



HOKKAIDO UNIVERSITY

Title	Phase separation promotes Atg8 lipidation and vesicle condensation for autophagy progression
Author(s)	Fujioka, Yuko; Tsuji, Takuma; Kotani, Tetsuya et al.
Citation	Nature structural & molecular biology, 32(11), 2285-2295 https://doi.org/10.1038/s41594-025-01678-3
Issue Date	2025-09-16
Doc URL	https://hdl.handle.net/2115/98060
Rights	This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature' s AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: http://dx.doi.org/10.1038/s41594-025-01678-3
Type	journal article
File Information	huscap_Fujioka_Manuscript.pdf



Phase separation promotes Atg8 lipidation and vesicle condensation for autophagy progression

Yuko Fujioka^{1*}, Takuma Tsuji¹, Tetsuya Kotani², Hiroyuki Kumeta³, Chika Kakuta², Junko Shimasaki², Toyoshi Fujimoto⁴, Hitoshi Nakatogawa² & Nobuo N. Noda^{1,5*}

¹Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan

²Cell Biology Center, Institute of Integrated Research, Institute of Science Tokyo, Yokohama 226-8501, Japan

³Faculty of Advanced Life Science, Hokkaido University, Sapporo 001-0021, Japan

⁴Research Institute for Diseases of Old Age, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan

⁵Institute of Microbial Chemistry (BIKAKEN), Tokyo 141-0021, Japan

*e-mail: fujioka@igm.hokudai.ac.jp (Y.F.), nn@igm.hokudai.ac.jp (N.N.N.)

Abstract

Upon starvation, the autophagy-initiating Atg1 complex undergoes phase separation to organize the pre-autophagosomal structure (PAS) in *Saccharomyces cerevisiae*, from which autophagosome formation is considered to proceed. However, the physiological roles of the PAS droplet remain unclear. Here we show that core Atg proteins are recruited into early PAS droplets that are formed by phase separation of the Atg1 complex with different efficiencies in vitro. The Atg12–Atg5–Atg16 E3 ligase complex for Atg8 lipidation is the most efficiently condensed in the droplets via specific Atg12–Atg17 interaction, which is also important for the PAS targeting of the E3 complex in vivo. In vitro reconstitution demonstrates that E3-enriched early PAS droplets promote Atg8 lipidation and that Atg8 coating of the vesicle membrane is both necessary and sufficient for their condensation into the droplets. These data suggest that the PAS functions as an efficient production site for lipidated Atg8 and pools membrane seeds to drive autophagosome formation.

Main text

Phase separation is a general mechanism that reversibly condenses biomolecules such as proteins and nucleic acids in the cytoplasm and the nucleoplasm^{1,2}. The resultant condensates are often called liquid droplets and play crucial roles in various cellular phenomena using the liquid-like property, the typical role of which is to promote or inhibit biochemical reactions by condensing or excluding specific enzymes³. Although phase separation was initially studied as a mechanism of condensing proteins and RNA, recent studies revealed that phase separation is also important for membrane-related phenomena such as clustering synaptic vesicles (SVs) at synapses⁴, maintaining the integrity of the Golgi ribbon⁵, generating autophagosomes in the cytoplasm⁶, and gathering autophagic cargo proteins⁷⁻¹⁰. However, the molecular mechanisms of how phase separation drives membrane-related functions remains largely unknown.

Autophagosome is a double membrane-bound organelle that is de novo generated upon induction of autophagy, a lysosomal degradation system important for cellular homeostasis¹¹. In budding yeast, autophagy-related (Atg) proteins are known to be recruited at the pre-autophagosomal structure (PAS), which functions as a site of autophagosome formation¹². We recently revealed that the PAS behaves as a liquid droplet¹³. Upon nutrient starvation, dephosphorylation of Atg13 triggers the formation of the autophagy-initiating Atg1 complex consisting of Atg1, Atg13, Atg17, Atg29, and Atg31, which undergoes phase separation to organize the early PAS droplets by multivalent interactions between Atg13 and Atg17. Early PAS droplets then transform into mature PAS droplets by recruiting downstream Atg proteins that include the phosphatidylinositol (PI)-3 kinase complex consisting of Vps34, Vps15, Vps30/Atg6, Atg14, and Atg38, a lipid transfer protein Atg2, PI 3-phosphate (PI3P) binding proteins Atg18 and Atg21, and the Atg8-phosphatidylethanolamine (PE) conjugation (Atg8 lipidation) system consisting of a ubiquitin-like protein Atg8, the E1 enzyme Atg7, the E2 enzyme Atg3, the E3 enzyme complex consisting of the Atg12–Atg5 protein conjugate and Atg16, the E2 enzyme Atg10 for Atg12–Atg5 conjugation, and the deconjugase Atg4 that delipidates Atg8^{11,14}. Moreover, the PAS droplets recruit a lipid scramblase Atg9-containing vesicles (termed Atg9 vesicles) as an initial membrane seed for generating autophagosomal precursor membranes¹⁵⁻¹⁷. However, little is known about the specific roles that PAS droplets play in initial steps of autophagosome formation.

Here, we introduced downstream Atg proteins into early PAS droplets in vitro and demonstrated that PAS droplets function as a site of Atg8–PE production by condensing the E3 complex. Moreover, we found that PAS droplets incorporate membrane vesicles in an Atg8-lipidation dependent manner, which could function as a mechanism of gathering membrane seeds for initial autophagosomal membrane generation. These data suggest that the PAS droplets drive autophagosome formation by performing a number of direct functions.

Results

The Atg12–Atg5–Atg16 complex is most efficiently condensed in early PAS droplets in vitro

We previously showed that the Atg1 complex undergoes phase separation to form liquid-like droplets in vitro (termed early PAS droplets hereafter), which function as a scaffold of the PAS assembly in yeast cells¹³. The mature PAS consists of not only the Atg1 complex, but also other various Atg proteins, which are important for progression of autophagy. We purified core Atg proteins and complexes as follows: 1) the Atg1 complex consisting of Atg1 (kinase-dead D211A mutant was used to prevent droplet dissolution in the presence of ATP in later analysis), Atg13, Atg17, Atg29, and Atg31; 2) Atg8 and Atg12 conjugation systems including Atg8, Atg4 (processing and deconjugating enzyme for Atg8), Atg7 (E1 for Atg8 and Atg12), Atg3 (E2 for Atg8), the Atg12–Atg5 conjugate complexed with Atg16 (the Atg12–Atg5–Atg16 complex; E3 for Atg8), and Atg10 (E2 for Atg12); 3) the autophagy-specific PI-3 kinase (PI3K) complex consisting of Vps34, Vps15, Atg6/Vps30, Atg14, and Atg38; 4) lipid transfer protein Atg2; and 5) phosphoinositide binding proteins Atg18 and Atg21 (Extended Data Fig. 1a). For fluorescence imaging, the proteins were expressed as fusion constructs bearing either a SNAP tag (Atg2, 4, 7, 16, 18, 21, 38) or a GFP tag (Atg8) except for Atg3 and Atg10, which were fluorescently labeled by chemical modification after purification. We verified that adding the SNAP tag does not substantially affect autophagy activity, as demonstrated by the Pgk1–GFP processing assay in yeast (Extended Data Fig. 1b). SNAP-fusion proteins were fluorescently labeled with a SNAP ligand after purification. These proteins were individually mixed with pre-formed early PAS droplets and observed by fluorescence microscopy (Fig. 1). Intriguingly, efficiency of condensation into early PAS

droplets varied widely from protein to protein: the Atg12–Atg5–Atg16 complex showed the highest condensation efficiency, followed by Atg2, Atg21, Atg8, and Atg18. The condensation of Atg3, Atg4, Atg7, Atg10, and PI3K was not significant compared with SNAP or GFP. These data indicate that there is a preference for the protein that early PAS droplets accommodate.

A portion of Atg12 IDR mediates specific interaction with Atg17

We then studied the mechanism of how the Atg12–Atg5–Atg16 complex was efficiently condensed in the droplets. We conducted fluorescence recovery after photobleaching (FRAP) analysis and confirmed that the Atg12–Atg5–Atg16 complex condensed within the droplets rapidly exchanges with the surrounding solution (Extended Data Fig. 1c). This observation indicates that the Atg12–Atg5–Atg16 complex does not form strong interactions with droplet constituents, suggesting the involvement of weaker contacts, typically mediated by intrinsically disordered regions (IDRs). We previously showed that the Atg12–Atg5 conjugate interacts with Atg17 *in vivo*, for which the N-terminal IDR of Atg12 is important¹⁸. Moreover, comprehensive protein complex prediction in yeast suggested that Atg12 IDR binds to Atg17¹⁹. We used AlphaFold2²⁰ to predict the Atg12–Atg17–Atg29–Atg31 complex structure and confirmed that Atg12 IDR, especially residues 42–62, bind to Atg17 without interfering with Atg29–Atg31 binding or homo-dimerization of Atg17 (Fig. 2a). In order to predict the detailed interactions, residues 38–63 of Atg12 were used for prediction of Atg17-bound structure (Fig. 2b). Residues 42–62 of Atg12 assume an α -helical conformation and bind to the hydrophobic patch of Atg17 consisting of Ile147, Ile154, Tyr303, Ile306, Phe307, Ile310, Leu313, and Phe317 using the side-chains of Val43, Leu47, Phe50, Arg53, Leu54, and Leu57. In addition, hydrophilic interactions were observed between Ser60 and Asp61 of Atg12 and Lys164 and Tyr303 of Atg17 and between Ser51 and Ser55 of Atg12 and Gln157 of Atg17. Hereafter, we call the residues 42–62 of Atg12 as Atg17-binding helix (17BH).

We then performed NMR analyses on Atg12^{17BH} fused to a GB1 tag, which showed that Atg12^{17BH} has a partial α -helical conformation and that the NMR signals of Atg12^{17BH} were much more severely attenuated than those of the GB1 moiety upon Atg17 titration (Fig. 2c and Extended Data Fig. 2). These data suggest that Atg12^{17BH} is actually involved in the interaction with Atg17, presumably in the α -helical conformation. *In vitro* pulldown assay using GST-fused Atg12^{17BH} and Atg17 confirmed their direct interaction, and this interaction

was severely impaired by Ala-substitution at most of the residues of Atg12 and Atg17 that were predicted to be involved in the Atg12–Atg17 interaction (Fig. 2d–2g). We then analyzed the interaction using isothermal titration calorimetry and showed that the binding affinity between Atg12^{17BH} and Atg17 was 3.8 μ M, which was severely impaired by Ala substitution at Leu47 or Leu54 of Atg12 (Fig. 2h). We further analyzed the interaction of the Atg12–Atg5 conjugate with Atg17 by coimmunoprecipitation experiments and confirmed that the Atg17 residues important for Atg12^{17BH} binding in vitro are also important for the interaction in vivo (Fig. 2i). These results indicate that the predicted Atg12–Atg17 interaction is sufficiently reliable and that Atg12^{17BH} forms specific direct interaction with Atg17 both in vitro and in vivo.

Specific Atg12–Atg17 interaction is necessary and sufficient for being condensed in early PAS droplets

We next studied whether the specific interaction between Atg12^{17BH} and Atg17 is responsible for the condensation of the Atg12–Atg5–Atg16 complex in early PAS droplets. We first confirmed that the condensation of the E3 complex in the droplets was significantly impaired by deletion of Atg12 IDR and more severely impaired by deletion of whole Atg12 from the E3 complex (Fig. 3a,b), indicating that Atg12 IDR is necessary for this process. Although GFP alone was not condensed, fusion of Atg12^{17BH} to GFP dramatically promoted its condensation in the droplets, indicating that Atg12^{17BH} functions as a targeting sequence to early PAS droplets (Fig. 3c). Furthermore, Ala substitution at Val43, Leu47, Leu54, or Leu57 in Atg12^{17BH} severely impaired the condensation of the fusion protein in the droplets (Fig. 3c and Extended Data Fig. 3a). Taken together, these data suggest that the specific Atg12–Atg17 interaction as predicted by AlphaFold2 is necessary and sufficient for the condensation of the Atg12–Atg5–Atg16 complex in early PAS droplets.

Specific Atg12–Atg17 interaction mediates Atg21-independent PAS targeting of the Atg12–Atg5–Atg16 complex in vivo

We previously reported that the Atg12–Atg5–Atg16 complex targets to the PAS via two distinct mechanisms, one is via the Atg16–Atg21–PI3P interaction and another via the Atg12–Atg5 conjugate–Atg17 interaction. We speculated that the specific Atg12–Atg17 interaction we characterized here is responsible for the latter mechanism. We observed the PAS localization of the Atg12–Atg5–Atg16

complex by monitoring GFP–Atg5 in *atg12Δ atg17Δ atg21Δ* cells co-expressing Atg5–GFP, Atg12, and Atg17-2xmCherry. Colocalization of GFP–Atg5 with Atg17–2xmCherry was observed in cells expressing wild-type Atg12 and Atg17, which was significantly reduced in cells expressing wild-type Atg17 and Atg12 mutants (V43A, L47A, L54A, or L57A) or expressing wild-type Atg12 and Atg17 mutants (Q157A K164A or I306A) (Fig. 3d,e and Extended Data Fig. 3b-d). These data, together with the binding data, suggest that the Atg12–Atg5–Atg16 complex targets to the PAS via specific Atg12–Atg17 interaction in the absence of Atg21.

