS, as opposed to ATG8–PE, is more resistant to ATG4 cleavage suggests that ATG8–PS is only a minor fraction of the total lipid conjugated ATG8 [60]. PS may have an additional role during ATG8 conjugation since negatively charged lipids aid the recruitment of the ATG12–Atg5-Atg16 complex to the membrane, which stimulates ATG8 conjugation to PE [61].

As discussed above PI3P generated by the class III PI3K complex 1 is critical for the early stages of isolation membrane formation but is also likely to be required during their expansion. Since PI3P is generated from PI, it is reasonable to assume that PI is present in the isolation membrane and that its levels will impact autophagosome formation. The WD40 repeat proteins of the WIPI family directly bind PI3P and PI3,5P2 [31, 62] and become recruited to the expanding isolation membrane, likely in complex with ATG2A/B to facilitate isolation membrane expansion and finally closure [63–65]. WIP2 was also shown to recruit the ATG12–ATG5-ATG16L1 complex to the isolation membrane, thus linking PI3P to the ATG8 conjugation machinery [66, 67].

A further aspect of how lipids may impact autophagosome formation is at the level of their shape. Thus, it was shown that ATG3 preferentially binds highly curved membranes [58]. The *S. cerevisiae* Atg12–Atg5-Atg16 complex also prefers highly curved membranes [61]. In both cases the curvature is detected due to a higher abundance of membrane defects, aiding the insertion of hydrophobic regions of proteins into the membrane. Lipids with small headgroups such as phosphatidic acid (PA) [68] and/or bulky acyl chains will have similar effects and may promote membrane binding and insertion of ATG3 and the Atg12–Atg5-Atg16 complex and thus autophagosome formation.

Surprisingly, by using quick-freezing and freeze-fracture replica labelling (QF-FRL), it was shown that the distribution of PI3P in autophagosomal membranes is different between yeast and mammals [69]. In yeast, PI3P is more abundant in the luminal than in the cytoplasmic leaflet, while PI3P in mammals is exclusively present in the cytoplasmic leaflet of the autophagosome. In yeast, PI3P is generated equally in both leaflets and the asymmetric distribution is generated by unilateral hydrolysis of PI3P by cytoplasmic phosphatase Ymr1p and Sjl3p. Proper distribution of PI3P in autophagosomes seems to be essential for the subsequent closure and fusion with vacuoles. The difference in PI3P distribution suggests that autophagy could involve different processes in yeast and mammals.

Role of phospholipids in autophagosome-lysosome fusion

In mammals autophagosomes are formed throughout the cytoplasm, while lysosomes are localized at the perinuclear region. Thus autophagosomes have to be transported to the lysosome before they can fuse. The movement of autophagosomes is mediated by the microtubule cytoskeleton [51, 70]. FYCO1 (FYVE and coiled-coil domain containing 1) has

been shown to bind to LC3, PI3P as well as the small GTPase Rab7 and is involved in this process. Endogenous FYCO1 is localized on perinuclear cytosolic vesicles, but is also distributed in the cell peripheral region in a microtubule dependent manner upon starvation. FYCO1 functions as an adaptor protein between autophagosomes and microtubule plus end-directed molecular motors, since FYCO1 depleted cells show the accumulation of autophagosomes at perinuclear clusters. [71]. The kinesin proteins interacting with FYCO1 remain to be defined in the future.

How and when do autophagosomes become competent for fusion with lysosomes? Some hints come from a yeast study. In yeast, dephosphorylation and thus clearance of PI3P by the PI3P phosphatase, Ymr1 on the completed autophagosome are essential for autophagosomevacuole fusion [72]. Clearance of PI3P triggers the dissociation of the ATG machinery from the outer autophagosomal membrane making them competent for fusion with the vacuole. Mammalian forms of PI3P phosphatases such as myotubularin-related protein 3 (MTMR3) and MTMR14/Jumpy appear to have different functions during autophagy [40, 41]. Knockdown of MTMR3 and MTMR14/Jumpy increase autophagosomes formation indicating these phosphatases are negative regulators of autophagy and function at early stage of autophagosome formation.

