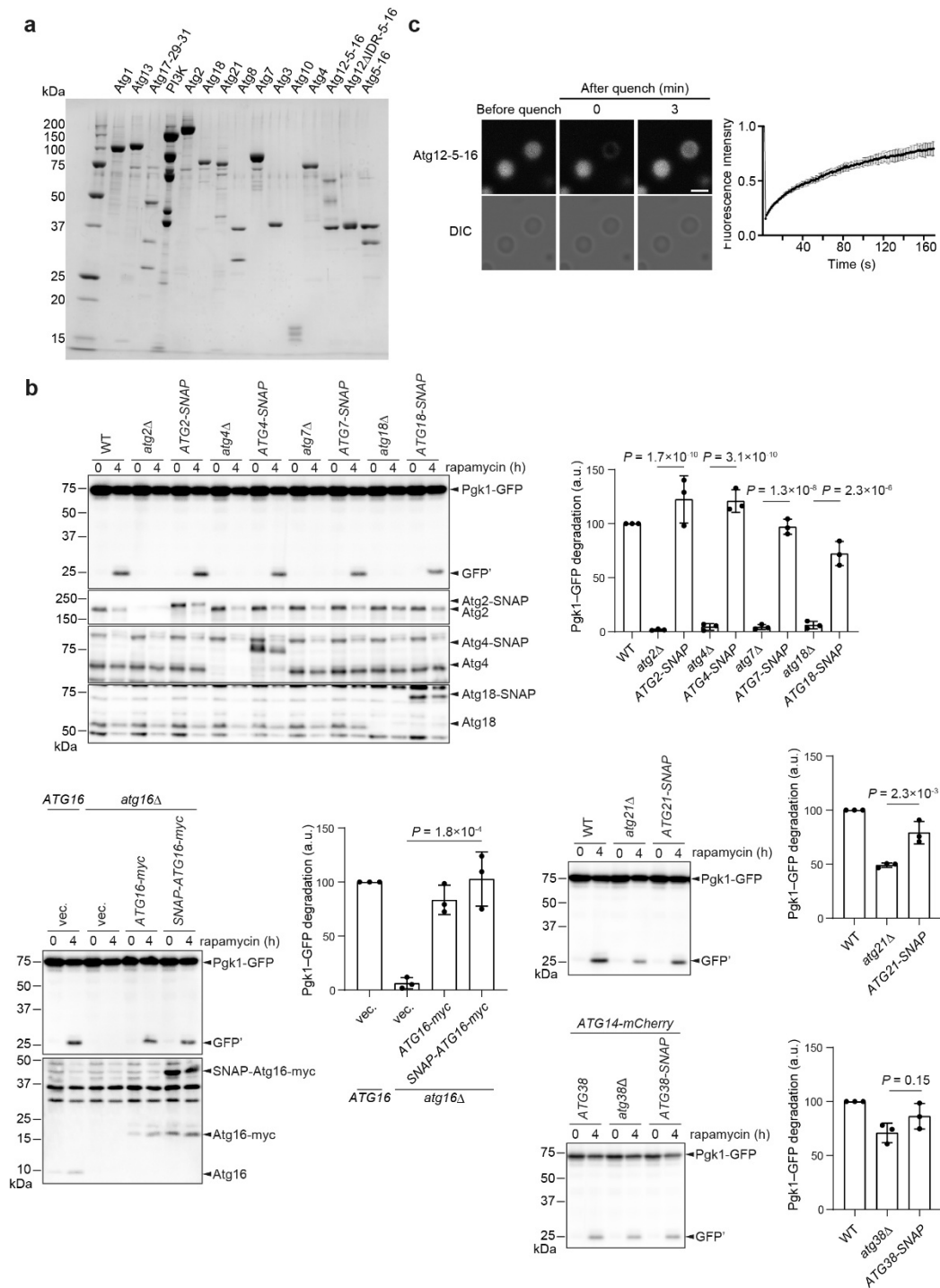




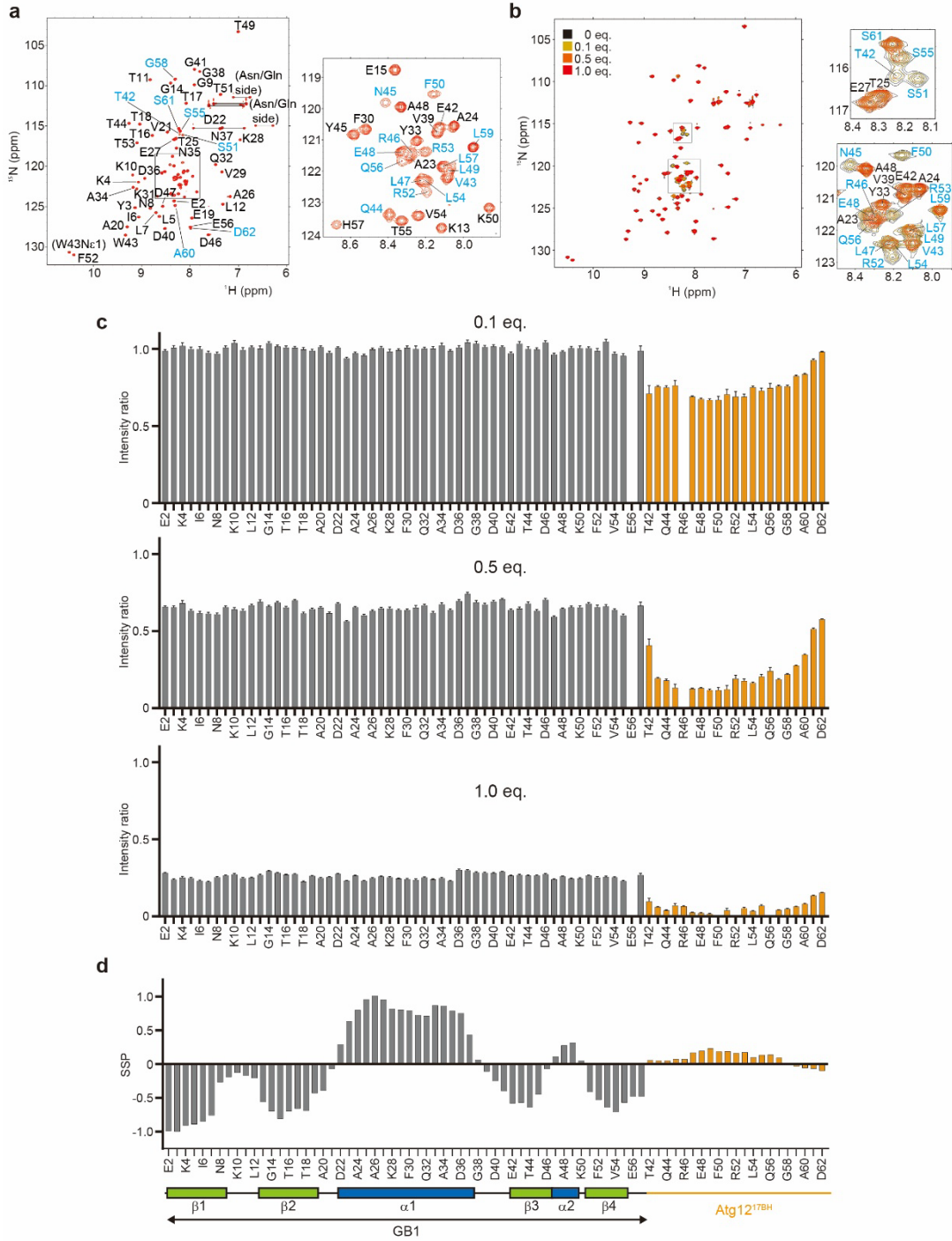
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Title	Phase separation promotes Atg8 lipidation and vesicle condensation for autophagy progression
Author(s)	Fujioka, Yuko; Tsuji, Takuma; Kotani, Tetsuya et al.