The Atg1 complex and Atg21 cooperatively promote lipidation of Atg8 in vitro

The findings that the early PAS droplet specifically condenses the Atg12–Atg5–Atg16 complex, the E3 enzyme for Atg8 lipidation, led us to consider the possibility that it could act as a reaction site for Atg8 lipidation. We then performed in vitro Atg8 lipidation reaction using Atg8, Atg7 (E1), Atg3 (E2), Atg12–Atg5–Atg16 (E3), liposomes mimicking the lipid composition of Atg9 vesicles (phosphatidylcholine (PC):PI:PE:phosphatidylserine (PS):phosphatidic acid (PA)=41:44:7:7:2)²¹, and MgATP in the presence or absence of early PAS droplets, which were confirmed to be formed in this reaction condition (Fig. 4a). We also studied the effect of Atg21 on Atg8 lipidation because Atg21 was reported to recruit the Atg12–Atg5–Atg16 complex to PI3P-containing membranes via binding to both Atg16 and PI3P and promote Atg8 lipidation^{22,23}. Importantly, early PAS droplets efficiently and significantly promoted Atg8 lipidation, and this stimulatory effect persisted in the presence of Atg21 (Fig. 4b,c, Extended Data Fig. 4a). By contrast, Atg21 alone enhanced Atg8 lipidation, but the increase did not initially reach statistical significance. A more detailed time-course analysis, however, demonstrated that Atg21 indeed accelerates Atg8 lipidation to a statistically significant extent (Fig. 4d,e, Extended Data Fig. 4b). We also confirmed that Atg1, Atg13, and Atg17–Atg29–Atg31, when provided individually, did not significantly promote Atg8 lipidation (Fig. 4f,g, Extended Data Fig. 4c). These results indicate that the cells employ multiple factors to promote the robust production of Atg8-PE, namely, early PAS droplets and Atg21, with the former exerting the stronger effect.

We then studied the effect of early PAS droplets and Atg21 on the efficiency of Atg8 delipidation by Atg4 in vitro. After performing Atg8 lipidation reaction using E1, E2, E3, and MgATP on PI3P-containing liposomes, the

reaction was stopped by treatment with an ATP eliminating reagent (Extended Data Fig. 4d) and further supplemented with Atg4 and either early PAS droplets, Atg21, or buffer solution (Fig. 4h,i, Extended Data Fig. 4e). The band intensity of Atg8–PE was markedly reduced upon incubation with Atg4, indicating that the used Atg4 was active and delipidated Atg8 in this reaction condition. Addition of early PAS droplets significantly inhibited the delipidation reaction by Atg4, irrespective of Atg21's presence. By contrast, Atg21 alone had little effect on the delipidation reaction. We further compared Atg8 lipidation reactions with and without Atg4 (Fig. 4j,k, Extended Data Fig. 4f). Atg4 significantly lowered the accumulation of Atg8–PE in every condition except when both early PAS droplets and Atg21 were present. These results indicate that, in vitro, early PAS droplets both stimulate Atg8 lipidation and inhibit Atg4-mediated delipidation, while Atg21 enhances lipidation; when their effects are combined, lipidated Atg8 accumulates to the same level regardless of Atg4's presence.

Specific Atg12–Atg17 interaction is important for Atg8 lipidation and autophagy in the absence of Atg21

We then studied the roles of the specific Atg12–Atg17 interaction in Atg8 lipidation and autophagy activity in yeast cells. Because the Atg16–Atg21 interaction redundantly functions in the recruitment of the Atg12–Atg5–Atg16 complex to the PAS¹⁴, we used *atg12Δ* strains with or without *atg21* deletion. In both *atg12Δ* and *atg12Δ atg21Δ* strains, Atg8–PE was not observed at all, which was recovered by expression of wild-type Atg12 (Fig. 5a,b). Expression of Atg12 mutants that are defective in Atg17-binding could also recover Atg8–PE formation in the case of *atg12Δ* strains, whereas it failed to recover Atg8–PE formation in the case of *atg12Δ atg21Δ* strains. These data suggest that the Atg12–Atg17 interaction is crucial for Atg8 lipidation in the absence of Atg21 in vivo. We also measured bulk autophagy activity by monitoring the processing of Pgk1–GFP, a non-selective autophagy cargo that releases free GFP upon delivery to the vacuole via autophagy²⁴. In both *atg12Δ* and *atg12Δ atg21Δ* strains, free GFP was not observed at all, which was recovered by expression of wild-type Atg12 (Fig. 5c,d). Expression of Atg12 mutants that are defective in Atg17-binding could also recover GFP processing in the case of *atg12Δ* strains, whereas it failed to recover GFP processing in the case of *atg12Δ atg21Δ* strains. These data suggest that the Atg12–Atg17 interaction is crucial for autophagy activity in the absence of Atg21, which is consistent with the levels of Atg8–PE.

In vivo analyses showed that Atg8–PE is sufficiently formed without the specific Atg12–Atg17 interaction in the presence of Atg21. We then studied the Atg21-mediated recruitment of the E3 complex to early PAS droplets in vitro (Fig. 5e and Extended Data Fig. 5). Consistent with Fig. 3a, lack of Atg12^{IDR} severely, and lack of Atg12 more severely impaired the condensation of the E3 complex in early PAS droplets. Atg21 alone was not efficiently localized at early PAS droplets and failed to promote the recruitment of the E3 complex lacking Atg12 or its IDR. On the other hand, PI3P-containing liposomes interacted with the surface of early PAS droplets and promoted the targeting of Atg21 to the droplet surface, and importantly, the combination of Atg21 and PI3P-containing liposomes recruited the E3 complex lacking Atg12 or its IDR efficiently to the droplet surface. These data suggest that Atg21 and PI3P-containing liposomes together can recruit the E3 complex to early PAS droplets without the Atg12–Atg17 interaction.

Atg8 lipidation promotes incorporation of lipid vesicles into early PAS droplets in vitro

We finally observed the behavior of liposomes with lipid compositions mimicking Atg9 vesicles during the Atg8 lipidation reaction in the presence of early PAS droplets using freeze fracture replica electron microscopy, in which deep etching was performed so that the protein droplets embedded in ice were exposed (Extended Data Fig. 6a; the procedure is summarized in Fig. 6a). Then PS, a component of the liposomes, as well as Atg8–PE was immune-stained. It should be noted that unlipidated Atg8 was not detected since it is removed during the washing steps in this protocol. For reference, images of liposomes outside the droplets are shown in Extended Data Fig. 6b. As shown in Fig. 6b,c, PS and Atg8–PE signals were accumulated inside the droplets only when incubated with MgATP. We also detected PS signals inside the droplets when Atg8 lipidation reaction was performed in the presence of Atg21 and PI3P-SUVs but in the absence of the Atg12–Atg17 interaction (Extended Data Fig. 6c). These data clearly demonstrate that liposomes are condensed within early PAS droplets by Atg8 lipidation via either the Atg12–Atg17 interaction or the Atg16–Atg21 interaction pathway (Extended Data Fig. 6d).

We next investigated how Atg8 lipidation promotes the incorporation of liposomes into early PAS droplets. Because replica EM is labor-intensive and low-throughput, we sought to develop a simpler fluorescence imaging-based assay to analyze liposome incorporation into droplets. Screening a panel of fluorescent

lipids revealed that liposomes labeled with any of three probes, octadecyl rhodamine B chloride (R18), BODIPY, or NBD-PE, were incorporated into early PAS droplets in an Atg8 lipidation-dependent manner (Fig. 7a,b, Extended Data Fig. 7a). FRAP analysis showed that the recovery of fluorescence of R18-liposomes at early PAS droplets was much slower than that of the Atg12–Atg5–Atg16 complex (Extended Data Fig. 1c, 7b), indicating that liposomes internalized into the droplets are tightly constrained and thus display little mobility. Tracking R18-labeled liposomes after the onset of the Atg8 lipidation reaction, we found that they first attached to the droplet surface and then gradually migrated into its interior (Fig. 7c). This behavior suggests that Atg8-PE is produced on liposomes by the E3 complex localized at the droplet surface, after which the Atg8–PE-coated liposomes are internalized within the droplet.

We then used R18-labeled liposomes in subsequent experiments to quantify the extent of droplet incorporation. Because Atg8 is positively charged (calculated pI = 8.9), its binding coats the negatively charged liposome surface with positive charge, which could enhance electrostatic interactions with the negatively charged early PAS droplets (calculated pI of the Atg1 complex = 5.6) and thereby promote liposome incorporation. To test this hypothesis, we prepared liposomes containing the cationic lipid DOTAP at various percentages and added them to early PAS droplets. Liposomes with 7 % or 25 % DOTAP showed no effect, and although 50 % DOTAP increased liposome binding to the droplet surface, it did not enhance their internalization (Extended Data Fig. 7c). Thus, the promotion of liposome uptake by Atg8 lipidation cannot be achieved merely by altering the liposome's surface charge. Atg8 is known to recognize Atg8-interaction motifs (AIMs) with high specificity, and it has been reported to bind the AIM in Atg1²⁵. We therefore tested whether AIM recognition by Atg8 promotes liposome incorporation into early PAS droplets. A canonical AIM peptide (EEEWEEL) and a binding-defective mutant (EEEAEAA) were synthesized, and we confirmed that the AIM peptide, but not its mutant, severely impaired the Atg1-Atg8 interaction by in vitro pulldown assay (Extended Data Fig. 7d). We then studied each peptide's effect on the Atg8 lipidation-dependent entry of liposomes into early PAS droplets. As shown in Extended Data Fig. 7e, neither peptide impeded liposome internalization, indicating that AIM recognition by Atg8 is not the primary mechanism driving liposome uptake into the droplets.

The Atg8 lipidation reaction is catalyzed by Atg7 (E1), Atg3 (E3), and the Atg12–Atg5–Atg16 complex (E3). After Atg8–PE is produced, these proteins

assemble with Atg8–PE on the membrane into higher-order complexes that help shape the isolation membrane²⁶. We therefore examined whether liposome uptake into droplets can be driven by Atg8 alone or requires the E1–E2–E3 enzymes as well. To this end, we tested several non-enzymatic strategies for attaching Atg8 to liposomes and discovered that fusing a membrane-binding peptide from the MARCKS effector domain (MED) to the C-terminus of Atg8 enables tight binding of Atg8 to liposomes without causing severe aggregation (Fig. 7d,e). Whereas Atg8 alone had little effect, the addition of Atg8–MED dramatically enhanced both the binding and internalization of liposomes to early PAS droplets (Fig. 7f,g). Taken together with our earlier findings, these results demonstrate that the attachment of Atg8 to the liposome surface is both necessary and sufficient for liposome internalization into the early PAS droplets.

Discussion

In budding yeast, starvation induces phase separation of the Atg1 complex into early PAS droplets, which lead to the construction of the mature PAS and initiate autophagosome formation¹³. However, why the PAS adopts a droplet state and what molecular functions this condensate confers remain unclear. Here, we show that the early PAS droplets condense the Atg12–Atg5–Atg16 E3 complex, but not the Atg4 deconjugase, via the specific interaction of the Atg12^{17BH} with Atg17, thereby promoting Atg8 lipidation *in vitro*. The early PAS droplets also inhibited the delipidation of Atg8 by Atg4, which is consistent with previous *in vivo* observations showing that Atg4 is responsible for deconjugating Atg8–PE produced at the organelles other than the PAS, even though the mechanism remains unknown^{27–29}. Atg4 is neither enriched nor excluded within early PAS droplets *in vitro* (Fig. 1b,c), and the mechanism underlying the suppression of its activity remains unclear. One possibility is that the droplet environment reduces Atg4's access to Atg8–PE, a hypothesis that will require further analysis. This dual function of the PAS will enable local production of sufficient amounts of Atg8–PE for autophagy progression as suggested by efficient Atg8–PE production even in the existence of Atg4 *in vitro* (Fig. 4j,k). A role of phase separation in promotion of ubiquitination has been reported for HECT-type E3 Itch³⁰. It will be interesting to see if the dual function of phase separation observed for the Atg8 conjugation system is also used in ubiquitin and other ubiquitin-like conjugation systems.