PI3P also participates in fusion process through PI3P binding proteins. In mammals, recent work identified the PI3P binding protein TECPR1 as a tethering factor mediating autophagosome-lysosome fusion [73]. TECPR1 depleted cells show impaired autophagic flux, the accumulation of autophagic vacuoles and autophagic substrates including p62 and lipidated LC3. TECPR1 was originally identified through its interaction with ATG5 [74]. Although the phagophore localization of the ATG12–ATG5-ATG16 complex is dependent on PI3P [50], the authors show that TECPR1 forms a complex with ATG12-ATG5 in a mutually exclusive manner with ATG16. Furthermore, they show that TECPR1 is localized on lysosomes/autolysosomes and recruits the ATG12-ATG5 conjugate to enable binding to PI3P, possibly facilitating autophagosome maturation and autophagosome-lysosome fusion (Figure 3). However, another study showed that TECPR1 also functions in phagophore biogenesis and maturation during selective autophagy [73]. These discrepancies might either reflect the dual roles of TECPR1 or different biological contexts.

In addition to PI3P, PI4P generation on autophagosomes is also critical for autophagosome-lysosome fusion in mammals [75]. Phosphatidylinositol 4-phosphate kinase type II α (PI4KIIα) is normally localized in the perinuclear region and trans-Golgi network (TGN). Upon starvation, PI4KIIα exits from the TGN and is distributed in the cytoplasm. Some of the PI4KIIα is localized on autophagosomes in a palmytoylation-dependent manner. Consistent with this, PI4P undergoes a similar redistribution and depletion of PI4KIIα reduces the concentration of PI4P in autophagosomes. Interestingly, PI4KIIα interacts with GABARAP and GABARAPL2, but not with LC3. Depletion of GABARAP inhibits PI4KIIα translocation to autophagosomes, while depletion of PI4KIIα does not affect GABARAP distribution, indicating that GABARAP functions upstream of PI4KIIα in this context. Knockdown of either GABARAP or PI4KIIα shows defective large autophagosomes and impaired degradation of LC3 and p62 due to defective autophagosomal fusion with lysosome. Importantly, the fusion defect is rescued by introduction of PI4P, but

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not by PI(4,5)P2, suggesting that PI4P generation, but not downstream metabolites, is essential for autophagosome-lysosome fusion (Figure 3). The exact role of PI4P in the fusion needs to be clarified in future studies.

Contrary to the roles of phospholipids in the autophagosomal membrane, it was unclear whether the specific composition of phospholipids in the lysosomal membrane is essential for autophagosome-lysosome fusion. Recently a phosphoinositide phosphatases, inositol polyphosphate-5 phosphatase E (INPP5E) has been identified as a novel regulator of autophagy, promoting the fusion step [76]. Inhibition of INPP5E causes the accumulation of autophagosomes as defined by LC3 puncta due to the impairment of autophagosomelysosome fusion. Importantly, knockdown of INPP5E does not affect the endocytic pathway and the integrity of lysosomes, further highlighting the specific role of INPP5E during the autophagosome-lysosome fusion step. Notably, some of the INPP5E protein is localized at lysosomes where it mediates the conversion of PI(3,5)P2 to PI3P in the lysosomal membrane, which in turn is crucial for the fusion with autophagosomes. Previously, it was shown that knockdown of the phosphoinositide kinase PIKfyve decreases the PI(3,5)P2 levels on lysosomes and causes severe autophagy defects due to the loss of lysosomal function [77]. Therefore, proper levels of PI(3,5)P2 in the lysosomal membrane seems to be required for its function and fusion with autophagosomes during autophagy. INPP5E activity on lysosomes is ultimately required for phosphorylation of cortactin/CTTN, which leads to actin polymerization followed by autophagosome-lysosome fusion (Figure 3). Mutations in INPP5E are known to be linked to Joubert syndrome, a rare brain abnormality [78] and these mutant forms of INPP5E could not rescue the autophagy defects caused by INPP5E knockdown in neuronal cells, strongly suggesting that the impairment of autophagy could be causal for this disease [76]. Since INPP5E is predominantly expressed in neuronal cells, future studies need to clarify if a similar mechanism functions during the autophagosome-lysosome fusion process in other cell types as well. Taken together, although several specific PIs both in autophagosome and lysosome have been shown to participate in autophagosome-lysosome fusion, one should address the exact role of each PIs, the timeline of the function of these PIs and how these different phospholipids cooperatively function during the fusion process in upcoming research.