Citation	Nature structural & molecular biology, 32(11), 2285-2295 https://doi.org/10.1038/s41594-025-01678-3
Issue Date	2025-09-16
Doc URL	https://hdl.handle.net/2115/98060
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Type	journal article
File Information	ExtendedData.pdf, Source data

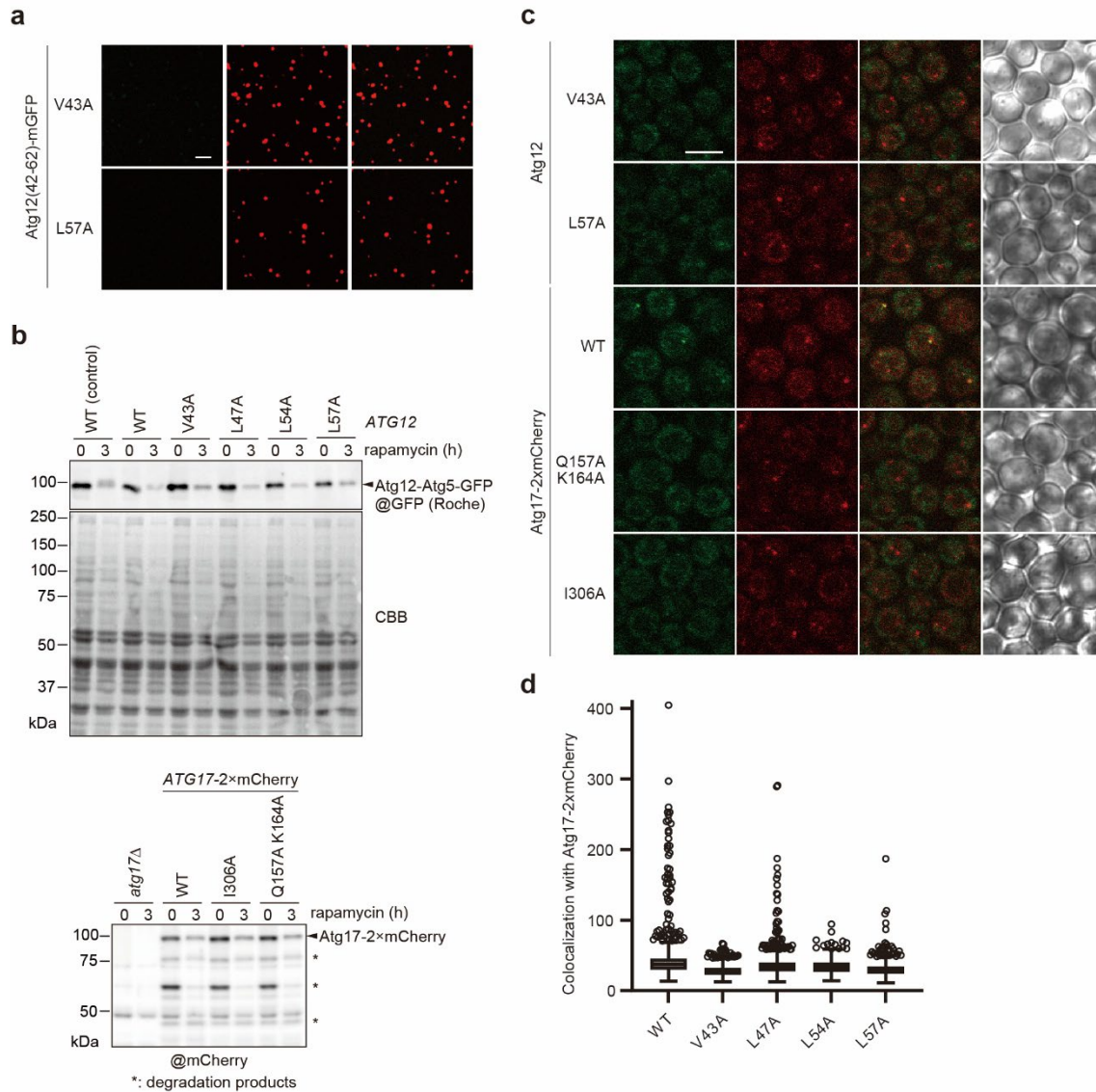




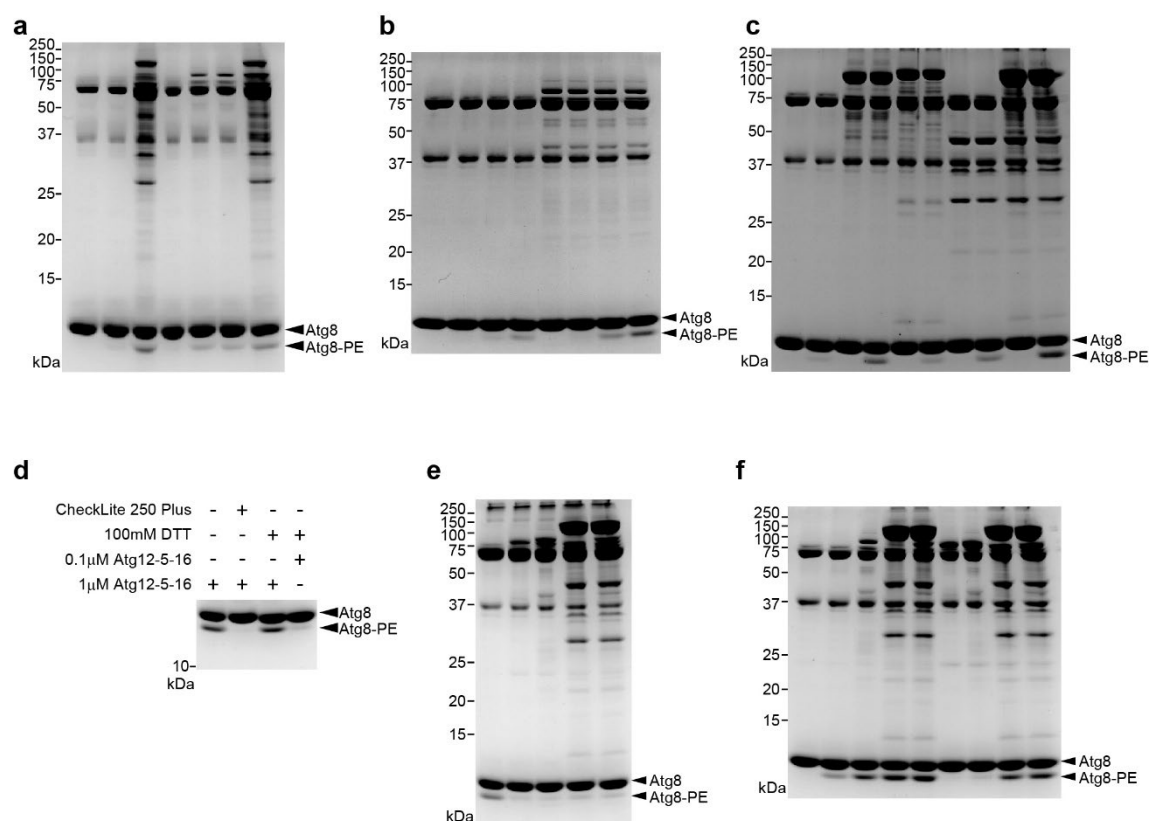
Extended Data Fig. 1. Purification of recombinant proteins and characterization of tagging effects on autophagy. **a**, SDS-PAGE image of the proteins used for Fig. 1 experiments. **b**, Cells expressing Pgc1-GFP were treated with rapamycin and examined by immunoblotting using an antibody against GFP. The band intensities of Pgc1-GFP and its cleaved fragments (GFP') were measured using Fiji. The proportion (%) of GFP' relative to the total signal (Pgc1-GFP + GFP') was calculated and regarded as the degradation of Pgc1-GFP. Bars represent means $\pm$ SD, $n = 3$ biological replicates. (a two-sided Tukey's multiple comparisons test with adjustments for multiple comparisons). **c**, FRAP analysis of the Atg12-Atg5-Atg16 complex at Early PAS droplets. Bars represent means $\pm$ SD, $n = 3$ technical replicates. Scale bar = 2 μ m.