As the earliest event of autophagosome formation, it has been proposed

that the PAS droplet recruits vesicles, such as Golgi-derived Atg9 vesicles and autophagy-specific COPII vesicles, which may originate from the ER–Golgi intermediate compartment (ERGIC) recently reported in yeast, a compartment known to serve as an ideal sorting station to generate autophagy-specific COPII vesicles in mammals. These vesicles are then used as membrane seeds to generate the initial autophagic membranes^{13,15,31-33}. However, this process leaves many open issues. Even the precise localization of seed vesicles at the PAS droplet, whether it is the surface or interior, has not been determined due to the small size of the PAS. Using the reconstitution system, we clearly demonstrate that the early PAS droplets internalize membrane vesicles mimicking the lipid composition and size of Atg9 vesicles in the Atg8 lipidation-dependent manner (Fig. 6b,c, 7a-c), proposing that Atg9 vesicles would also localize at the interior of the PAS droplet upon autophagy induction. This phenomenon is reminiscent of the incorporation of SVs into phase separated synapsin condensates at the presynapse^{4,34}. Synapsin condensates selectively incorporate SVs but not other organelles such as mitochondria and endoplasmic reticulum despite their localization in close proximity. This selectivity could be attributed to the small size of SVs (~45 nm), their characteristic high content of negatively charged lipids (~20 mol%), or the membrane proteins such as VAMP2 and synaptophysin embedded in SVs, although the actual mechanism is still unclear⁴. The mechanism by which the early PAS droplets incorporate Atg9 vesicles, which are similar to SVs in size (30-60 nm) and in their high content of negatively charged lipids (~53 mol%), presumably with selectivity, is also not yet fully understood. Our in vitro analyses show that surface attachment of Atg8 is necessary and sufficient for liposome internalization into early PAS droplets, whereas neither AIM recognition by Atg8 nor a membrane positive charge drives this process (Fig. 7f,g, Extended Data Fig. 7c-e). The mechanism of Atg8-mediated liposome uptake therefore warrants further investigation.

Then, what are the roles for Atg9 vesicle condensation in the PAS droplets? It is known that enforced contact between lipid vesicles promotes their fusion in vitro³⁵. It could be speculated that Atg9 vesicles trapped within narrow PAS droplets are forced to interact closely with each other, which promote their homotopic fusion to generate initial autophagic membranes in concert with PAS-localizing factors such as Atg8–PE, which is known to perturb membranes and promote their tethering and hemi/full-fusion in vitro³⁶⁻³⁹. Alternatively, as in canonical membrane-fusion processes, SNARE proteins may also facilitate the

fusion of Atg9 vesicles, and their activity could act synergistically with the fusion-promoting effect of the droplets. These hypotheses represent important questions that must be addressed in future studies. The reconstitution study presented here provides the basis for unlocking these secrets and leading to a complete understanding of the mechanisms of autophagosome formation.

Acknowledgements

We thank Yohei Ohashi and Roger L Williams for providing the plasmids to purify the PI3K proteins; Hayashi Yamamoto for providing the plasmids for expressing the Atg17 mutants; Yuki Ishikawa, Konomi Marumo, Sonoko Kurazono, and Elijah William Caldwell for assistance with the protein preparation; Ayaka Saito for assistance with EM sample preparation; and Yuta Ogasawara for assistance with fluorescence imaging quantification. We thank the Open Facility, Global Research Facility Alliance Center, Office for Integrated Technical Core Hub, Hokkaido University for allowing us to conduct the analysis of Replica using JEM-1400plus TEM. This work was supported in part by JSPS KAKENHI Grant Number: JP21H05731, JP23H02429, JP23H04923, JP23K27122 to Y.F.; JP22K06818, JP22H04654 to T.T.; JP22K06123, JP25K09544 to T.K.; JP22H00446 to T.F.; JP22K19282 to H.K.; JP19H05708, JP25H01322 to H.N.; JP23K20044 to H.N. and N.N.N.; JP19H05707, JP24H00060, JP25H00966, JP25H01320, JP25H01321 to N.N.N.; PRIME, Japan Agency for Medical Research and Development Grant number JP20gm6410009 to Y.F.; CREST, Japan Science and Technology Agency Grant number JPMJCR20E3 to N.N.N.; a grant from the Nagase Science and Technology Foundation to H.N.; and grants from the Takeda Science Foundation to Y.F., H.N. and N.N.N.

Author Contributions Statement

Y.F. and N.N.N. conceived the project. Y.F. performed *in vitro* experiments. T.T. and T.F. performed replica electron microscopy. H.K. performed NMR analysis. Y.F., T.K., J.S., C.K. and H.N. performed yeast experiments. All authors analyzed the data. Y.F. and N.N.N. wrote the manuscript with input from all other authors. Y.F. and N.N.N. supervised the work.

Competing Interests Statement

The authors declare no competing interests.

Figure legends

Figure 1. Atg12–Atg5–Atg16 complex is efficiently condensed in early PAS droplets

a, Schematic drawing of the in vitro experiments. **b**, Fluorescence images of early PAS droplets supplemented with each Atg protein. Scale bar = 10 μ m. **c**, Quantification of **b**. Bars indicate the mean of n=3 technical replicates. *** $P = 4.4 \times 10^{-4}$ (SNAP vs. Atg18–SNAP), **** $P = 1.4 \times 10^{-8}$ (SNAP vs. Atg2–SNAP), 7.5×10^{-8} (SNAP vs. Atg21–SNAP), 1.8×10^{-14} (SNAP vs. Atg12–5–16–SNAP), 3.7×10^{-6} (SNAP vs. GFP–Atg8), 1.1×10^{-6} (GFP vs. GFP–Atg8), n.s., not significant (SNAP vs. PI3K–SNAP, Atg7–SNAP, Atg4–SNAP, GFP, Atg3, and Atg10), using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons.

Figure 2. Structural basis of the Atg12–Atg17 interaction

a, AlphaFold2 structure of Atg12 bound to the Atg17–Atg29–Atg31 complex. Atg17, Atg29, and Atg31 are colored in yellow, dark gray, and light gray, respectively, whereas Atg12 is colored based on the predicted local distance difference test (pLDDT) value. **b**, Close-up view of the interactions between Atg12 and Atg17. Top: Atg17 is shown with the surface model with coloring based on the electrostatic potentials (blue and red indicate positive and negative potentials, respectively). Bottom: Atg12 and Atg17 are shown with the ribbon model, in which the side-chains of the residues involved in Atg12–Atg17 interaction are shown with the stick model. Possible hydrogen bonds are shown with a broken line. **c**, Atg17–Atg31C titration experiment of GB1–Atg12^{17BH} by NMR. Top: a portion was extracted from Extended Data Fig. 2b. GB1-derived signals were labeled in black, and Atg12-derived signals were labeled in blue. Bottom: the signal intensity ratio before and after the addition of 0.5 molar equivalents of Atg17–Atg31C was extracted from Extended Data Fig. 2c, and GB1 is shown in gray and Atg12 in orange. The signal-to-noise ratio of the signals was used to calculate the error bars (n=1 experiment). **d**, In vitro pulldown assay between GST–Atg12 mutants and Atg17–Atg29–Atg31. **e**, Quantification of **d**. Bars indicate the mean of n=3 technical replicates. **** $P = 6.4 \times 10^{-11}$ (WT vs. L43A), 9.6×10^{-12} (WT vs. L47A), 1.7×10^{-11} (WT vs. R53A), 6.5×10^{-12} (WT vs. L54A), 1.9×10^{-11} (WT vs. L57A), 1.6×10^{-11} (WT vs. S61A D62A), n.s., not significant (WT vs. F50A), using a two-sided Tukey's multiple comparisons test with adjustments for multiple

comparisons. **f**, In vitro pulldown assay between GST–Atg12 and Atg17–Atg29–Atg31 mutants. **g**, Quantification of **f**. Bars indicate the mean of $n=3$ technical replicates. **** $P = 1.1 \times 10^{-10}$ (WT vs. Q157A K164A), 7.8×10^{-11} (WT vs. I306A), 1.5×10^{-8} (WT vs. L313A), using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. **h**, ITC results were obtained by titration of the GB1–Atg12^{17BH} into a solution of the Atg17–Atg31C complex. **i**, Coimmunoprecipitation of Atg5–FLAG and Atg17 mutants from yeast cells treated with rapamycin for 2 h. This experiment was repeated twice with similar results.

Figure 3. Atg12–Atg17 interaction is responsible for the in vitro and in vivo condensation of the E3 complex in the droplet

a, Fluorescence images of the early PAS droplets supplemented with each Atg protein. Atg12–5–16 images are the same as those used in Fig. 1b. Scale bar = 10 μm . **b**, Quantification of fluorescence intensity of SNAP–Atg16 in the droplets in **a**. Bars indicate the mean of $n=3$ technical replicates. * $P = 1.04 \times 10^{-2}$, **** $P = 6.3 \times 10^{-6}$ (Atg12-5-16 vs. Atg5-16), 4.0×10^{-5} (Atg12–5–16 vs. Atg12 Δ IDR–5–16), using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. **c**, Fluorescence images of the early PAS droplets supplemented with GB1–Atg12^{17BH}. Scale bar = 10 μm . **d**, Fluorescence images of GFP–Atg5 and mCherry–Atg17 in yeast cells. Scale bar = 5 μm . **e**, Quantification of the colocalization of GFP–Atg5 with mCherry–Atg17 at the PAS puncta in **d**. The box represents the interquartile range (IQR) with the median indicated by a horizontal line; whiskers extend to the most extreme data points within $1.5 \times \text{IQR}$ from the quartiles, and points beyond are plotted as outliers. Number of PAS puncta: $n = 1,548$ (WT), 1,990 (V43A), 1,815 (L47A), 1,710 (L54A), and 1,829 (L57A). **** $P < 1.0 \times 10^{-15}$, using a two-sided Dunn's multiple comparisons test with adjustments for multiple comparisons.

Figure 4. Early PAS droplets promote Atg8 lipidation and inhibit Atg8–PE delipidation in vitro

a, Observation of the early PAS droplets in the reaction solution using differential interference contrast microscopy. The left and right images correspond to reaction conditions 1 and 2 in **b**, respectively. **b**, Effect of the early PAS droplets, Atg21, and PI3P on the Atg8 lipidation reaction. In **b**, **d**, **f**, **h**, and **j**, the reaction solutions were subjected to urea-SDS-PAGE, followed by Coomassie brilliant blue staining. **c**, Quantification of the ratio between Atg8–PE

and total Atg8 in **b**. $*P = 1.7 \times 10^{-2}$ (4 vs. 6), 1.2×10^{-2} (5 vs. 6), $***P = 2.0 \times 10^{-4}$ (1 vs. 2), $****P = 5.9 \times 10^{-5}$ (1 vs. 6), n.s., not significant, using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. **d**, Time-course observation of the effect of Atg21 on the Atg8 lipidation reaction. **e**, Quantification of the ratio between Atg8–PE and total Atg8 in **d**. $*P = 1.3 \times 10^{-2}$, $**P = 4.2 \times 10^{-3}$, n.s., not significant, using a two-sided Šídák's multiple comparisons test with adjustments for multiple comparisons. **f**, Effect of Atg1, Atg13, Atg17–Atg29–Atg31, and the early PAS droplets on the Atg8 lipidation reaction. **g**, Quantification of the ratio between Atg8–PE and total Atg8 in **f**. $**P = 5.8 \times 10^{-3}$ (2 vs. 5), 1.9×10^{-3} (4 vs. 5), $***P = 2.2 \times 10^{-4}$ (3 vs. 5), 1.5×10^{-4} (1 vs. 5), n.s., not significant, using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. **h**, Effect of the early PAS droplets and Atg21 on the Atg8–PE delipidation reaction. **i**, Quantification of the ratio between Atg8–PE and total Atg8 in **h**. $*P = 1.3 \times 10^{-2}$ (2 vs. 4), 1.1×10^{-2} (2 vs. 5), n.s., not significant, using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. **j**, Effect of the early PAS droplets and Atg21 on the Atg8–PE delipidation reaction under the existence or absence of Atg4. **k**, Quantification of the ratio between Atg8–PE and total Atg8 in **j**. $*P = 4.2 \times 10^{-2}$, $**P = 3.3 \times 10^{-3}$, $****P = 2.8 \times 10^{-6}$, n.s., not significant, using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. Data are shown as means $\pm$ SD in **c**, **e**, **g**, **i**, **k**, $n = 3$ technical replicates.