Outlook

At its core, autophagosome formation is a membrane sculpting process and it therefore not surprising that there is an extensive cross talk between lipids and the autophagic protein machinery. Consequently, individual lipids, mostly phosphorylated forms of PI, have been shown to be required for multiple steps during the life of an autophagosome. However, in order to fully comprehend the role of lipids in autophagy the lipid composition of autophagosome precursors and of completed autophagosomes will have to be known, which is currently not the case. To this end, methods to purify autophagosomes to a high degree and subsequent lipidomics are essential. Improvement of imaging techniques allowing the visualization of lipids using electron microscopy and super resolution microscopy will also help to study the distribution and dynamics of lipids during autophagosome formation. In our view this is one of the major challenges in autophagy research that must be overcome in order to fully appreciate the role of lipids during this process.

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Acknowledgements

S.M. is supported by ERC grant (No.646653), by the FWF (No. P25546-B20) and by the EMBO Young Investigator Program. Y.T. is supported by The Ministry of Education, Culture, Sports, Science and Technology (MEXT) and Japan Agency for Medical Research and Development (AMED).

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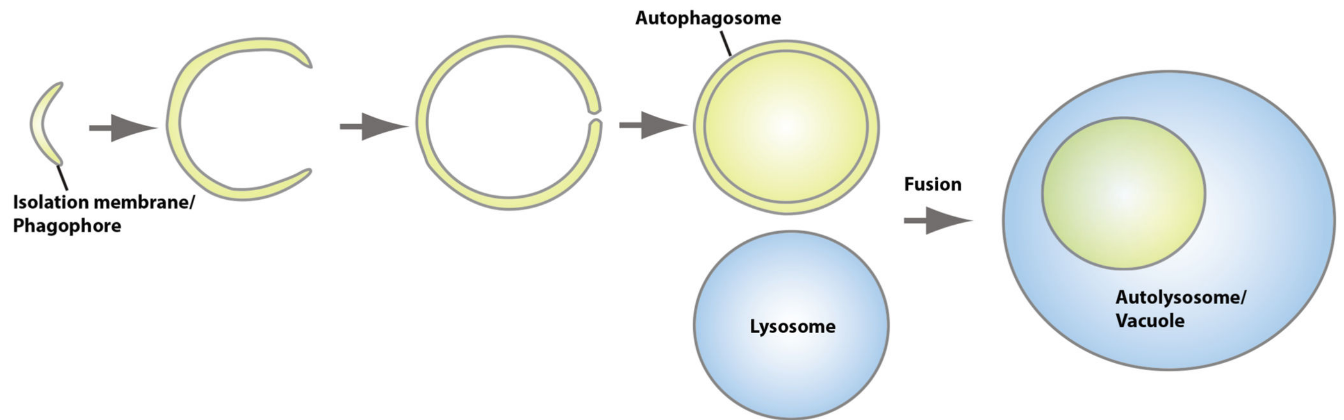

Figure 1. The major steps of autophagosome formation

Upon induction of autophagy, small membrane structures referred to isolation membranes (or phagophores) are generated. These structures expand and gradually enclose cytoplasmic material as they grow. Upon closure of the isolation membranes autophagosomes are formed, which fuse with lysosomes to form autolysosomes, within which the inner membrane and the cargo are degraded.