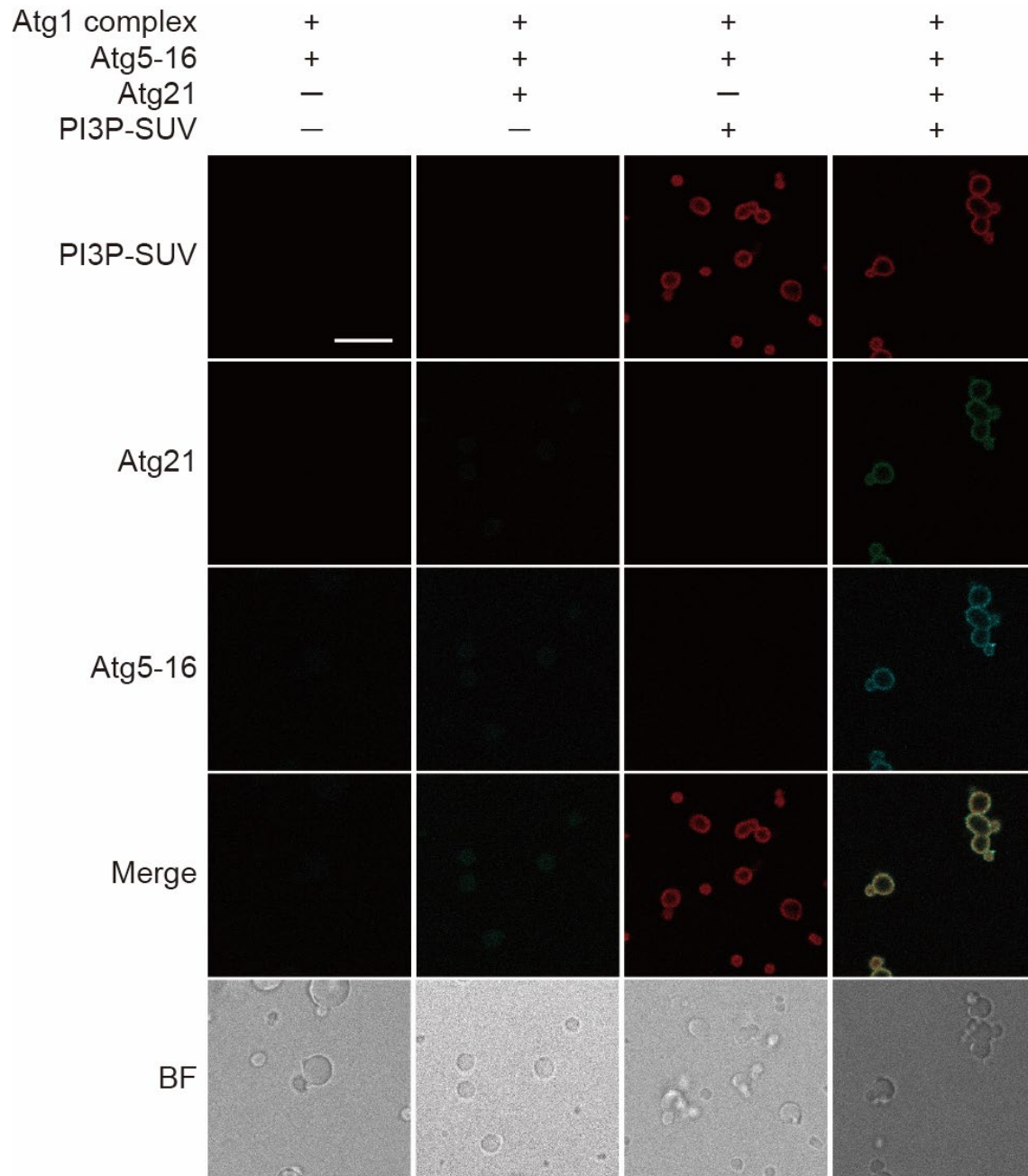
Extended Data Fig. 2. NMR analysis of GB1-Atg12^{17BH}. **a**, Main chain assignment results are shown on $[^1\text{H}-^{15}\text{N}]$ heteronuclear single quantum coherence spectroscopy (HSQC); GB1-derived signals are labeled in black, and Atg12-derived signals are labeled in blue. **b**, Superposition of $[^1\text{H}-^{15}\text{N}]$ HSQC obtained by titration experiments. **c**, Signal intensity ratios before and after the addition of Atg17-Atg31C are plotted by residue (GB1 is shown in gray and Atg12 in orange, 0.1, 0.5, and 1.0 molar equivalents from top to bottom). The signal-to-noise ratio of the signals was used to calculate the error bars ($n=1$ experiment). **d**, Secondary structure propensity analysis results (GB1 is shown in gray and Atg12 in orange). The secondary structure position in the GB1 structure is attached.



