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REVIEW

Molecular structures and function of the autophagosome-lysosome fusion machinery

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ABSTRACT

Macroautophagy (also known as autophagy) plays a pivotal role in maintaining cellular homeostasis. The terminal step of the multi-step autophagy degradation pathway involves fusion between the cargo-laden, double-membraned autophagosome and the lytic organelle lysosome/vacuole. Over the past decade, various core components of the molecular machinery that execute this critical terminal autophagy event have been identified. This review highlights recent advances in understanding the molecular structures, biochemical functions, and regulatory mechanisms of key components of this highly sophisticated machinery including the SNARE fusogens, tethering factors, Rab GTPases and associated guanine nucleotide exchange factors, and other accessory factors.

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Introduction

Macroautophagy (hereafter referred to as autophagy) plays a key role in the maintenance of cellular homeostasis by removing protein aggregates, generating nutrients during stress, and turning over damaged organelles. The multi-step autophagic degradation process begins with the formation of a crescent-shaped membrane structure known as the phagophore. The phagophore wraps

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around a cargo and self-fuses into a double-membrane transport vesicle called the autophagosome. The cargo-laden autophagosome ultimately fuses with the lysosome or vacuole where the content is degraded inside the lumen by lysosomal enzymes and the macromolecules are recycled by lysosomal permeases. The discovery of the ATG (autophagy-related) genes by yeast genetic screens in the 1990s propelled molecular investigations of the autophagy pathway. Subsequent biochemical, structural, and cell biology-based studies on the core ATG proteins have generated insights into the molecular mechanism of autophagy initiation and autophagosome biogenesis in different model organisms and in mammalian cells. In recent years, there has been significant progress made in understanding the molecular details of late autophagy events. The most notable findings are the identification of specific Rab (Ras-associated binding) GTPases, guanine nucleotide exchange factors (GEF), SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, and tethering factors that function collaboratively in a similar fashion as their counterparts in conventional intracellular trafficking pathways to mediate fusion between the autophagosome and lysosome/vacuole (Table 1). Several excellent recent reviews have covered major findings in this area of autophagy research.[1–5] This review is dedicated to describing advances made in

Table 1. Components of the autophagosome-lysosome/vacuole fusion machinery.

	Saccharomyces cerevisiae	Drosophila melanogaster	Caenorhabditis elegans	Homo sapiens
SNAREs	Ykt6, Vam3, Vti1,	Syntaxin 17, Snap29, Vamp7 Ykt6	Syntaxin 17, SNAP-29, VAMP-7/8 YKT-6	Syntaxin 17, SNAP29, VAMP7/8 YKT6, SNAP29, Syntaxin-7
Rab and Rab regulators	Ypt7, Mon-Ccz1	Rab7, Mon1-Ccz1-Bulli	RAB-7, SAND-1-CCZ-1	RAB7A, MON1 -CCZ1-RMC1 RAB2 RAB39A
Tethering factors	HOPS complex (Vps11, Vps16, Vps18, Vps33a, Vps39, Vps41)	HOPS complex (Vps11, Vps16A, Vps18, Vps33A, Vps39, Vps41) Epg5	HOPS complex (VPS-11, VPS-16, VPS-18, VPS-33, VPS-39, VPS-41) EPG-5	HOPS complex (VPS11, VPS16, VPS18, VPS33A, VPS39, VPS41) EPG5
Atg8 proteins	Atg8	Atg8a, Atg8b	LGG-1, LGG-2	LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2
Other factors		dBruce Plekhm1		ATG14L TECPR1 BRUCE PLEKHM1

understanding the biochemical features and molecular structures of components of the autophagic fusion machinery.

SNARE proteins

Similar to other membrane trafficking processes, SNARE proteins represent the main driver of autophagome-lysosome/vacuole fusion in autophagy.[6] In particular, various SNARE proteins localized to opposing membranes, and when brought within a distance of 10nm, are capable of "zippering" themselves into a four-helical bundle that can bring two membranes closer and reduce the energy barrier for membrane fusion.[7,8] Based on the presence of a conserved glutamate (Q) or arginine (R) residue in the highly conserved "zero layer", SNARE proteins can be classified into three Q-SNARE proteins – Qa, Qb, and Qc – and one R-SNARE protein.[9] Despite considerable sequence divergence, their mechanism of action is conserved; different sets of SNARE proteins assemble into complexes (i.e., QabcR-complex) in a combinatorial fashion that has been suggested to contribute to fusion specificity.[10,11] All SNARE complexes contain tail-anchored members, which have cytosolic N-terminal fragments and a single transmembrane domain (TMD) or double TMDs at the end of the C-terminus.[12,13] Some SNARE proteins are cytosolic and collaborate with membrane-anchored members for the formation of SNARE protein complexes.[14]