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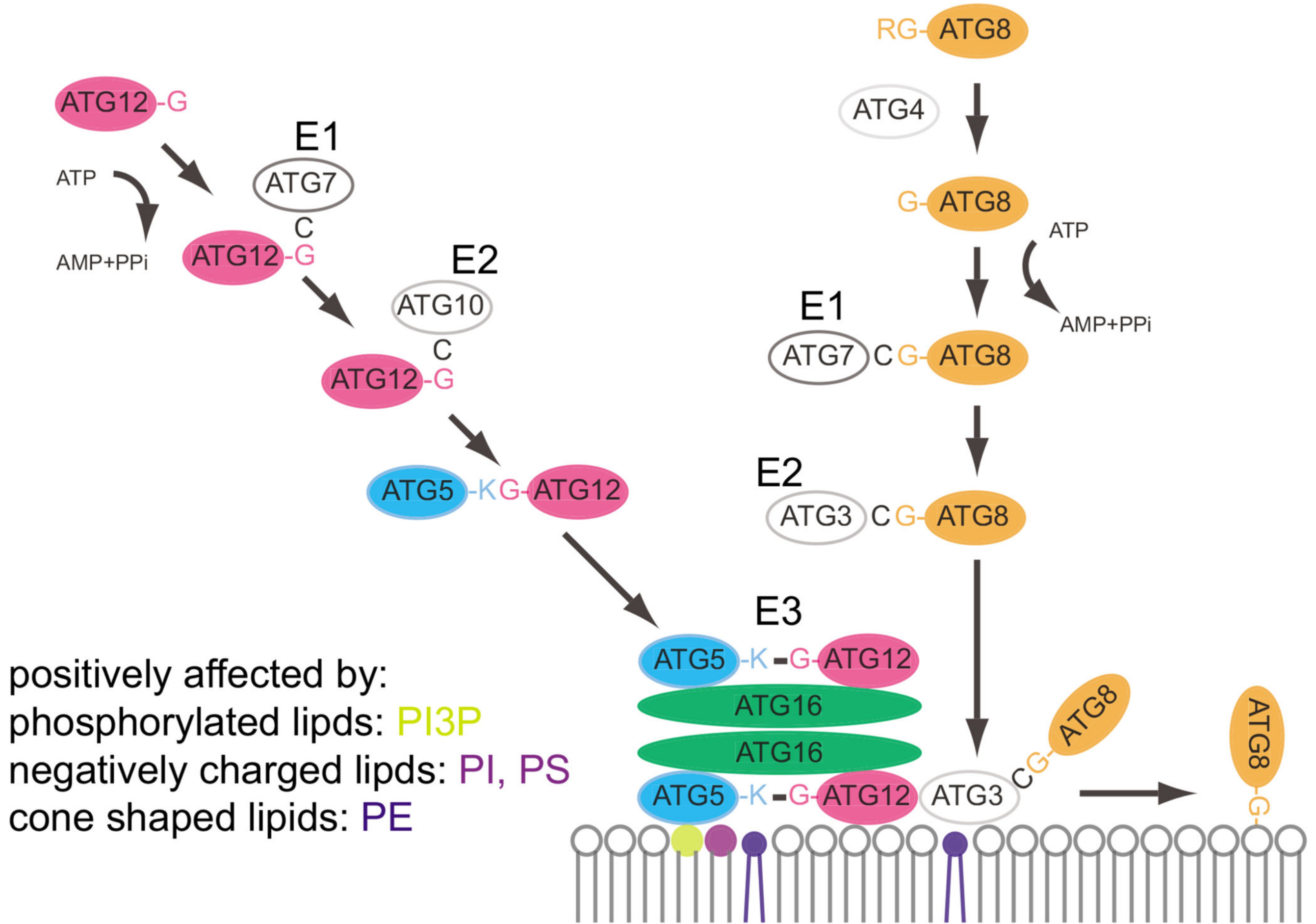

Figure 2. The ubiquitin-like protein conjugation machinery in autophagy

ATG8 proteins, including the LC3 and GABARAP families in mammalian cells, are initially synthesized as precursors with C-terminal extensions. These become cleaved off by the ATG4 cysteine proteases exposing a C-terminal glycine residue. Subsequently, ATG8 proteins are activated by the E1-like ATG7 protein under consumption of ATP. From ATG7, ATG8 proteins are transferred to the E2-like ATG3 and further to the headgroup of the membrane lipid phosphatidylethanolamine (PE). This last step is massively promoted by the ATG12–ATG5-ATG16 complex. This complex is itself the product of an ubiquitin-like conjugation reaction during which the ubiquitin-like ATG12 is transferred via its C-terminal glycine residue to an internal lysine residue of ATG5. The ATG12–ATG5 conjugate subsequently forms a non-covalent complex with ATG16. Lipids that positively influence the recruitment of the ATG12–ATG5-ATG16 complex and ATG3 are highlighted.