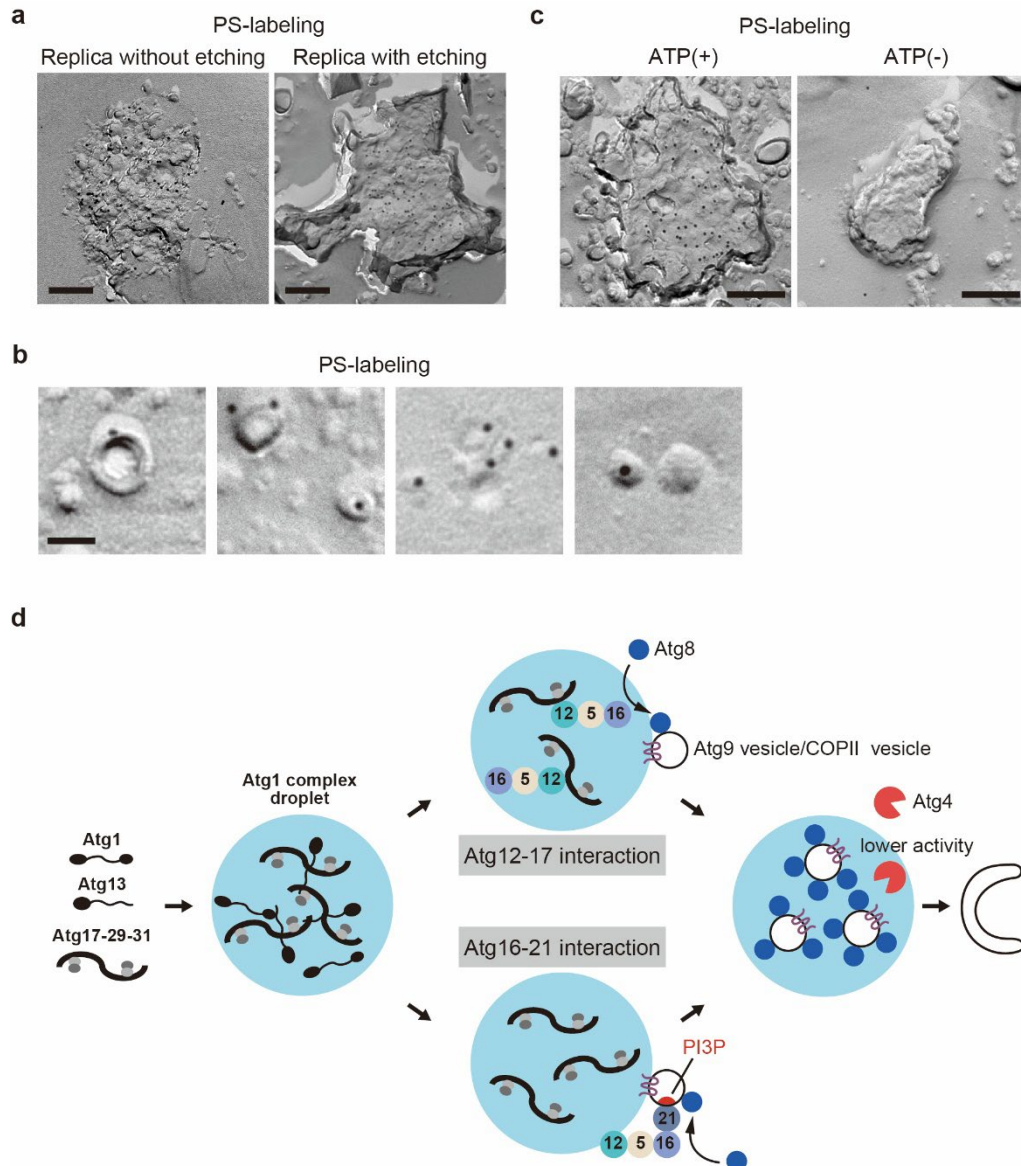
Extended Data Fig. 3. Specific Atg12^{17BH}-Atg17 interaction is important for the condensation at the droplet both in vitro and in vivo. **a**, Condensation of GFP-Atg12^{17BR} at the early PAS droplets was impaired by mutations. Scale bar = 10 μ m. **b**, Cells expressing Atg12 or Atg17 mutants were treated with rapamycin, and the amounts of Atg12-Atg5-GFP and Atg17-2xmCherry were analyzed by immunoblotting using anti-GFP and anti-mCherry antibodies. **c**, PAS targeting of the Atg12-Atg5-Atg16 complex was impaired by mutations. Scale bar = 5 μ m. **d**, Overall view of Fig. 3e. 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×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).



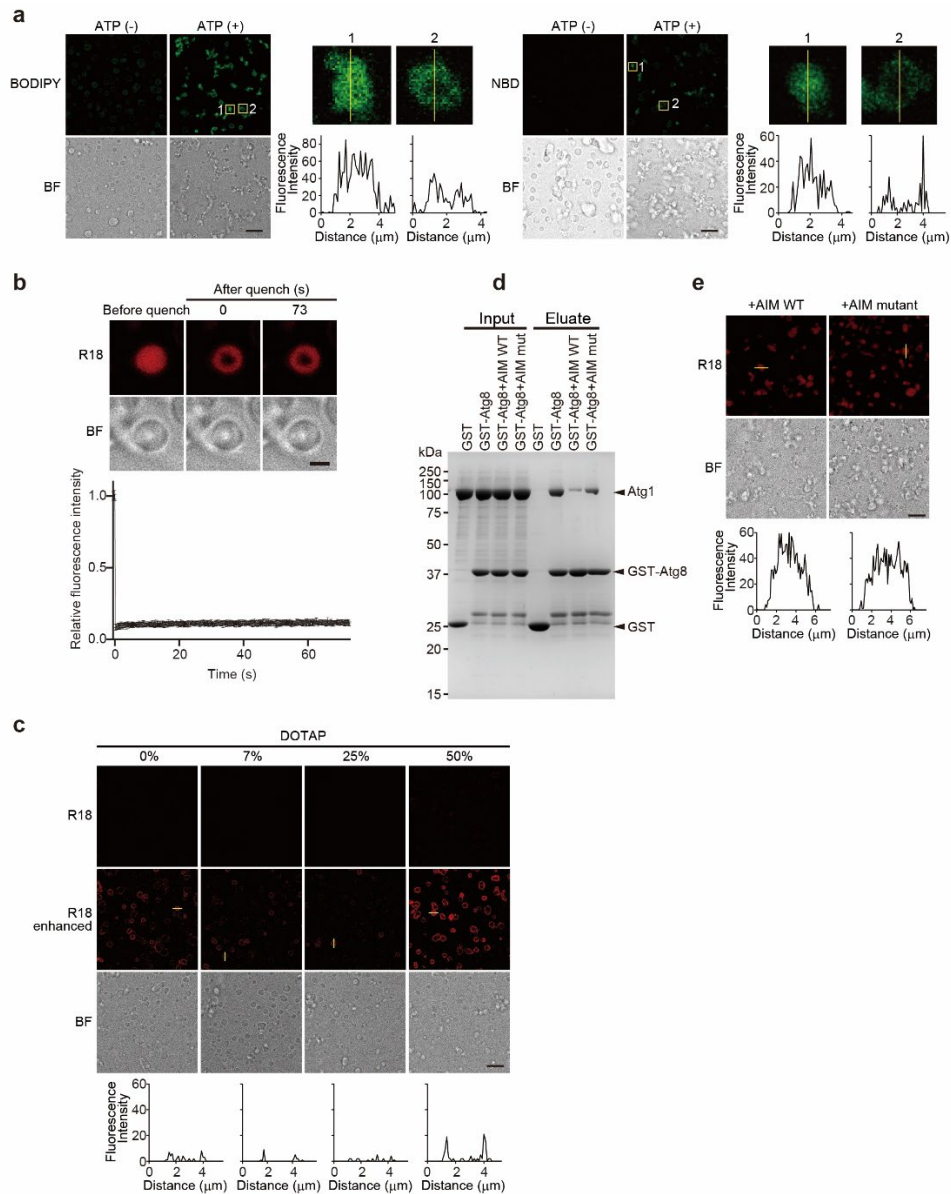
Extended Data Fig. 4. Effect of the PAS droplets, Atg21, and PI3P on Atg8 lipidation and dephosphorylation. **a**, Overall gel image of Fig. 4b. **b**, Overall gel image of Fig. 4d. **c**, Overall gel image of Fig. 4f. **d**, Investigation of conditions for terminating the Atg8 lipidation reaction. Treatment with 100 mM DTT largely suppressed Atg8 lipidation when the E3 concentration was 0.1 μ M, but could not halt it when the E3 concentration