It has been suggested that Vam3, Vam7, Ykt6, and Vti1 are involved in autophagosome-vacuole fusion in yeast and that vesicle-associated membrane protein 7 (VAMP7), VAMP8, and Vti1 are responsible for autophagosome-endolysosome fusion in mammals (Table 1).[15–17] Meanwhile, a set of SNARE proteins, including syntaxin 17 (STX17), synaptosomal-associated protein 29 (SNAP29) on autophagosomes, and VAMP8 on lysosomes, have been found to form SNARE complexes that are essential for the fusion between autophagosomes and lysosomes.[18,19] Crystallographic analysis revealed that the SNARE domains of STX17, SNAP29, and VAMP8 form a helical bundle that resembles other previously characterized trans-SNARE complexes[20] (Figure 1). The Q-SNARE protein STX17 translocates to the outer membrane of the completed autophagosome, and the packed hairpin-like structure causes the exposure of hydrophobic residues.[18] In contrast to other SNARE proteins, the unique hairpin-like double TMDs localize STX17 to autophagosomes and reduce fusion rate by increasing the protein-membrane mismatch.[21] Besides STX17 and SNAP29 on autophagosomes and VAMP8 on endolysosomes, recently, another independent set of SNAREs, YKT6/SNAP29 on autophagosomes and syntaxin 7 (STX7) on endolysosomes, has also been proposed to mediate lysosome–autophagosome fusion.[22] Meanwhile, for the late stage autophagosome–vacuole fusion, the R-SNARE Ykt6 on the autophagosome and the Q-SNAREs Vam3, Vam7, and Vti1 on the vacuole have been recently found to play an important role in yeast[23] and in *Drosophila melanogaster*.[24]

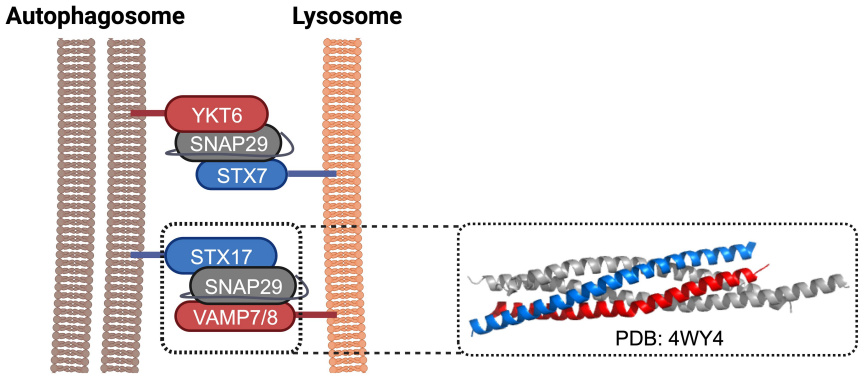

Figure 1. Mammalian autophagic SNARE proteins. Two sets of SNAREs capable of forming trans-SNARE complexes have been implicated in autophagosome-lysosome fusion in mammalian cells: (1) syntaxin 17 (STX17)/ SNAP29/ VAMP7(8) and (2) Ykt6/ SNAP29/ syntaxin 7 (STX7). The Qa, Qbc, and R SNAREs are coloured in blue, grey, and red, respectively. The crystal structure of the complex formed by the SNARE domains of STX17, SNAP29, and VAMP8 is displayed on the right.

Post-translational modifications (PTMs), such as phosphorylation, of SNAREs have been shown to regulate membrane fusion by impacting SNARE zipping.[25] Particularly, PTMs at the residues of SNARE domain facing towards ionic layers can block SNARE zipping.[26] O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT) mediates the O-GlcNAcylation of SNAP29 and regulates autophagy in a nutrient-dependent manner. In mammalian cells, OGT knockdown, or the mutation of O-GlcNAc sites in SNAP29, promotes the formation of a SNAP29-containing a SNARE protein complex, increases fusion between autophagosomes and both endosomes and lysosomes, and promotes autophagic flux.[27] It has been reported that O-GlcNAc-modified SNAP29 reduces the binding affinity with partner SNARE proteins and thus attenuates the assembly of the SNAP29-containing SNARE protein complex. O-GlcNAc-modification of SNAP29 could create steric hindrance that affects SNARE assembly and function, thus preventing the untimely or ectopic formation of the SNARE protein complex. Remarkably, the depletion of ogt-1 has a similar effect on autophagy in *Caenorhabditis elegans*, while the expression of an O-GlcNAc-defective SNAP29 mutant facilitates the autophagic degradation of protein aggregates.[28] Most recently, the fusion blocked by the O-GlcNAcylation of SNAP29 has been shown to promote apoptosis via ROS production.[29] The phosphorylation on VAMP8 has been shown to influence membrane fusion.[25] It was recently shown that VAMP8 plays an important role in forming a prefusion state of lysosomal clusters, in which multiple lysosomes form clusters around individual autophagosomes, setting the stage for membrane fusion.[30] Using a phosphorylation mimic for C-terminal residues of VAMP8, researchers observed