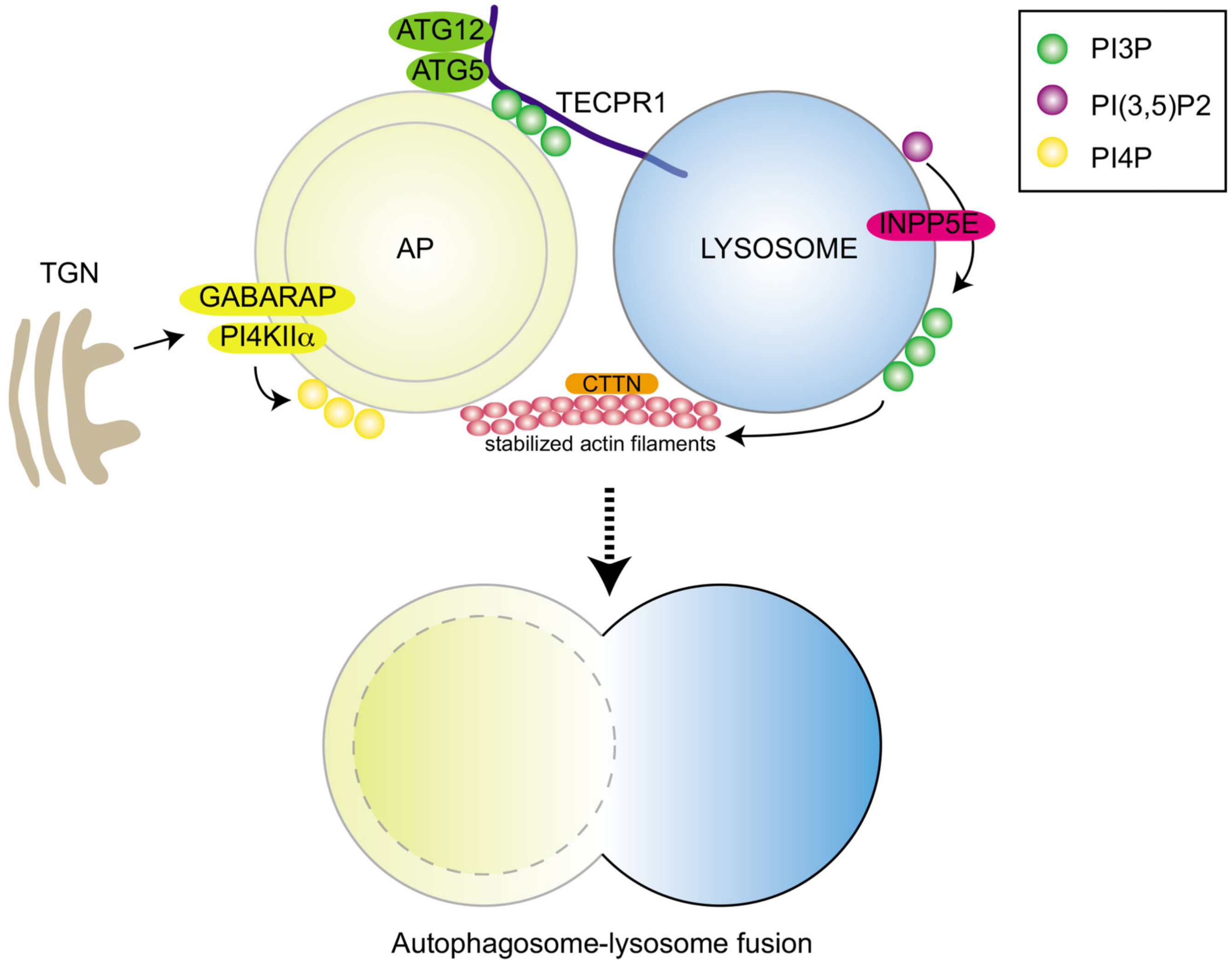

Figure 3. Roles of phosphoinositides in autophagosome-lysosome fusion

TECPR1 on lysosome interacts with ATG12-ATG5 and binds to PI3P on autophagosome, thus functioning as a tethering factor mediating autophagosome-lysosome fusion. GAPARAP recruits palmytoylated PI4KIIα from TGN (Trans Golgi Network) to autophagosomes and the production of PI4P by PI4KIIα is essential for autophagosome-lysosome fusion. Some INPP5E are localized on lysosome and are converting PI(3,5)P2 to PI3P, leading to the activation of cortactin/CTTN and stabilization of actin filaments which are required for autophagosomal fusion. Exact orders of these events need to be clarified in future.

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