Figure 5. Atg12–Atg17 interaction is important for autophagy in the absence of Atg21

a, Observation of Atg8–PE accumulation in yeast cells. **b**, Quantification of the ratio between Atg8–PE and total Atg8 in **a**. Bars represent means $\pm$ SD, $n = 4$ biological replicates. $*P = 1.6 \times 10^{-2}$ (WT in *atg12* Δ vs. WT in *atg12* Δ *atg21* Δ), 1.7×10^{-2} (WT vs. V43A in *atg12* Δ *atg21* Δ), $***P = 3.5 \times 10^{-4}$ (WT vs. L47A in *atg12* Δ *atg21* Δ), $****P = 1.5 \times 10^{-11}$ (WT vs. vec in *atg12* Δ), 7.9×10^{-6} (WT vs. vec in *atg12* Δ *atg21* Δ), 6.9×10^{-5} (WT vs. L54A in *atg12* Δ *atg21* Δ), 9.3×10^{-5} (WT vs. L57A in *atg12* Δ *atg21* Δ), n.s., not significant, using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. **c**, Pgk1–GFP processing assay. **d**, Quantification of Pgk1–GFP degradation in **c**. Bars represent means $\pm$ SD, $n = 3$ biological replicates. $****P < 1.6 \times 10^{-13}$ (WT vs. vec in *atg12* Δ), $****P = 3.7 \times 10^{-6}$ (WT in *atg12* Δ vs. WT in *atg12* Δ *atg21* Δ), 9.8

$\times 10^{-7}$ (WT vs. vec in *atg12 Δ atg21 Δ*), 2.1×10^{-5} (WT vs. V43A in *atg12 Δ atg21 Δ*), 4.6×10^{-7} (WT vs. L47A in *atg12 Δ atg21 Δ*), 2.9×10^{-7} (WT vs. L54A in *atg12 Δ atg21 Δ*), 2.2×10^{-7} (WT vs. L57A in *atg12 Δ atg21 Δ*), n.s., not significant, using a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons. **e**, In vitro Atg21 and PI3P-dependent targeting of the Atg12 Δ IDR–Atg5–Atg16 complex in the early PAS droplets observed by fluorescence microscopy. Scale bar = 10 μ m.

Figure 6. Early PAS droplets incorporate Atg8-coated vesicles via two distinct pathways

a, Schematic drawing of the procedures for freeze-fracture replica electron microscopy. **b**, Atg8 lipidation-dependent detection of the phosphatidylserine signals in the Atg1 complex droplet observed by freeze-fracture replica electron microscopy. The right graph shows the number of colloids per area of each droplet. Number of droplets analyzed: n = 14 for ATP (-) and 16 for ATP (+). **** $P = 1.4 \times 10^{-8}$, using a two-sided Mann-Whitney test. Scale bar = 200 nm. **c**, Atg8 lipidation-dependent detection of Atg8 signals in the Atg1 complex droplet observed by freeze-fracture replica electron microscopy. The right graph shows the number of colloids per area of each droplet. Number of droplets analyzed: n = 10 for ATP (-) and 11 for ATP (+). **** $P = 5.7 \times 10^{-6}$, using a two-sided Mann-Whitney test. Scale bar = 200 nm.

Figure 7. Atg8 coating is important for the condensation of the vesicles into the early PAS droplets.

a, Observation of R18-containing liposomes at the early PAS droplets with or without Atg8 lipidation reaction. All R18 fluorescence images in Fig. 7 and Extended Data Fig. 7 were acquired on the same microscope under identical settings (laser power, gain, and scan speed). Scale bar = 10 μ m. **b**, Enlarged views of the boxed region in **a** and the corresponding line profile along the yellow line. Yellow lines = 5.5, 4.7, 4.3, and 6.1 μ m from left to right. **c**, Time course of the R18 signal at the droplet. The graph on the right shows the line profile along the yellow line. Yellow lines = 4.6 μ m for top panels and 5.2 μ m for bottom panels. **d**, Schematic drawing of the procedures for the flotation assay. **e**, Flotation assay of liposomes in the existence of Atg8 or Atg8-MED fusion. This experiment was repeated three times with similar results. **f**, Observation of R18-containing liposomes at the early PAS droplets, in the absence or presence

of Atg8 or Atg8-MED. Scale bar = 10 μm . **g**, Enlarged views of the boxed region in **f** and the corresponding line profile along the yellow line. Yellow lines = 6.6, 7.4, 6.5, 5.9, 8.3, and 10.3 μm from left to right.

References

1. Banani, S.F., Lee, H.O., Hyman, A.A. & Rosen, M.K. Biomolecular condensates: organizers of cellular biochemistry. *Nat. Rev. Mol. Cell Biol.* **18**, 285-298 (2017).
2. Boeynaems, S. et al. Protein Phase Separation: A New Phase in Cell Biology. *Trends Cell Biol.* **28**, 420-435 (2018).
3. Zhang, Y., Narlikar, G.J. & Kutateladze, T.G. Enzymatic Reactions inside Biological Condensates. *J. Mol. Biol.* **433**, 166624 (2021).
4. Sansevrino, R., Hoffmann, C. & Milovanovic, D. Condensate biology of synaptic vesicle clusters. *Trends Neurosci.* **46**, 293-306 (2023).
5. Zhang, Y. & Seemann, J. RNA scaffolds the Golgi ribbon by forming condensates with GM130. *Nat. Cell Biol.* **26**, 1139-1153 (2024).
6. Fujioka, Y. & Noda, N.N. Biomolecular condensates in autophagy regulation. *Curr. Opin. Cell Biol.* **69**, 23-29 (2021).
7. Zhang, G., Wang, Z., Du, Z. & Zhang, H. mTOR Regulates Phase Separation of PGL Granules to Modulate Their Autophagic Degradation. *Cell* **174**, 1492-1506 e1422 (2018).
8. Yamasaki, A. et al. Liquidity Is a Critical Determinant for Selective Autophagy of Protein Condensates. *Mol. Cell* **77**, 1163-1175 (2020).
9. Ma, X. et al. CCT2 is an aggrephagy receptor for clearance of solid protein aggregates. *Cell* **185**, 1325-1345 e1322 (2022).
10. Sun, D., Wu, R., Zheng, J., Li, P. & Yu, L. Polyubiquitin chain-induced p62 phase separation drives autophagic cargo segregation. *Cell Res.* **28**, 405-415 (2018).
11. Nakatogawa, H. Mechanisms governing autophagosome biogenesis. *Nat. Rev. Mol. Cell Biol.* **21**, 439-458 (2020).
12. Suzuki, K. et al. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J.* **20**, 5971-5981 (2001).
13. Fujioka, Y. et al. Phase separation organizes the site of autophagosome formation. *Nature* **578**, 301-305 (2020).
14. Noda, N.N. & Inagaki, F. Mechanisms of Autophagy. *Annu Rev Biophys* **44**, 101-122 (2015).
15. Yamamoto, H. et al. Atg9 vesicles are an important membrane source during early steps of autophagosome formation. *J. Cell Biol.* **198**, 219-233 (2012).

16. Matoba, K. et al. Atg9 is a lipid scramblase that mediates autophagosomal membrane expansion. *Nat. Struct. Mol. Biol.* **27**, 1185-1193 (2020).
17. Mari, M. et al. An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. *J. Cell Biol.* **190**, 1005-1022 (2010).
18. Harada, K. et al. Two distinct mechanisms target the autophagy-related E3 complex to the pre-autophagosomal structure. *Elife* **8**(2019).
19. Humphreys, I.R. et al. Computed structures of core eukaryotic protein complexes. *Science* **374**, eabm4805 (2021).
20. Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583-589 (2021).
21. Sawa-Makarska, J. et al. Reconstitution of autophagosome nucleation defines Atg9 vesicles as seeds for membrane formation. *Science* **369**(2020).
22. Juris, L. et al. PI3P binding by Atg21 organises Atg8 lipidation. *EMBO J.* **34**, 955-973 (2015).
23. Munzel, L. et al. Atg21 organizes Atg8 lipidation at the contact of the vacuole with the phagophore. *Autophagy* **17**, 1458-1478 (2021).
24. Torggler, R., Papinski, D. & Kraft, C. Assays to Monitor Autophagy in *Saccharomyces cerevisiae*. *Cells* **6**(2017).
25. Nakatogawa, H. et al. The autophagy-related protein kinase Atg1 interacts with the ubiquitin-like protein Atg8 via the Atg8 family interacting motif to facilitate autophagosome formation. *J. Biol. Chem.* **287**, 28503-28507 (2012).
26. Alam, J.M. et al. Complete set of the Atg8-E1-E2-E3 conjugation machinery forms an interaction web that mediates membrane shaping. *Nat. Struct. Mol. Biol.* **31**, 170-178 (2024).
27. Nakatogawa, H., Ishii, J., Asai, E. & Ohsumi, Y. Atg4 recycles inappropriately lipidated Atg8 to promote autophagosome biogenesis. *Autophagy* **8**, 177-186 (2012).
28. Nair, U. et al. A role for Atg8-PE deconjugation in autophagosome biogenesis. *Autophagy* **8**, 780-793 (2012).
29. Yu, Z.Q. et al. Dual roles of Atg8-PE deconjugation by Atg4 in autophagy. *Autophagy* **8**, 883-892 (2012).
30. Li, J. et al. Feedback regulation of ubiquitination and phase separation of HECT E3 ligases. *Proc Natl Acad Sci U S A* **120**, e2302478120 (2023).
31. Shima, T., Kirisako, H. & Nakatogawa, H. COPII vesicles contribute to autophagosomal membranes. *J. Cell Biol.* **218**, 1503-1510 (2019).
32. Tojima, T., Suda, Y., Jin, N., Kurokawa, K. & Nakano, A. Spatiotemporal dissection of the Golgi apparatus and the ER-Golgi intermediate compartment in budding yeast. *Elife*

- 13(2024).
33. Ge, L., Melville, D., Zhang, M. & Schekman, R. The ER-Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. *Elife* **2**, e00947 (2013).
 34. Milovanovic, D., Wu, Y., Bian, X. & De Camilli, P. A liquid phase of synapsin and lipid vesicles. *Science* **361**, 604-607 (2018).
 35. Bailey, A.L. & Cullis, P.R. Liposome Fusion. *Current Topics in Membranes* **44**, 359-373 (1997).
 36. Nakatogawa, H., Ichimura, Y. & Ohsumi, Y. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* **130**, 165-178 (2007).
 37. Maruyama, T. et al. Membrane perturbation by lipidated Atg8 underlies autophagosome biogenesis. *Nat. Struct. Mol. Biol.* **28**, 583-593 (2021).
 38. Knorr, R.L. et al. Membrane morphology is actively transformed by covalent binding of the protein Atg8 to PE-lipids. *PLoS One* **9**, e115357 (2014).
 39. Rogov, V.V. et al. Atg8 family proteins, LIR/AIM motifs and other interaction modes. *Autophagy Rep* **2**(2023).

Methods

Construction of expression plasmids

Expression plasmids encoding Atg1^{D211A}, Atg17–Atg29–Atg31, and Atg17–Atg31C (residues 174–196) were constructed as previously described⁴⁰. An expression plasmid encoding N-terminal His-tagged and C-terminal SNAP-Twin-Strep-tagged Atg13 was constructed as previously described¹³. The expression plasmids encoding the processed form of Atg8^{K26P}, Atg7, Atg3, and Atg10 were constructed as previously described^{41–44}. Expression plasmids encoding the Atg12–Atg5 conjugate, Atg12ΔIDR (residues 100–186)–Atg5 conjugate, and Atg5 were constructed as previously described^{45,46}. The expression plasmids encoding the Atg7–SNAP, Atg4 (residues 28–494)–SNAP, SNAP–Atg16 were constructed as previously described^{26,37}.

To construct the expression plasmids encoding N-terminal GST-tagged Atg12^{17BH}, N-terminal GST-tagged SNAP, N-terminal GST-tagged mGFP, N-terminal GST-tagged and C-terminal SNAP-tagged Atg38, N-terminal GST-tagged and C-terminal mGFP-tagged Atg12^{17BH}, and N-terminal GST and mGFP-tagged Atg8^{K26P}, the genes were amplified by polymerase chain reaction (PCR)

and cloned into pGEX6p-1 vectors. To construct an expression plasmid encoding N-terminal GB1-His-tagged Atg12^{17BH}, the gene was amplified by PCR and cloned into a pGBHPS vector⁴⁷. To construct an expression plasmid encoding the C-terminal SNAP-tagged Atg21 with an HRV3C protease recognition site followed by a His tag and a SUMO tag, the genes were amplified by PCR and cloned into a pE-SUMOpro Amp vector. To construct an expression plasmid encoding Atg8^{K26P} with an N-terminal GST tag and C-terminal MED (MARCKS effector domain; KKKRFSFKKSFKLSGFSFKKNKK), the genes were amplified by PCR and cloned into a pGEX6p-1 vector. To construct yeast expression plasmids encoding Atg2 and Atg18, respectively, followed by Twin-Strep, SNAP, and His tags, the genes were amplified by PCR and cloned into a pRS426-based multi-copy plasmid under the control of the GAL1 promoter. The cloning procedure was performed using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). The mutations that resulted in the indicated amino acid substitutions were introduced via PCR-mediated site-directed mutagenesis. To confirm the identity of the constructs, they were all sequenced.