a decrease of fusion in an ensemble lipid mixing assay and an increase of unfused lysosomes associated with autophagosomes.[30] These results suggest that phosphorylation not only reduces spontaneous fusion for minimizing autophagic flux under normal conditions, but also preassembles multiple lysosomes to increase the fusion probability for resuming autophagy upon stimulation. A parallel study identified mTOR as the kinase for VAMP8 phosphorylation.[31] STX17 contains sites for both acetylation[32] and phosphorylation.[33] Two residues, K219 and K223, on STX17 are acetylated by CREB binding protein under normal conditions to block SNARE zippering and thereby minimize autophagy flux. Similar to the VAMP8 dephosphorylation, upon stimulation, the deacetylation of STX17 by HDAC2 can release the brake of SNARE zipping and significantly increase autophagy.[32] The phosphorylation of STX17 at the residue S200 by TBK1 is mainly for regulating the formation of the autophagy initiation ATG13 complex.[33] Finally, ULK1 keeps the inactivated YKT6 through phosphorylation until the completion of autophagosome formation,[34–36] which prevents the formation of premature complexes.

Tethering factors

SNARE proteins alone are not sufficient to drive membrane fusion and require other accessary factors to facilitate this process. Perhaps the most important accessory factors are tethering factors, which are recruited to specific membranes by Rab GTPases and phosphoinositide lipids to mediate initial engagement between an incoming vesicle and its target organelle.[37,38] The action of tethering factors is thought to bring the two compartments to a sufficiently close distance to allow trans-SNARE assembly. It has also been postulated that tethering factors confer fusion efficiency and specificity by binding/chaperoning distinct SNARE proteins and/or SNARE subassemblies to promote trans-SNARE formation.[39] Studies in conventional membrane trafficking pathways have identified two major groups of tethering factors: long coiled-coil tethers and multi-protein tethering complexes (MTC's).[38] Amongst these, the HOPS (homotypic fusion and protein sorting) complex, an MTC that mediates endosomal trafficking,[40] has been demonstrated to function in autophagosome-lysosome tethering.[41,42] Recent investigations in mammalian cells have identified several other noncanonical tethering factors that are critical to autophagosome-lysosome fusion (Table 1, Figure 2).

The HOPS complex

Conserved from yeast to humans, HOPS mediates the fusion of late endosomes and autophagosomes with lysosomes/vacuoles by tethering membranes, chaperoning, proofreading SNARE's and promoting trans-SNARE assembly.[43] Yeast HOPS binds the Q-SNAREs Vam3, Vam7, and Vti1 and