was 1 μ M. In contrast, treatment with an ATP-eliminating agent (CheckLite 250 Plus) completely stopped Atg8 lipidation even at an E3 concentration of 1 μ M. This experiment was performed once. **e**, Overall gel image of Fig. 4h. **f**, Overall gel image of Fig. 4j.



Extended Data Fig. 5. In vitro Atg21 and PI3P-dependent targeting of the Atg5–Atg16 complex to the PAS droplets observed by fluorescence microscopy. Representative images from n = 3 experiments. Scale bar = 10 μ m.



Extended Data Fig. 6. Replica electron microscopy of the early PAS droplets. **a**, Replica electron microscopy with or without etching. Representative images from $n = 1$ experiment (for without etching) and $n > 3$ experiments (for with etching). Scale bar = 200 nm. **b**, Replica electron microscopy of liposomes outside the droplets. Representative images from $n > 3$ experiments. Scale bar = 50 nm. **c**, Replica electron microscopy in the presence of Atg21 but in the absence of Atg12–Atg17 interaction. Representative images from $n = 1$ experiment. Scale bar = 200 nm. **d**, Proposed model of the processes in the PAS droplet during autophagy initiation.



Extended Data Fig. 7. Mechanistic analysis of liposome uptake into droplets. **a**, Observation of BODIPY or NBD-PE-containing liposomes at the early PAS droplets with or without Atg8 lipidation reaction. Fluorescence images obtained with and without ATP were acquired on the same microscope under identical imaging settings (laser power, gain, and scan speed). This experiment was performed once. Scale bar = 10 μm . Yellow lines = 5.1, 4.3, 4.7, and 5.7 μm from left to right. **b**, FRAP analysis of R18-liposomes at Early PAS droplets. Scale bar = 2 μm . Bars in the graph represent means $\pm$ SD, $n = 5$ technical replicates. **c**, Observation of R18-containing liposomes with various concentrations of DOTAP at the early PAS droplets. Bottom shows the line profile along the yellow line. This experiment was performed once except for 50% DOTAP, which was performed three times with similar results. Bar = 10 μm . **d**, Effect of AIM WT or AIM mutant treatment on the interaction between GST-Atg8 and Atg1 analyzed by in vitro pulldown assay. This experiment was performed once. **e**, Observation of R18-containing liposomes with AIM WT or AIM mutant at the early PAS droplets. Bottom shows the line profile along the yellow line. This experiment was performed once. Scale bar = 10 μm .