Protein expression and purification

Atg1^{D211A}, Atg17-Atg29-Atg31, Atg17-Atg31C, and their mutants with the amino acid mutations indicated in the main text were purified as previously described³¹. N-terminal His-tagged and C-terminal SNAP-Twin-Strep-tagged Atg13 was purified as previously described¹³. The processed forms of Atg8^{K26P}, Atg7, Atg3, and Atg10 were purified as previously described⁴¹⁻⁴⁴. Atg7-SNAP, Atg4 (residues 28-494)-SNAP, SNAP-Atg16, and Atg12-Atg5-SNAP-Atg16 were purified as previously described^{26,37}. Atg12 Δ IDR-Atg5-SNAP-Atg16 and Atg5-SNAP-Atg16 were purified in a similar manner as that of the full-length or non-tagged proteins, respectively^{26,48}.

To express the recombinant proteins in *Escherichia coli*, the BL21(DE3) strain was used as the expression host. GST-Atg38-SNAP was purified using a COSMOGEL® GST-Accept column (Nacalai). Subsequently, the GST was excised with human rhinovirus 3C (HRV3C) protease, and the eluate was desalted using a Bio-Rad Bio-Gel P-6 Desalting Cartridge (Bio-Rad). Subsequently, Atg38-SNAP was applied to a GST-Accept column to remove the excised GST. The expression of the PI3K complex I (composed of Vps34, Vps30, Vps15, and Atg14) was performed as previously described⁴⁹. During the incubation of the lysate with immunoglobulin G (IgG) beads, Atg38-SNAP was

added to form the PI3K complex I pentamer. Subsequent purification steps were conducted in a similar manner as those of a previously described method¹⁶.

The GST–SNAP, GST–mGFP, GST–mGFP–Atg8^{K26P}, GST–Atg12 (residues 42–62), and GST–Atg12 (residues 42–62)–mGFP were initially purified using a GST-Accept column. Subsequently, the samples were desalted using a Bio-Gel P-6 desalting cartridge. Phosphate-buffered saline (PBS) was used as the buffer. For the purification of GST–SNAP, GST–mGFP, GST–mGFP–Atg8^{K26P}, and GST–Atg12^{17BH}–mGFP, GST was excised using HRV3C protease. Subsequently, the samples were subjected to another GST-Accept column purification step, to remove the excised GST. Finally, the protein was purified on a HiLoad 26/60 Superdex 200 PG column (Cytiva), with elution performed using 20 mM HEPES [pH 7.0] and 150 mM NaCl. The GST–Atg8 and GST–Atg8^{K26P}-MED were purified using a GST-Accept column.

Atg21–SNAP–HisSUMO was purified via affinity chromatography with a Ni-NTA column (Qiagen). After affinity chromatography, the HisSUMO tag at the C-terminus was excised with HRV3C protease. Subsequently, Atg21–SNAP was purified on a HiLoad 26/60 Superdex 200 PG column eluted with 20 mM HEPES [pH 7.0] and 250 mM NaCl.

Atg2 and Atg18 expression were performed using BJ3505 yeast cells, which lack endogenous Atg2 and Atg18 genes⁵⁰. The yeast cells harboring the Atg2–Twin–Strep–SNAP–His expression plasmid were cultured in a SD + CA + AT medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.5% casamino acids, and 2% glucose with 0.002% adenine sulfate and 0.002% tryptophan at 30°C. The expression of the protein was induced by the addition of galactose at a final concentration of 4%. The subsequent purification was performed as previously described⁵⁰, except for the following amendments: The sample that had been eluted from the HisPur Ni-NTA resin (Thermo Fisher Scientific) and subsequently dialyzed was applied to the Strep-Tactin® XT 4Flow® high-volume resin (IBA LifeSciences). The resin was then washed with buffer ST, which consisted of 100 mM Tris-HCl [pH 8.0], 500 mM NaCl, and 10% glycerol. Following elution from the resin with buffer ST containing 50 mM biotin, the eluate was subjected to dialysis against a buffer consisting of 20 mM HEPES [pH 7.0] and 250 mM NaCl. Similarly, Atg18–Twin–Strep–SNAP–His was expressed and prepared. The yeast cells were suspended in a solution of 100 mM Tris-HCl [pH 8.0] and 500 mM NaCl. After the addition of phenylmethylsulfonyl fluoride (PMSF) to the lysate at a final concentration of 1

mM, the yeast cells were disrupted with zirconia beads, centrifuged, and avidin was added to the resulting supernatant at a final concentration of 50 µg/mL. The lysate was then applied to a Strep-Tactin XT high-capacity resin column. Following the resin washing step, Atg18–Twin-Strep–SNAP–His was eluted using a buffer comprising 100 mM Tris-HCl [pH 8.0], 500 mM NaCl, and 50 mM biotin. The protein was then subjected to a HiLoad 26/60 Superdex 200 PG column and eluted with 20 mM HEPES [pH 7.0] and 150 mM NaCl.

The SNAP-tags of Atg2, Atg4, Atg7, Atg16, Atg18, Atg21, and Atg38 were labeled with SNAP-Surface Alexa Fluor 488 (New England BioLabs), the SNAP-tag of Atg13 was labeled with SNAP-Surface 549 (New England BioLabs), and the SNAP-tag of Atg16 for Fig. 5e was labeled with SNAP-Surface 647 (New England BioLabs), in accordance with the manufacturer's instructions. Atg13-SNAP used in Fig. 1b was supplemented with the SNAP-Surface Block (New England BioLabs) after labeling.

Atg3 and Atg10 were chemically labeled with Alexa Fluor 488 C5-Maleimide (Thermo Fisher) in a 2:1 molar ratio. The mixtures were incubated on ice for more than 1 h, after which they were applied to a PD SpinTrap™ G-25 (Cytiva) to exclude any residual dye.

Immunoprecipitation

The *Saccharomyces cerevisiae* strains used in this study are listed in Supplementary Table 1^{51,52}. Cells carrying the pRS316-derived plasmids expressing Atg17 and its mutants were cultured in SD + CA + AT to a mid-log phase, converted to spheroplasts, and incubated at 30°C in 0.5 × SD + CA + AT containing 1 M sorbitol and 0.2 µg/mL rapamycin (LC Laboratories) for 2 h. The spheroplasts were solubilized in IP buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM EGTA, and 50 mM NaF) containing 2 mM PMSF, 2×cOmplete Protease Inhibitor Cocktail (Roche), and 1% n-dodecyl-β-maltoside (DDM). The samples were clarified by centrifugation at 2,000 ×g for 5 min. The supernatants were further centrifuged at 15,000 ×g for 15 min. The resulting supernatants (input) were incubated with NHS FG-beads (Tamagawa Seiki) conjugated with anti-FLAG M2 antibody (F1804; Sigma-Aldrich) at 4°C for 2 h while rotating. The beads were washed three times with IP buffer containing 0.1% DDM. The bound proteins were eluted with SDS sample buffer (100 mM Tris-HCl [pH 7.5], 2% SDS, 10% glycerol, and a trace amount of bromophenol blue) at 65°C for 10 min. After removing the beads, DTT was added to the eluates to a

final concentration of 20 mM. The samples were analyzed by immunoblotting using antibodies against Atg5 and Atg17^{53,54}.

Pgk1–GFP cleavage assay

Yeast cells harboring the pRS316-derived plasmids were cultured in SD + CA + AT medium, while cells without the plasmid were cultured in SD + CA + ATU medium (SD + CA + AT with 0.002% uracil). All cells were grown to the mid-log phase and treated with rapamycin (LC Laboratories). Samples for immunoblotting analysis were prepared as previously described⁵⁵. For immunoblotting, antibodies against GFP (11814460001; Roche), Atg2⁵⁶, Atg4²⁷, Atg5⁵³, Atg16⁵³ and Atg18⁵⁷ were used.

Analysis of Atg12–Atg5–GFP and Atg17–2×mCherry expression levels

Cells cultured in SD + CA + ATU were treated with rapamycin (LC Laboratories) when the optical density at 600 nm (OD₆₀₀) reached approximately 4. Samples for immunoblotting analysis were prepared as previously described⁵⁵. For immunoblotting, antibodies against GFP and mCherry (600-401-379-RTU; Rockland) were used.

Analysis of Atg8–PE formation

Yeast cells harboring the pRS316-derived plasmids expressing Atg12 and its mutants were cultured in SD + CA + AT to the mid-log phase, treated with 1 mM PMSF for 10 min, and then treated with rapamycin (LC Laboratories). Samples for immunoblotting analysis were prepared as previously described⁵⁵. Lipidated Atg8 (Atg8–PE) was separated from the unmodified form by urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)⁵⁸ and detected using antibodies against Atg8 (anti-Atg8-2)²⁷.

Imaging of yeast cells

Yeast cells were cultured until the OD₆₀₀ reached 4.0–6.0. Rapamycin (LC Laboratories) was then added to the cell culture medium to a final concentration of 0.2 µg/mL, and the cells were cultured for an additional 3 hours to induce autophagy. The cells were imaged on a glass bottom dish (Mattek) with an FV4000RS confocal laser scanning microscope (EVIDENT) equipped with a UPLXAPO60XO, NA 1.42 Oil objective (EVIDENT) or an FV3000RS confocal laser scanning microscope (EVIDENT) equipped with a UPLSAPO60XO, NA

1.42 Oil objective (EVIDENT).

Liposome preparation

Soy PI (#840044C), dioleoylphosphatidylethanolamine (PE) (#850725C), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) (#850375C), 18:1 dioleoylphosphatidylserine (PS) (#840035C), 1,2-dioleoyl-sn-glycero-3-phosphate (PA) (#840875C), 18:1 PI3P (#850150P), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (#890890C), 18:1 Cy-5.5 PE (#810336), and 18:1-06:0 NBD PC (#810132P) were purchased from Avanti Polar Lipids. β -BODIPY™ FL C12-HPC (#D3792) was purchased from Invitrogen. Octadecyl Rhodamine B Chloride (R18) (#O0512) was purchased from TCI. Phospholipids and fluorescence dyes/DOTAP, if applicable, were dissolved in chloroform at appropriate proportions in glass vials. Liposomes mimicking the lipid composition of Atg9 vesicles were prepared with the following molar percentages: PC:PI:PE:PS:PA = 41.1:44.3:6:7:1.6 (mol%). Liposomes containing PI3P were prepared with the following molar percentages: PC:PI:PI3P:PE:PS:PA = 41.1:39.3:5:6:7:1.6 (mol%). For DOTAP-containing liposomes, the lipid compositions were as follows: 0% DOTAP (0:7:92.9:0.1 mol% DOTAP:PS:PC:R18), 7% DOTAP (7:7:85.9:0.1 mol% DOTAP:PS:PC:R18), 25% DOTAP (25:7:67.9:0.1 mol% DOTAP:PS:PC:R18), and 50% DOTAP (50:7:42.9:0.1 mol% DOTAP:PS:PC:R18). The solvent was then evaporated under a stream of nitrogen while rotating the vials to form a thin lipid film. Samples were dried further in a desiccator under vacuum for 16 hours. The resulting lipid film was suspended in a buffer (20 mM Tris-HCl [pH 7.5] and 150 mM NaCl) at a final concentration of 3 mM phospholipid by vigorous mixing. Samples were then sonicated three times for 30 sec to obtain small unilamellar liposomes.

Reconstitution and microscopy of protein-rich droplets

Liquid droplets of the Atg1 complex were formed by the dilution of proteins from a stock solution into the buffer as previously described¹³, with minor modifications. Briefly, liquid droplets of 4 μ M Atg1 complex (Atg1^{D211A}, Atg13–SNAP, Atg17–Atg29–Atg31) were formed by the dilution of proteins from a stock solution into the buffer (50 mM HEPES [pH 7.0] and 250 mM NaCl) with subsequent incubation at 25°C for 5 min. Then 0.5 μ M of each Atg protein/complex was added to the liquid droplets and incubated at 25°C for 15 min for Fig. 1b and 3a, Extended Data Fig. 1c; 3 μ M of Atg12^{17BH}–mGFP was added to the liquid droplets

and incubated at 25 °C for 5 min for Fig. 3c, Extended Data Fig. 3a; 0.3 μ M of Atg5–SNAP–Atg16 or Atg12 Δ IDR–SNAP–Atg16 with or without Atg21–SNAP and 0.5 mM of PI3P-containing liposomes (PC:PE:PI:PS:PA:PI3P at a ratio of 41.1:6:39.3:7:1.6:5 (mol%)) were added to the liquid droplets and incubated at 25 °C for 15 min for Fig. 5e. For Fig. 7a-c, Extended Data Fig. 7b, liquid droplets of 4 μ M of the Atg1 complex (Atg1^{D211A}, Atg13–SNAP, Atg17–Atg29–Atg31) were formed by the dilution of proteins from a stock solution into the buffer (50 mM HEPES [pH 7.0] and 250 mM NaCl) with subsequent incubation at 25 °C for 10 min. The solution was then mixed with Atg8 lipidation reaction solution (purified 2 μ M of Atg7, 2 μ M of Atg3, 2 μ M of Atg12–Atg5–Atg16, 20 μ M of the C-terminal glycine-exposed form of Atg8^{K26P}, and 0.75 mM liposomes containing 0.1 mol% R18 in a reaction buffer consisting of 20 mM HEPES [pH 7.0], 150 mM NaCl, and 2 mM DTT with or without 2 mM ATP and 2 mM MgCl₂) in equal volumes and further incubated at 25 °C for 20 min. For Extended Data Fig. 7a, liposomes were prepared by adding NBD PC or β -BODIPY FL C12-HPC instead of R18. For Extended Data Fig. 7c, following a 10-min reaction, the droplet solution was combined with an equal volume of 0.75 mM DOTAP-containing liposomes and incubated for an additional 20 min at 25 °C. For Extended Data Fig. 7e, after a 10-min reaction, the droplet solution was mixed with an equal volume of Atg8 lipidation reaction solution containing 143 μ M AIM peptide (>50-fold molar excess over Atg1) and further incubated at 25 °C for 20 min. For Fig. 7 f,g, following a 10-minute reaction, the droplet solution was combined in an equal volume with 1.7 μ M of GST–Atg8^{K26P}–MED and 0.75 mM liposomes, which had been supplemented with HRV3C protease immediately prior to mixing. This resulting mixture was then incubated for an additional 30 min. These samples were mixed in a microtube and imaged on a glass bottom dish (Mattek) coated with 3% bovine serum albumin (BSA) (Wako).