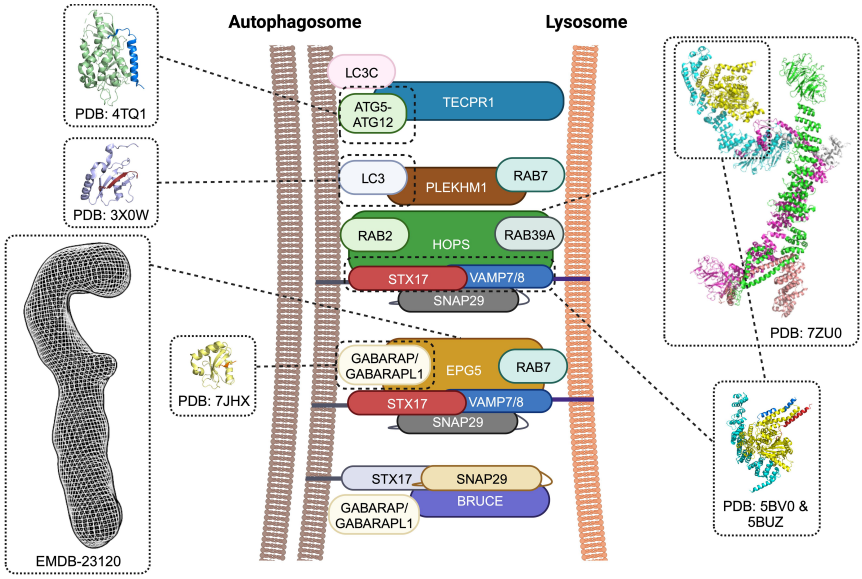

Figure 2. Accessory factors that function in autophagosome-lysosome fusion. TECPR1 binds LC3C to promote fusion between autophagosomes and lysosomes. The crystal structure of the human TECPR1 AIR domain in complex with human ATG5 (TECPR1 AIR domain in blue, ATG5 in light green) showed that TECPR1 binds to the same interface that ATG5 uses to bind ATG16L. The Rab7 effector PLEKHM1 functions as a multivalent adaptor to mediate autophagosome-lysosome fusion. Human PLEKHM1 binds LC3 proteins through a conserved LIR domain and this interaction has been visualized by X-ray crystallography (LC3 in light blue, PLEKHM1 LIR motif in brown). The highly conserved HOPS complex facilitates autophagosome-lysosome fusion by tethering membranes, chaperoning and proofreading SNAREs, and promoting trans-SNARE assembly. The overall architecture of yeast *Saccharomyces cerevisiae* HOPS was visualized by cryo-EM and an atomic model of the core complex was generated based on the cryo-EM density map (Vps33a in yellow, Vps16 in cyan, Vps11 in green, Vps8 in magenta, Vps39 in salmon, Vps41 in grey). Two crystal structures of *Chaetomium thermophilum* Vps33-Vps16 subassembly in complex with the Qa-SNARE Vam3 and the R-SNARE Nyv1 revealed how the SM-module of HOPS bind SNAREs. A composite model generated from these two crystal structures is shown in the inset with Vam3 coloured in blue and Nyv1 coloured in red. EPG5 is thought to tether autophagosomes to lysosomes through binding LC3/GABARAP proteins and Rab7. The overall architecture of full-length human EPG5 has been visualized by negative stain electron microscopy (density map coloured in grey). X-ray crystallography revealed how the LIR2 motif of human EPG5 (coloured in red) interacts with GABARAPL1 (coloured in yellow).

R-SNARE Nyv1[44] while mammalian HOPS binds the autophagosomal Qa-SNARE STX17[41,42] with conflicting data regarding its ability to bind the autophagosomal R-SNARE Ykt6.[22,23] HOPS is thought to be recruited to autophagosomes, late endosomes, and lysosomes/vacuoles directly by Ypt7 in yeast or indirectly by the small GTPase RAB7 through the adaptor protein PLEKHMI in higher eukaryotes.[45] Phosphoinositide lipids might also play

a role in HOPS recruitment as this complex shows an affinity for PI(3)P, PI(3,5)P2, and PI(4,5)P2 *in vitro*.[46] In mammalian cells, HOPS has also been shown to interact with the GABARAP subfamily of Atg8 autophagosome surface proteins and to bind lysosomes via the ARL8B small GTPase and the BORC complex.[45,47,48]

The hexameric HOPS complex is composed of two HOPS-specific subunits (Vps39 and Vps41) and four core subunits (Vps11, Vps18, Vps16, and Vps33) shared with the related CORVET complex that functions in early endosomal trafficking.[43] X-ray crystallography generated the first structural information on HOPS, and in particular, the Vps33 subunit that serves as a SM (Sec1/Munc18) protein that chaperones SNARE proteins and facilitates trans-SNARE assembly. The first crystal structures of human Vps33a and Vps33 from the thermophilic fungus *Chaetomium thermophilium* not only showed that this HOPS subunit adopts an overall fold similar to other structurally characterized SM proteins but also revealed that this SM protein does not contain a binding groove for the N-terminal peptide of Qa-SNARE.[49] The subsequent crystal structures of Vps33 proteins in complex with the C-terminal domain (CTD) of the HOPS subunit, which is the first structural data obtained for a HOPS subassembly, demonstrated that Vps33 binds the alpha-solenoid of the Vps16 CTD via an extended surface.[50] Lastly, a landmark crystallographic study generated structural snapshots of Vps33 in complex with the SNARE domains of the R-SNARE Nyv1 or the Qa-SNARE Vam3. These structures revealed that the SNARE motifs of Nyv1 and Vam3 adopt helical structures and bind to overlapping sites. When the two structures were superimposed, the two SNARE domains are arranged in a half-zippered configuration with their zero-layer residues located proximal to one another.[51] This finding led to the proposal that Vps33 not only templates the folding of the two complementary SNARE domains from the Qa- and R-SNAREs but also guides them toward complex assembly (Figure 2). Future studies will focus on delineating how Vps33 assists the observed assembly intermediate to incorporate the third SNARE and to transition into the full assembly, and to determine if mammalian VPS33a exerts similar SNARE chaperoning/templating function on its cognate autophagic SNARE's STX17 and VAMP7/8.

The other five HOPS subunits share a similar overall architecture featuring N-terminal beta-propeller domains followed by extended alpha-solenoids similar to those found in clathrin, COP-II cage components, and coat nucleoporins. Except for the N-terminal beta-propeller of yeast Vps18[52], experimental structural information has been difficult to obtain for HOPS subunits through the X-ray crystallographic approach. The development of procedures to isolate native and overexpressed HOPS from yeast cells has facilitated structural studies of this MTC in its fully assembled state by the single-particle electron microscopy (EM) approach. The first negative stain EM investigation of full yeast HOPS, which involves stabilization by mild chemical crosslinking, revealed a seahorse-like

architecture featuring a head region containing a deep cavity and attached to a tail region that contains a flexible lobe.[53] Further negative stain EM analysis of HOPS subassemblies enabled these investigators to deduce a subunit organization map of the complex, with the Vps33, Vps16, and Vps41 making up the head domain, Vps11 and Vps18 making up the extended tail, and Vps39 the tail-localized flexible lobe. They also demonstrated that the Vps41 and Vps39 subunits located at the tips of the complex can engage in interaction with Ypt7. Interestingly, a subsequent negative stain EM study on non-crosslinked yeast HOPS reported by a different research group showed that this complex adopts a more extended "spaghetti-dancer" architecture featuring a "head region" composed of two domains and three leg-like extensions.[54] As the proposed subunit organization of the two EM studies largely agrees with one another, one suggestion is that the two studies captured yeast HOPS in two different conformational states: open and closed.

The recent breakthrough single-particle cryo-EM investigation reported, for the first time, visualization of the complete yeast HOPS complex in its solution state (Figure 2). Although they only managed to obtain a medium-resolution (local resolution between 3.6 and 5 Å) composite map by combining several local-refined maps, these investigators were able to construct an atomic model of yeast HOPS by leveraging the power of the recently public-released machine learning-based AlphaFold2 structural prediction algorithm.[55] In particular, these investigators used AlphaFold2 to generate structural models of all HOPS subunits and then fit them into their cryo-EM density map. The refined structural model showed that yeast HOPS adopts a highly extended yet relatively rigid architecture of 430 Å in height and 130 Å in width, with an overall morphology resembling a baseball pitcher. The two core subunits Vps11 and Vps18 engage in interactions with one another through their elongated alpha-solenoids in an antiparallel fashion to form the rigid "body" or central core, while their N-terminal beta-propeller domains project to the periphery forming the "head" (Vps11) of one of the "feet" on the opposite side of the complex. On the other hand, the HOPS-specific units Vps39 and Vps41 are anchored to the core by their C-terminal helical regions through engaging in coiled-coil interactions with the extended C-terminal helix and the RING finger domain of Vps11 and Vps18 respectively. Even though the rest of Vps39 and Vps41 are poorly resolved in the cryo-EM map beyond the regions in contact with the core due to their inherent flexibility, the AlphaFold2-predicted structures suggested that Vps39 forms the second leg and feet while Vps41 forms one of the arms and hands. Lastly, the SNARE binding module subunits Vps16 and Vps33 form the second arm and hand holding the "baseball". More specifically, Vps16 extends to the lateral side of the core and engages in an interaction interface through part of its alpha-solenoid with a coiled coil formed by Vps18 and Vps41

and the N-terminal region of Vps18. The SM protein Vps33, however, is anchored to the alpha-solenoid of Vps16 and also contacts a structured loop of Vps18. These investigators also used AlphaFold2 to model the interaction between Vps39 and Vps41 and Ypt7 GTPase and found that Ypt7 binds the beta-propeller of Vps41 and the alpha-solenoid of Vps39, respectively. Based on their new structural findings, these investigators proposed a model in which the Vps41 and Vps39 recruit HOPS to opposing membranes to allow the rigid central core of HOPS to stably tether these membranes. In the next phase, Vps33 engages in interaction with the SNAREs to promote zippering with the slight flexibility of Vps41 and Vps39 allowing them to dampen the motion of HOPS as the opposing membranes are brought closer together.

Whether or not mammalian HOPS adopts a similar architecture remains unknown. However, it is worth mentioning that a very recent study proposed a "hook-up" model for mammalian HOPS complex assembly, which requires two HOPS sub-complexes docking on membranes via membrane-associated Rab GTPases.[56] The four-subunit subcomplex containing VPS16/VPS18/VPS33A/VPS41 binds with Rab39A on lysosomes via its VPS41, while the two-subunit HOPS subcomplex containing VPS39/VPS11 binds with Rab2 on autophagosomes via its subunit VPS39. Proper pairing with Rab2 and Rab39A enables HOPS complex assembly between autophagosome and lysosome for its tethering function, facilitating efficient membrane fusion driven by autophagic SNAREs.

EPG5

Originally discovered in the *C. elegans* genetic screen metazoan-specific autophagy factors,[57] EPG5 (Ectopic P-granules protein 5) is a Rab7 effector that functions as a tethering factor in autophagosome fusion with late endosome/lysosome.[58] Deficiency in EPG5 in *C. elegans*, mice, and humans leads to the accumulation of autophagosomes, amphisomes, and non-degradative autolysosomes and in turn impairment in autophagy.[58] Recombinant *C. elegans* EPG-5 and human hEPG5 promote assembly of the STX17-SNAP29-VAMP8 trans-SNARE complex *in vitro* and knockdown of EPG5 in HeLa cells reduces levels of STX17, SNAP29, VAMP7, and VAMP8[58]. EPG5 engages with autophagosomes through interaction with LC3/GABARAP family of ATG8 proteins via its two tandemly arranged LIR motifs, and is likely localized to the late/lysosomes through Rab7 and/or the R-SNARE VAMP7/8. EPG-5 deficiency leads to non-specific fusion of autophagosomes with different endocytic vesicles and the formation of abnormally large vesicles with mixed identities in *C. elegans*, indicating that this tethering factor plays a pivotal role in enforcing fusion specificity.[58] Mutations to human EPG5 cause an autosomal recessive severe multi-system disorder known as Vici syndrome.[59]