These experiments, except for Fig. 4a, 5e, 7 and Extended Data Fig. 7 were performed using an FV3000RS confocal laser scanning microscope (Olympus) equipped with a UPLSAPO60XO, NA 1.42 Oil objective (Olympus). The experiments for Fig. 4a, 5e, 7 and Extended Data Fig. 7 were performed using a STELLARIS 5 confocal laser scanning microscope (Leica) equipped with an HC PL APO CS2 63x, NA 1.40 Oil objective (Leica). Fig. 7c images were generated as a maximum-intensity projection of a z-stack consisting of 23 optical sections acquired at 0.3 μ m intervals, whereas all other images represent a single optical section. Quantitative analysis was performed with FIJI (ImageJ 1.54p).

In vitro pulldown assay

The purified proteins were incubated with GST-Accept beads at 4°C for 30 min. For Extended Data Fig. 7d, AIM peptide was added to the binding reaction at a final concentration of 110 μ M (equivalent to >50-fold the Atg1 concentration). After three washes with PBS, the proteins were eluted using 10 mM glutathione in 50 mM Tris-HCl [pH 8.0]. The samples were separated by SDS-PAGE. The protein bands were then detected by One Step CBB (BIO CRAFT).

Isothermal titration calorimetry analysis

Atg12^{17BH}-mGFP and Atg17-Atg31C were prepared in the same buffer, which consisted of 20 mM HEPES [pH 7.0] and 150 mM NaCl. The binding of Atg12^{17BH}-mGFP to Atg17-Atg31C was measured by ITC using a MICROCAL PEAQ-ITC calorimeter (Malvern) with stirring at 750 rpm. at 25°C. The titration of Atg12^{17BH, WT}-mGFP with Atg17-Atg31C involved 18 injections of 2 μ L of the Atg12^{17BH, WT}-mGFP solution (220 μ M) at intervals of 150 s into a sample cell containing 200 μ L of Atg17-Atg31C (22 μ M). The titration of the Atg12^{17BH, L47A}-mGFP with Atg17-Atg31C involved 18 injections of 2 μ L of the Atg12^{17BH, L47A}-mGFP solution (224 μ M) at intervals of 150 s into a sample cell containing 200 μ L of Atg17-Atg31C (21 μ M). The titration of the Atg12^{17BH, L54A}-mGFP with Atg17-Atg31C involved 18 injections of 2 μ L of the Atg12^{17BH, L54A}-mGFP solution (232 μ M) at intervals of 150 s into a sample cell containing 200 μ L of Atg17-Atg31C (23 μ M). The raw titration data were analyzed using the MicroCal PEAQ-ITC Analysis Software with a single-site binding model. The error of each parameter shows the fitting error.

Atg8 lipidation assay

Liquid droplets of the Atg1 complex were formed by the dilution of proteins from a stock solution into the buffer as previously described, with minor modifications.⁹ Briefly, liquid droplets of 4 μ M of the Atg1 complex (Atg1^{D211A}, Atg13-SNAP, Atg17-Atg29-Atg31) were formed by the dilution of proteins from a stock solution into the buffer (50 mM HEPES [pH 7.0] and 250 mM NaCl) with subsequent incubation at 30 °C for 10 min. During incubation, purified 2 μ M of Atg7, 2 μ M of Atg3, 0.2 μ M of Atg12-Atg5-Atg16, 20 μ M of the C-terminal glycine-exposed form of Atg8^{K26P}, 2 μ M of Atg21, and 0.75 mM of liposomes were combined in a reaction buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, and 2 mM DTT). After

the droplet formation reaction was complete, it was mixed with the lipidation reaction solution in equal volumes, to which 1 mM ATP and 1 mM MgCl₂ were added and incubated at 30°C for 5 min. The reaction was stopped by boiling in the SDS-PAGE sample buffer. The samples were subjected to urea-SDS-PAGE and stained with One Step CBB (BIO CRAFT).

Assay for Atg8–PE delipidation

For Fig. 4 h,i and Extended Data Fig. 4d,e, purified 2 μM of Atg7, 2 μM of Atg3, 0.2 μM of Atg12–Atg5–Atg16, 20 μM of the C-terminal glycine-exposed form of Atg8^{K26P}, and 0.75 mM of liposomes were combined in a reaction buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 2 mM DTT, 2 mM ATP, and 2 mM MgCl₂). The mixture was then incubated at 30°C for 10 min. After the lipidation reaction, 1/5 volume of CheckLite ATP Eliminating Kit (Kikkoman Biochemicals) was added to the reaction solution to quench the reaction for 30 min. For Extended Data Fig. 4d, an equal amount of DTT was added to a final concentration of 100 mM, instead of treatment with an ATP-eliminating reagent. Liquid droplets of the Atg1 complex were formed as described above. The droplets, 1 μM of Atg4, and 2 μM of Atg21 were mixed in equal volumes with the lipidation reaction solution and incubated at 30°C for 15 min. For Fig 4 j,k, and Extended Data Fig. 4f, purified 2 μM of Atg7, 2 μM of Atg3, 0.2 μM of Atg12–Atg5–Atg16, 20 μM of the C-terminal glycine-exposed form of Atg8^{K26P}, 0.75 mM of liposomes, 2 μM of Atg4, 2 μM of Atg21 were combined in a reaction buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 2 mM DTT, 2 mM ATP, and 2 mM MgCl₂). This mixture was then combined with an equal volume of liquid droplets of the Atg1 complex and incubated at 30°C for 5 min. The reaction was stopped by boiling in the SDS-PAGE sample buffer. The samples were subjected to urea-SDS-PAGE and stained with One Step CBB.

Quick-freezing and freeze-fracture replica labeling

Liquid droplets of 4 μM of the Atg1 complex (Atg1^{D211A}, Atg13–SNAP, Atg17–Atg29–Atg31) were formed by the dilution of proteins from a stock solution into the buffer (50 mM HEPES [pH 7.0] and 250 mM NaCl) with subsequent incubation at 25 °C for 10 min. The solution was then mixed in equal volumes with Atg8 lipidation reaction solution [containing 2 μM purified Atg7, 2 μM Atg3, 2 μM Atg12–Atg5–Atg16, 20 μM C-terminal glycine-exposed Atg8^{K26P}, and 0.75 mM liposomes in a reaction buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, and 2 mM

DTT with or without 1 mM ATP and 1 mM MgCl₂]. The reaction was carried out on an aluminum stub with the sample at 25 °C for 20 min. The specimens were quick-frozen using an HPM 010 high-pressure freezing machine according to the manufacturer's instructions (Leica Microsystems, Wetzlar, Germany). The frozen specimens were transferred to the cold stage of ACE900 (Leica Microsystems, Wetzlar, Germany), fractured at -102°C under a vacuum of $\sim 1 \times 10^{-6}$ mbar, and maintained at the same temperature for 3 min to induce sublimation of water from the fractured surface. Replicas were made by electron-beam evaporation in three steps: (1) carbon (6 nm thick) at a 90° angle to the specimen surface; (2) platinum-carbon (2 nm thick) at a 45° angle; and (3) carbon (10 nm thick) at a 90° angle. For PS labeling, thawed replicas were treated with 2.5% SDS in 0.1 M Tris-HCl [pH 8.0] containing 50 µg/mL proteinase K (Nacalai, Kyoto, Japan) at 60°C overnight. Replicas were then incubated at 4°C overnight with GST-tagged 2× eectin-2 PH domain (150 ng/mL) in 0.1 M phosphate buffer [pH 6.0] containing 1% BSA and 1% cold-water fish gelatin, followed by rabbit anti-GST antibody (5 µg/mL, Bethyl Laboratories, Texas, USA) and colloidal gold (10 nm)-conjugated protein A (PAG10; University of Utrecht, Utrecht, The Netherlands), for 40 min each at 37°C in 1% BSA in PBS⁵⁹. For Atg8 labeling, replicas were treated with 2.5% SDS in 0.1 M Tris-HCl [pH 8.0] at 60°C overnight and incubated with rabbit anti-Atg8-IN13 antibody³⁶ (kindly provided by Dr. Yoshinori Ohsumi of Institute of Science Tokyo), followed by PAG10. The labeled replicas were observed using a JEOL JEM-1400plus transmission electron microscope (Tokyo, Japan).

NMR experiments

Stable-isotope-labeled reagents were purchased from Cambridge Isotope Laboratories. [¹⁵N]GB1-His-Atg12(42-62) was overexpressed in a M9 minimal medium containing 1.0 g/l [¹⁵N] ammonium chloride. A 1/100th volume pre-culture solution was added to 4 L of LB medium and incubated at 130 rpm at 37°C until the OD₆₀₀ reached 0.5. Cells were harvested by centrifugation at 3,000 x g for 10 min at 25°C. The cells were resuspended in M9 medium devoid of NH₄Cl and glucose and incubated in 1 L of M9 medium for 40 min at 130 rpm at 37°C. Subsequently, NH₄Cl and glucose were added, and the culture was shaken for an additional 30 min. IPTG was then added to a final concentration of 1 mM, and the culture was shaken for 3 hours at 30°C. Cells were harvested by centrifugation, suspended in Ni-NTA wash buffer and boiled for 25 min. The lysate

was subjected to centrifugation at 16,000 x g for 20 min at 4°C, and the supernatant was applied to a Ni-NTA column. Following the washing step, the eluate was subjected to a HiTrap™ Desalting column, and eluted with 20 mM HEPES [pH 7.0], 150 mM NaCl.

All NMR experiments were performed using a Bruker 800 MHz NMR AVANCE NEO with a CPTCI probe, for which TopSpin 4.1.4 (Bruker co. ltd) was used as the measurement control software, or a Bruker 600 MHz NMR AVANCE III HD with a TBI probe, whose broadband channel tuned to ^{15}N , for which TopSpin 3.2 (Bruker co. ltd) was used as the measurement control software. The pulse programs used in the NMR experiments and their references, as well as the measurement parameters, are listed in the Supplementary Table 2. Fourier transform and chemical shift value calibrations were performed on TopSpin software. Sparky 3.113 (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco) was used to analyze the obtained spectra. The measurement temperature was 25°C. The NMR sample used for the main chain assignment was a $^{13}\text{C}/^{15}\text{N}$ -labeled 1.1 mM GB1–Atg12 sample, adjusted to pH 7.0 with 20 mM HEPES buffer containing 150 mM NaCl. The following standard spectral sets were used for main chain assignment; HNCO, HNCA, CBCA (CO)NH, HBHA (CO)NH, ^{15}N -NOESY (mix 150 msec). As a result, we were able to assign all main chain amide signals observed on [^1H - ^{15}N] HSQC. Details of the NMR experiments are summarized in the Supplementary method. Chemical shift information was registered with BMRB number 52732. All signals derived from GB1 and Atg12 were assigned (Extended Data Fig. 2a). The secondary structure propensities (SSP)⁶⁰ were calculated from the obtained chemical shift value information (Extended Data Fig. 2d). ^{15}N -labeled GB1–Atg12 adjusted to 0.1 mM was used for the titration experiments. Atg17–Atg31C adjusted to their respective ratios (0, 0.1, 0.5, and 1.0 molar equivalents) were added, and the [^1H - ^{15}N] HSQC was measured (Extended Data Fig. 2b). The signal intensity ratio before and after the addition of Atg17–Atg31C was plotted (Extended Data Fig. 2c).