Recent studies have generated new insights into the biochemical and structural properties of human EPG5. Negative stain EM analysis revealed that the ~290kDa human EPG5 adopts an overall shepherd staff architecture with a "hook" connected to a "finger region" and rigid shaft[60] (Figure 2). Its highly extended nature and overall length (375Å) are in line with that observed for MTCs in other membrane trafficking pathways. Biochemical pulldown analysis showed that EPG5 preferentially binds the GABARAP subfamily of ATG8 proteins. Although both LIR motifs within the tandem are required for maximal binding to GABARAPs, one LIR (LIR2) exhibits stronger binding affinity compared to the other (LIR1). The crystal structure of EPG5 LIR2 in complex with GABARAPL1 revealed that this LIR docks to the canonical LDS (LIR docking site) on this human ATG8 isoform[60] (Figure 2). Further negative stain EM studies suggested that this tandem LIR is localized to the concave side of the hook region of EPG5. Finally, mutagenesis studies showed that despite being the low-affinity binding site, LIR1 plays a more important role in mediating interaction with GABARAP. Collectively, these data led to a two-factor authentication model in which the initial binding of LIR1 to GABARAP licensed the subsequent docking and binding of LIR2 to lock EPG5 on autophagosomes into a tight interaction in preparation for subsequent fusion with the lysosome.[6]

Other accessory factors

ATG14/Barkor/ATG14L, an essential autophagy-specific regulator of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, is concentrated on the curved autophagic membrane enriched in PtdIns(3)P via the Barkor/ATG14(L) autophagosome targeting sequence (BATS) domain in a stress-inducible manner.[61,62] That finding indicates that Barkor/ATG14(L) functions as a membrane curvature sensor and targeting factor for PI3KC3 to autophagosomes recruited by STX17.[61] In addition to its localization to phagophores,[62–66] ATG14 also localizes to mature autophagosomes and controls the fusogenic activity of the autophagic SNARE protein complex, both spatially and temporally.[20,61,63,67] It has been previously shown that human ATG14 binds directly to the binary complex of STX17 and SNAP29 on autophagosomes and promotes full SNARE complex zippering to mediate the fusion between autophagosomes and lysosomes. ATG14 homo-oligomerization is required for SNARE protein binding and fusion promoting, yet is dispensable for PtdIns3K stimulation and autophagosome biogenesis.[20] These data suggest ATG14's pivotal role in autophagy-specific autophagosome–endolysosome fusion activity.

TECPR1 was initially identified component of the autophagy network via its interaction with Atg5 from proteomics-based interactome analysis.[68] TECPR1 co-localized with lysosomal LAMP-2 and deficiency in this protein leads to accumulation of autophagosomes. Subsequent analysis revealed

that TECPR1 interacts with the Atg12-Atg5 conjugate via the AIR (Atg12-Atg5-interacting region) domain located adjacent to a PH (pleckstrin homology) domain that mediates PI3P binding. TECPR1 binds LC3C to promote fusion between autophagosomes and lysosomes. The crystal structure of human TECPR1 AIR domain in complex with human ATG5 revealed that TECPR1 binds to the same interface as ATG16L which forms a complex with Atg12-Atg5 conjugate[69] (Figure 2). This leads to a working model in which Atg16L hands off the Atg12-Atg5 conjugate to TECPR1 and this interaction leads to a conformational change that exposes the PH domain of TECPR1 for binding PI3P on autophagosome to confer fusion specificity. Interestingly, three different research groups (Randow, Wu, Lystad) recently discovered that TECPR1 contains an N-terminal dysferlin domain that binds sphingomyelin and likely serves as an E3 ligase to mediate conjugation of ATG8 proteins to single membranes induced by membrane damage.[70–73]

A member of the inhibitor of apoptosis (IAP) family, BRUCE is a large-sized protein that was identified by an RNAi screen for autophagy factors. BRUCE localizes to both lysosomes and autophagosomes. Deficiency in BRUCE leads to the accumulation of autophagosomes upon starvation and in turn defective autophagy.[74] BRUCE binds the autophagic snares STX17 and SNAP29 and it engages GABARAP and GABARAPL1 in a LIR-independent manner.[74] Future studies will focus on mapping the GABARAP-binding site on BRUCE and on delineating how it engages in non-canonical interaction with Atg8 proteins.

PLEKHM1 (pleckstrin homology domain containing, family M [with RUN domain] member 1) is a Rab7 effector discovered from a proteomics analysis of interaction partners of active Rab7.[71] It serves as a multivalent adaptor to regulate fusion between autophagosome and lysosome by simultaneously binding Rab7, Arl8, LC3, and the HOPS complex.[75] PLEKHM1 binds LC3/GABARAP proteins through a conserved LIR domain. Crystallographic analysis revealed that PLEKHM1's LIR docks into the LDS of LC3B in a canonical fashion[45] (Figure 2). PLEKHMI has also been shown to interact with the C-terminal region of the HOPS subunit Vps39 via its RUN domain. Further biochemical and structural studies will be needed to understand the basis of this interaction.

Members of the Atg8 family undergo conjugation to phosphatidylethanolamine and this lipidation modification anchors these ubiquitin-like proteins to the inner and outer membranes of autophagosomes.[76] Recent imaging studies in mammalian cells revealed that the Atg8 conjugation system, more specifically the ATG3 E2-like enzyme, is important for the degradation of the autophagosome inner membrane after fusion with lysosomes.[77] Apart from binding and cooperating with tethering factors, Atg8 family members may participate in other aspects of autophagosome-lysosome fusion. *In vitro* synthetic liposomes-based studies showed that yeast Atg8 and mammalian LC3 and GABARAP proteins are capable of mediating membrane tethering and hemifusion.[78–83] More recently, it was demonstrated that mammalian LC3B and GABARAP show

distinct membrane curvature-dependent tethering activities with LC3B more efficeHowever, it remains to be confirmed that the intrinsic membrane modulation properties allow Atg8 proteins to directly mediate autophagosome-lysoosme/vacuole fusion.

RAB and RAB regulators

The highly conserved Rab family of proteins are the master regulators of intracellular transport in eukaryotic cells. These small GTPases function as molecular switches by cycling between an inactive, cytoplasmic GDP-bound state and an active, membrane-bound GTP-bound state.[84] Active Rabs localize to unique membrane surfaces and control transport by recruiting specialized effectors to their sites of action.[85] Within the Rab family, Rab7 and its homologue yeast Ypt7 are required for autophagosome-lysosome/late endosome fusion.[86] Early X-ray crystallographic analysis of yeast Ypt7 and subsequent structural studies of Rab7 in complex with different effectors showed that Rab7/Ypt7 adopts a similar fold as other Rabs/Ypt proteins but with slight conformational differences in the Switch I and II regions, the N-terminal and C-terminal regions, as well as several loops that define binding specificity to effectors.[87,88] Like all Rabs, the spatialtemporal activation and thereby membrane localization of Rab7/Ypt7 is tightly controlled by guanine nucleotide exchange factors (GEFs), a sequence divergent family of proteins and protein complexes that facilitate GDP to GTP exchange of their cognate Rabs.[90,89] The *bona fide* Rab7/Ypt7 GEF is the evolutionarily conserved heterodimeric Mon1-Ccz1, a member of the Tri Longin Domain (TLD) Rab GEF family. This complex is a critical component of the Rab cascade that controls controlling endosome maturation. Mon1-Ccz1 is recruited by the early endosome-localized Rab5 and PI(3)P and this subsequently displaces Rabex5 (Rab5 GEF) from endosomal membrane to stop positive feedback loop of Rab5 activation.[91] Mon1-Ccz1 then recruits and activates Rab7 to drive late endosome maturation and fusion events.

In autophagy, Mon1-Ccz1 is recruited to autophagosomes in a Rab5-independent fashion likely through interaction between the conserved LIR motif at the C-terminus Ccz1 C-terminus and Atg8 family of proteins that are anchored to the outer membrane of autophagosomes.[92] Recent structural studies have generated insights into how Mon1-Ccz1 activates Rab7/Ypt7 and potentially gets recruited to membrane surfaces. The crystal structure of the catalytic core composed of LD1 (Longin domain 1) of Mon1 and the LD1 of Ccz1 in complex with Ypt7 revealed how the switch regions of Ypt7 are remodelled by Mon1-Ccz1 to open up the nucleotide-binding pocket to promote displacement of bound nucleotide[93] (Figure 3). Regions outside of the core are believed to mediate membrane recruitment of Mon1-Ccz1. The recently reported cryo-EM