Flotation assay

We performed a flotation assay with some modifications to our previous report¹⁶. Briefly, 120 μl of proteoliposome solution (3 μM GST-Atg8 with or without MED, 1mM liposome with R18, 20 mM HEPES [pH 7.0], 150 mM NaCl) was mixed with 150 μl of Solution C (54% (w/v) iodixanol (OptiPrep, serumwerk) solution, 20 mM HEPES [pH 7.0], 150 mM NaCl). Then, 250 μl of this mixture was placed at the

bottom of a tube (eppendorf 1PC tube), and sequentially overlaid with 625 μ l of 25% iodixanol solution and 25 μ l of 5% iodixanol solution. Centrifugation was carried out in a fixed-angle rotor (S120AT2, Himac) at 100,000 r.p.m. for 70 min at 4 °C. Fractions (150 μ l each) of the separated samples were collected from the top to the bottom of the tube. The collected fractions were subjected to SDS-PAGE and stained with Oriole (Bio-Rad).

Statistics & Reproducibility

No statistical method was used to predetermine sample size but our sample sizes are similar to those reported in previous publications^{8,13,26}. Data distribution was assumed to be normal but this was not formally tested. Data points were not excluded from the analyses. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. Data are presented as mean $\pm$ SD or mean without SD. The number of replicates (n) is indicated in the figure legends.

Data Availability

Source data have been provided in Source Data. Microscopic images are available in figshare [<https://doi.org/10.6084/m9.figshare.29874152>]. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

40. Yamamoto, H. et al. The Intrinsically Disordered Protein Atg13 Mediates Supramolecular Assembly of Autophagy Initiation Complexes. *Dev. Cell* **38**, 86-99 (2016).
41. Noda, N.N. et al. Structural basis of Atg8 activation by a homodimeric E1, Atg7. *Mol. Cell* **44**, 462-475 (2011).
42. Yamada, Y. et al. Crystallization and preliminary X-ray analysis of Atg3. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **62**, 1016-1017 (2006).
43. Kumeta, H. et al. The NMR structure of the autophagy-related protein Atg8. *J. Biomol. NMR* **47**, 237-241 (2010).
44. Hanada, T. et al. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J. Biol. Chem.* **282**, 37298-37302 (2007).
45. Matsushita, M., Suzuki, N.N., Fujioka, Y., Ohsumi, Y. & Inagaki, F. Expression, purification and crystallization of the Atg5-Atg16 complex essential for autophagy. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **62**, 1021-1023 (2006).
46. Noda, N.N., Fujioka, Y., Hanada, T., Ohsumi, Y. & Inagaki, F. Structure of the Atg12-Atg5

- conjugate reveals a platform for stimulating Atg8-PE conjugation. *EMBO Rep* **14**, 206-211 (2013).
47. Kobashigawa, Y., Kumeta, H., Ogura, K. & Inagaki, F. Attachment of an NMR-invisible solubility enhancement tag using a sortase-mediated protein ligation method. *J. Biomol. NMR* **43**, 145-150 (2009).
 48. Matsushita, M. et al. Structure of Atg5-Atg16, a complex essential for autophagy. *J. Biol. Chem.* **282**, 6763-6772 (2007).
 49. Rostislavleva, K. et al. Structure and flexibility of the endosomal Vps34 complex reveals the basis of its function on membranes. *Science* **350**, aac7365 (2015).
 50. Osawa, T. et al. Atg2 mediates direct lipid transfer between membranes for autophagosome formation. *Nat. Struct. Mol. Biol.* **26**, 281-288 (2019).
 51. Thomas, B.J. & Rothstein, R. Elevated recombination rates in transcriptionally active DNA. *Cell* **56**, 619-630 (1989).
 52. Hitomi, K., Kotani, T., Noda, N.N., Kimura, Y. & Nakatogawa, H. The Atg1 complex, Atg9, and Vac8 recruit PI3K complex I to the pre-autophagosomal structure. *J. Cell Biol.* **222**(2023).
 53. Kuma, A., Mizushima, N., Ishihara, N. & Ohsumi, Y. Formation of the approximately 350-kDa Apg12-Apg5-Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast. *J. Biol. Chem.* **277**, 18619-18625 (2002).
 54. Kabeya, Y. et al. Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. *Mol Biol Cell* **16**, 2544-2553 (2005).
 55. Kotani, T. et al. A mechanism that ensures non-selective cytoplasm degradation by autophagy. *Nat Commun* **14**, 5815 (2023).
 56. Shintani, T., Suzuki, K., Kamada, Y., Noda, T. & Ohsumi, Y. Apg2p functions in autophagosome formation on the perivacuolar structure. *J. Biol. Chem.* **276**, 30452-30460 (2001).
 57. Obara, K., Sekito, T., Niimi, K. & Ohsumi, Y. The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. *J. Biol. Chem.* **283**, 23972-23980 (2008).
 58. Nakatogawa, H. & Ohsumi, Y. SDS-PAGE techniques to study ubiquitin-like conjugation systems in yeast autophagy. *Methods Mol Biol* **832**, 519-529 (2012).
 59. Tsuji, T. et al. Predominant localization of phosphatidylserine at the cytoplasmic leaflet of the ER, and its TMEM16K-dependent redistribution. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 13368-13373 (2019).
 60. Marsh, J.A., Singh, V.K., Jia, Z. & Forman-Kay, J.D. Sensitivity of secondary structure propensities to sequence differences between alpha- and gamma-synuclein: implications

for fibrillation. *Protein Sci.* **15**, 2795-2804 (2006).