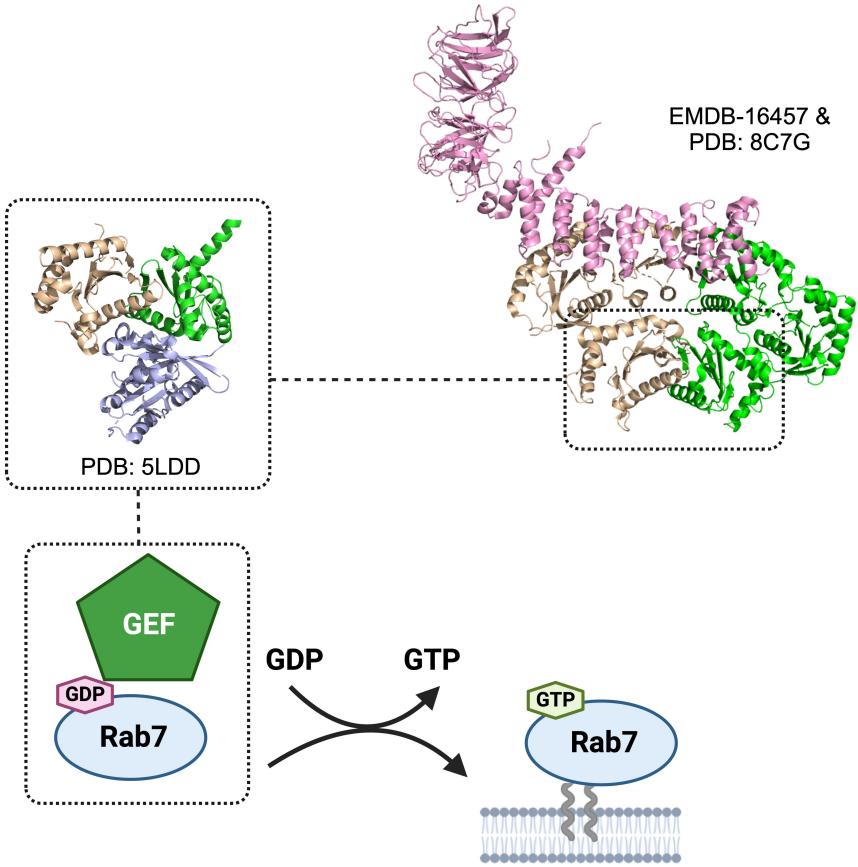

Figure 3. Rab7 regulator and its activator. Rab7 plays a key role in mediating late endosomal trafficking and autophagy and has been shown to localize to both the lysosome and the autophagosome to recruit different effectors. Rab7 is activated by a GEF which promotes displacement of GDP and loading of GTP. The GEF for Rab7 is the heterodimeric Mon1-Ccz1 complex. The crystal structure of the catalytic core Mon1-Ccz1 in complex with Rab7-orthologue Ypt7 from *Thermochaetoides thermophila* revealed how this GEF to promote nucleotide exchange (Mon1 in green, Ccz1 in gold, Ypt7 in blue). Mon1-Ccz1 in higher eukaryotes contain an additional component called RMC1 or Bulli. The cryo-EM structure of *Drosophila melanogaster* Mon1-Ccz1-Rmc1 complex revealed the third component adopts a leg-like architecture and binds to a region opposite to the Rab7 substrate binding site.

structure of the full fungal Mon1-Ccz1complex from *Chaetomium thermophilum* showed that both subunits contain 3 Longin domains (LS) arranged in a triangular fashion, with LD1 and LD3 from the two subunits projected towards one another to mediate.[94] This structural model also showed that the additional LDs did not affect the configuration of the LD1-LD1 catalytic core. Analysis of the electrostatic potential of the surface of the complex led to the identification of

a basic patch located in LD2 and LD3 of Mon1 that may serve as the PIP-containing membrane binding region of Mon1-Ccz1.

Interestingly, Mon1-Ccz1 in higher eukaryotes contain an additional non-TLD subunit called RMC1 or Bulli. The high-resolution structure of the *Drosophila melanogaster* Mon1-Ccz1-Rmc1 complex was reported by two separate research groups very recently[95,96] (Figure 3). Rmc1 was found to adopt a leg-like architecture composed of an N-terminal beta-propeller foot domain resembling the WD40 domain of yeast Atg18 but lacks the lipid binding motif and a C-terminal alpha-solenoid shin domain. Although Rmc1 binds to the opposite surface of the Rab7 substrate binding site and does not alter the structural configuration of catalytic core, the LD2 and LD2 domains of Mon1 and Ccz1 were observed to undergo significant conformational changes to accommodate the binding of the third subunit.

Summary

The identification of key components of the fusion machinery has generated a framework for dissecting the molecular mechanism of the autophagosome-lysosome/vacuole fusion process critical to the specific and efficient execution of autophagy. While many of the players involved in the autophagy fusion process are similar to (eg. Rabs, SNAREs) or even sometimes shared with (eg. HOPS) other intracellular transport pathways, unique factors have been uncovered that may function to provide an additional level of regulation of this process (eg. EPG5). Classical X-ray crystallographic-based studies have produced high-resolution snapshots of the human STX17-SNAP29-VAMP8 trans-SNARE complex central to autophagosome-lysosome fusion as well as a presumed intermediate containing fungal Vps33, the SM component of HOPS, in complex with two vacuolar SNAREs, and the fungal Mon1-Ccz1 catalytic core in complex with Ypt7. Further studies are still required to delineate the structural dynamics of these fusion components. The advent of single-particle cryo-EM and machine learning-based structural modeling have greatly facilitated structural investigations of all types of proteins and protein complexes. The recently reported molecular structure of yeast HOPS is a testament to the state of these cutting-edge technologies and illustrates how high-quality structural information can be obtained from a previously intractable dynamic assembly. We anticipate integrated structural approaches combining conventional X-ray crystallography, cryo-EM, and AlphaFold2 will produce exciting data to further our knowledge of the molecular structures and dynamics of other components of the autophagosome-lysosome /vacuole machinery. Future studies will also focus on delineating if, when, and how the different components are recruited to the fusion site and how they work collaboratively with one another to ensure the specificity and efficiency of fusion.

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