Figure 2

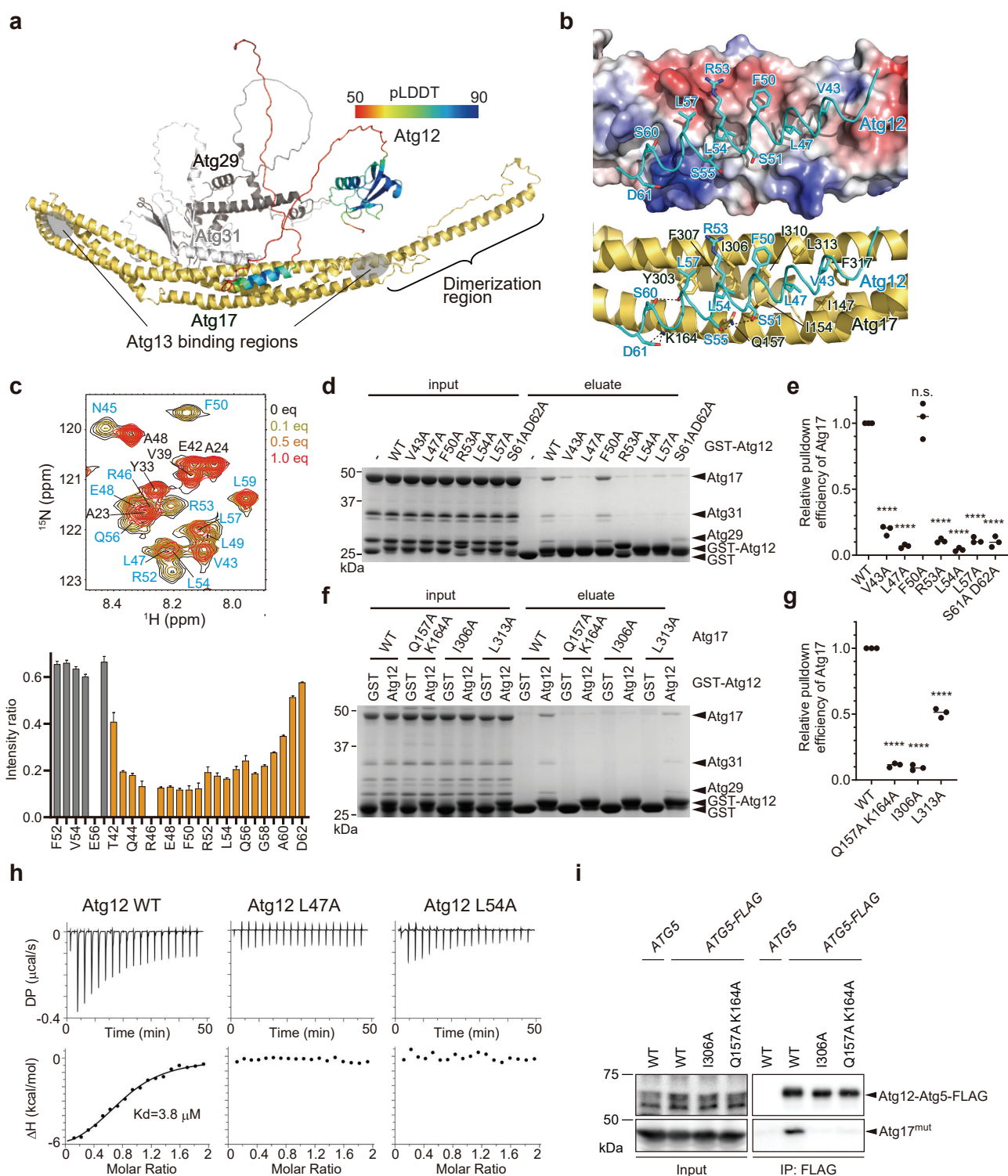


Figure 3

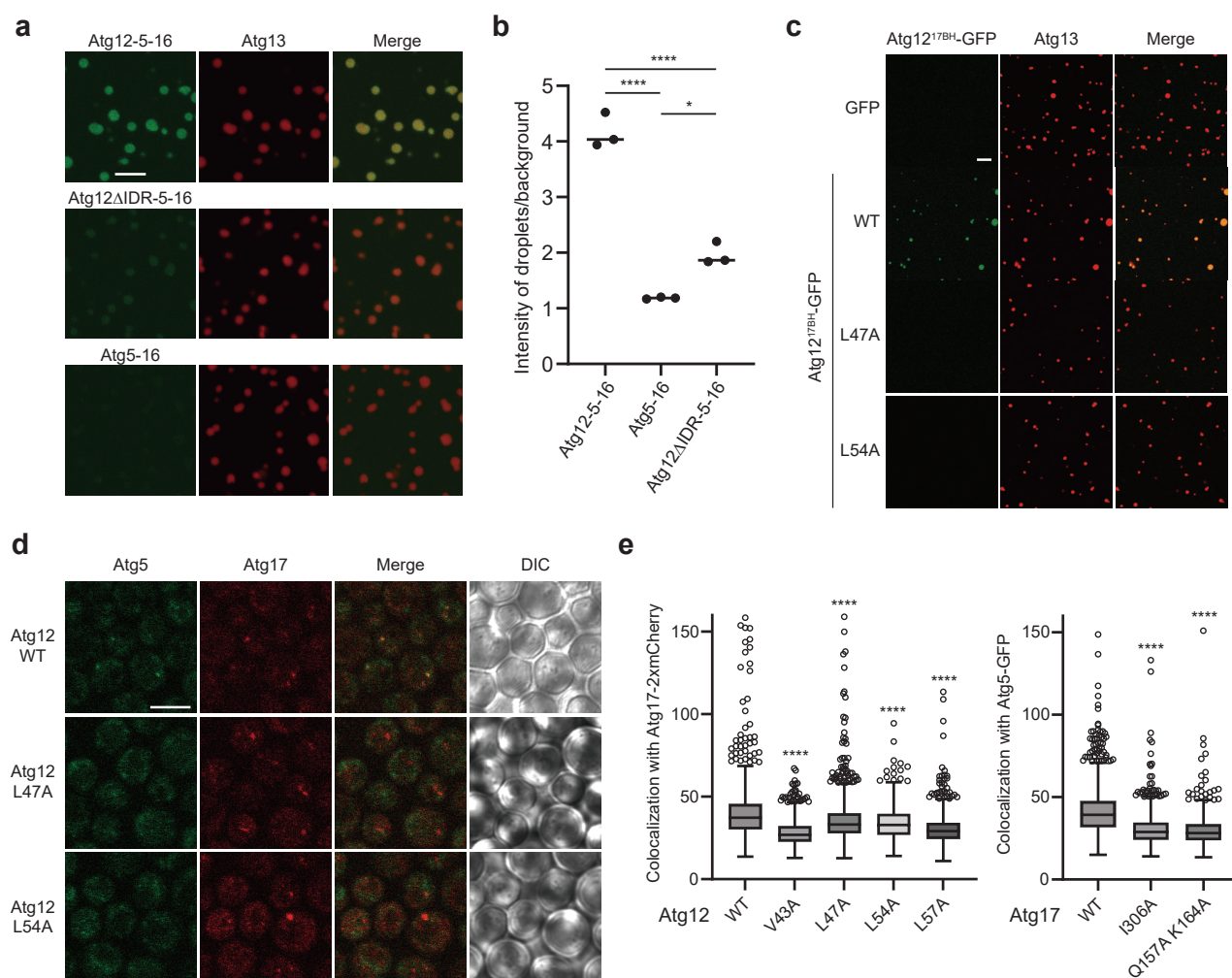


Figure 4

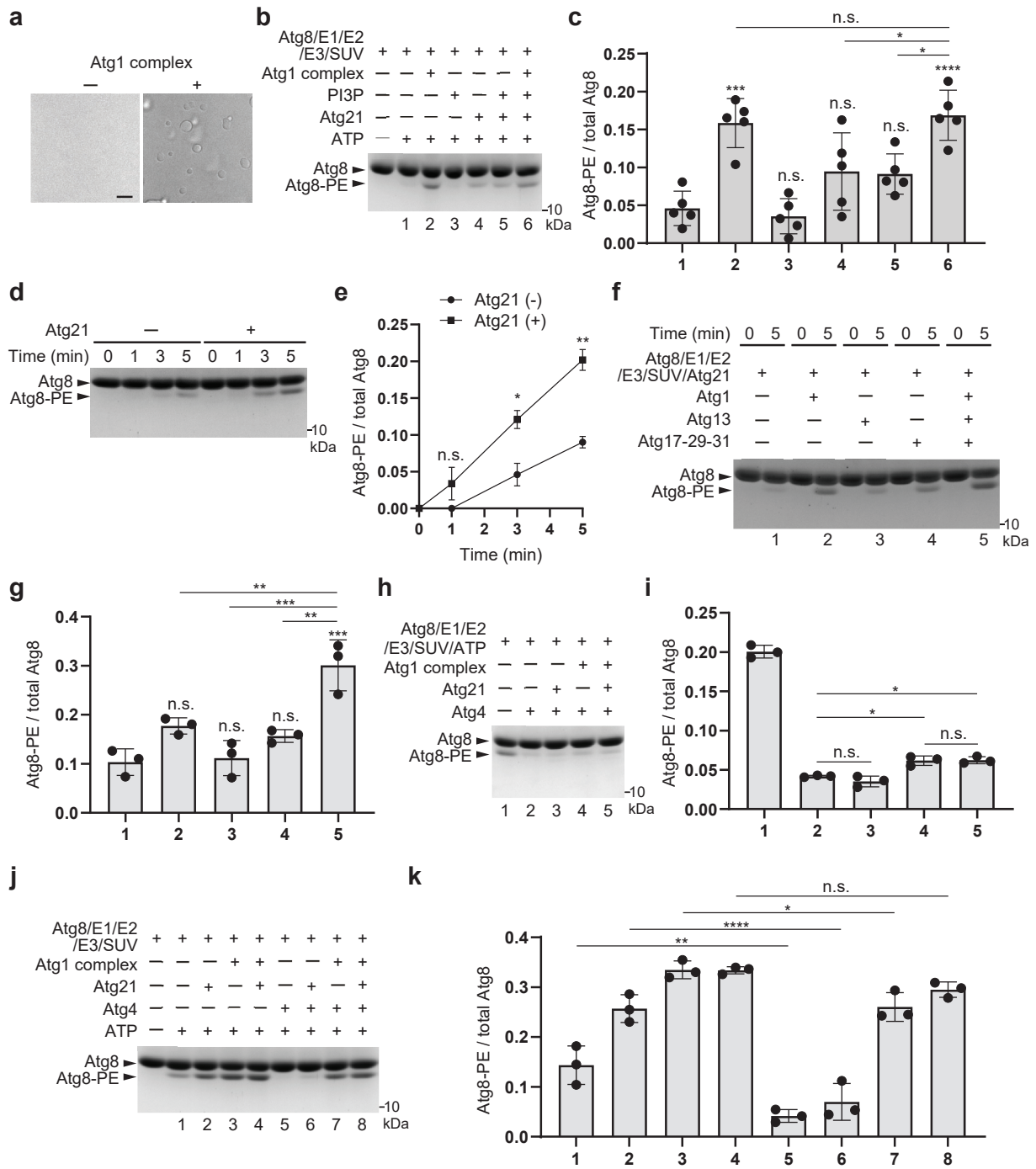


Figure 5

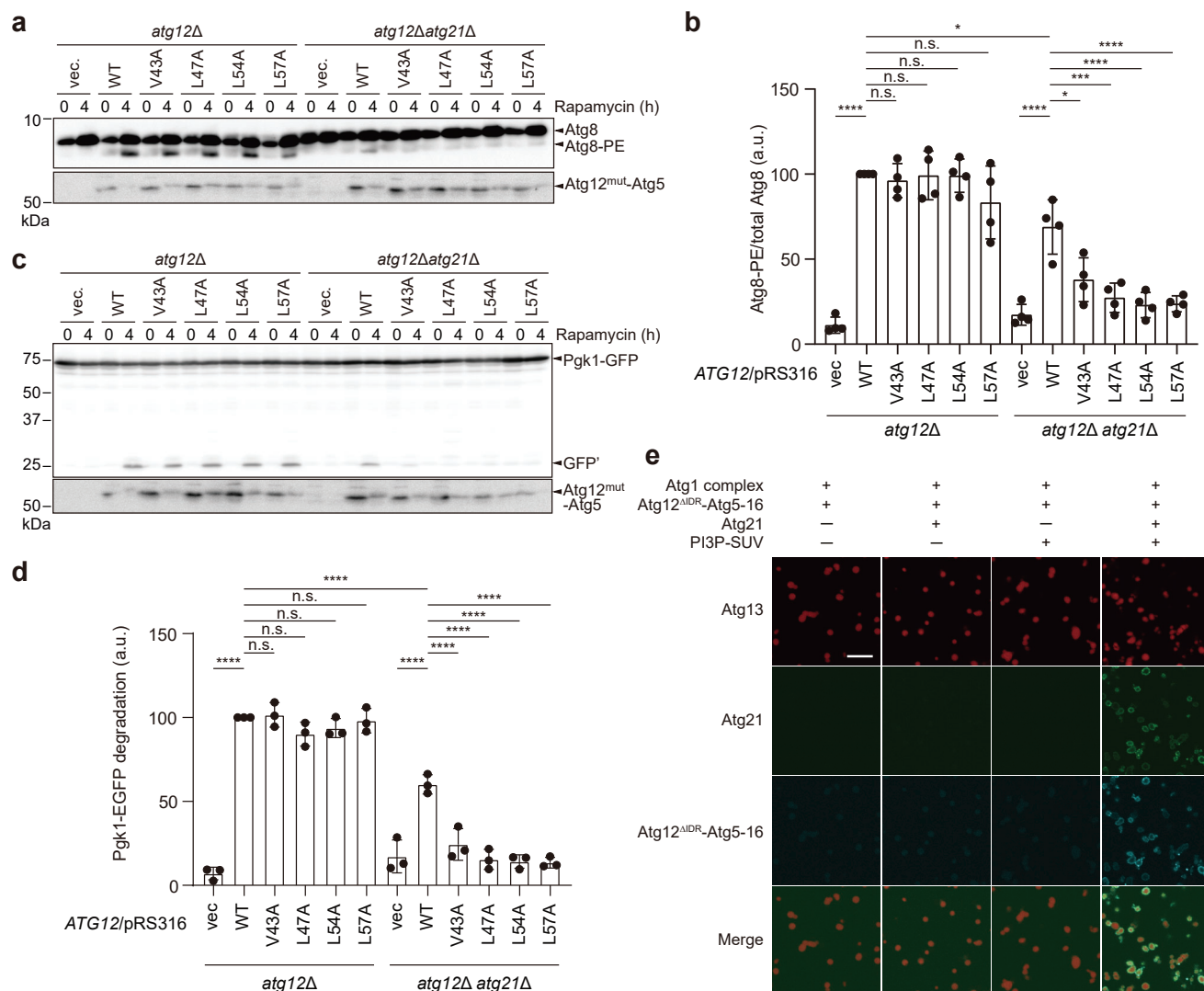


Figure 6

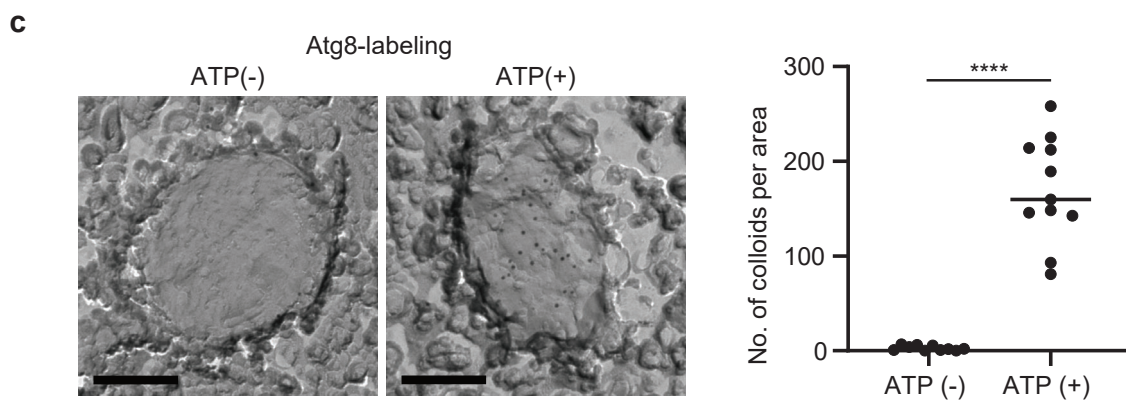
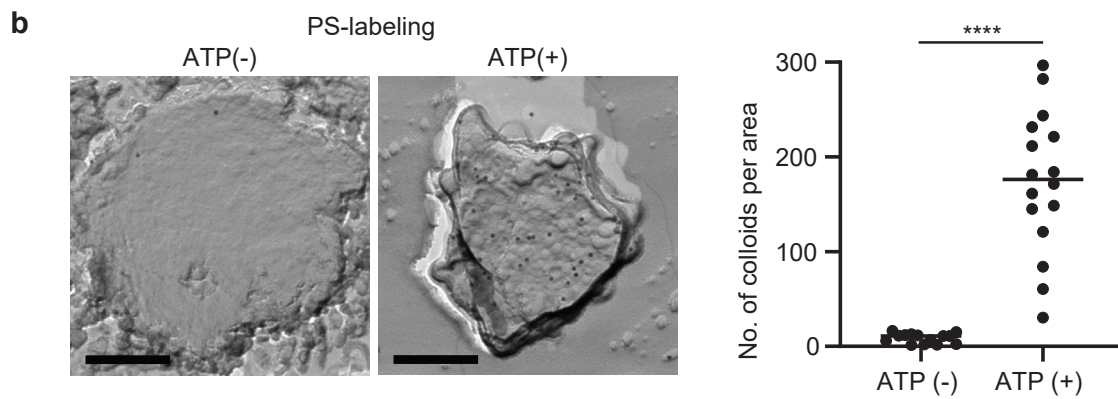
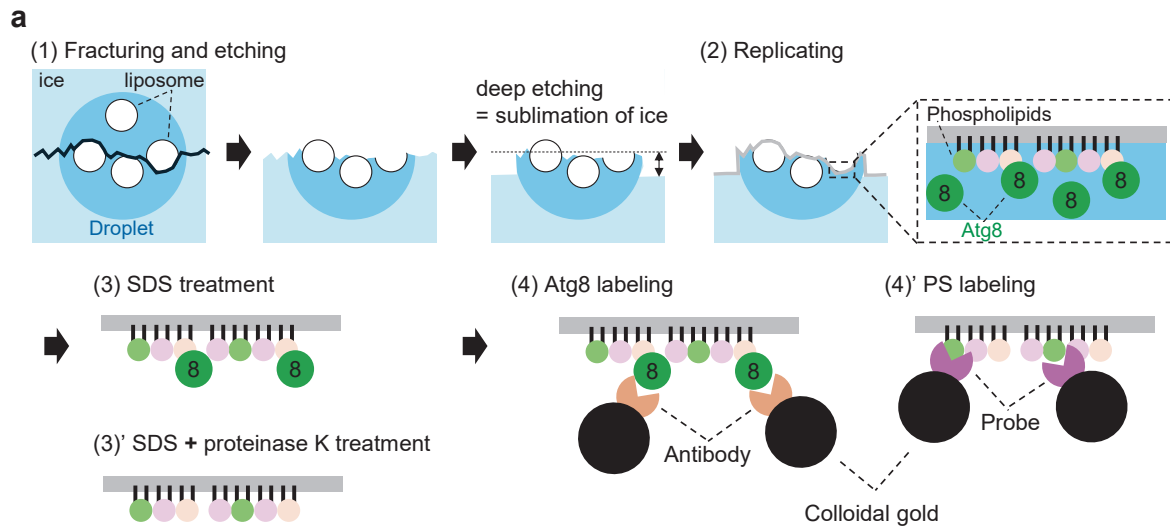


Figure 7

