Supplementary Figure 1.
Control experiments using yeast.

(a, b) QF-FRL of wild-type yeast in log phase. PtdIns(3)P was labeled with GST-PX. The plasma membrane, as well as the outer and inner mitochondrial membranes, exhibited negligible labeling in both PF and EF. Bars, 0.5 μm.

(c) QF-FRL of vps15Δ. Vps15p is an indispensable component of the PtdIns 3-kinase complex. No labeling of PtdIns(3)P was observed. Bar, 0.5 μm.
Supplementary Figure 2.
GFP or the PX domain of p40phox tagged with GFP were expressed in wild-type and \textit{vps34Δ} cells, and observed by structured illumination microscopy. In wild-type cells in log phase, GFP was distributed diffusely in the cytoplasm, whereas GFP-PX signal could be observed intensely along the vacuole surface, which was also labeled by FM4-64. Vacuolar concentration of GFP-PX was not observed in \textit{vps34Δ} cells lacking PtdIns(3)P. When cells expressing GFP-PX were incubated in S(–NC) in the presence of 1 mM PMSF, fluorescence was observed in the vacuolar lumen; this fluorescence was most likely derived from autophagic bodies. Bar, 10 μm.
Supplementary Figure 3.

Cryo-ultrathin sections of wild-type yeast cultured in S(-NC) medium for 4 h. Bars, 0.2 μm.

(a) GST-PX yielded labeling in the vacuolar and autophagic body membranes. However, as is evident at high magnification (see inset), most of the labeling was observed along the luminal side of the membrane and not on the cytoplasmic side.

(b) When GST-PX<sup>R58A</sup> was used instead of GST-PX, little labeling was observed, indicating that the labeling was derived from PtdIns(3)P.
Supplementary Figure 4.

Huh7 cells treated with 0.25 μM Torin1 and 0.4 μM bafilomycin A1 for 1 h.

(a) Confocal microscopy showing GFP-LC3 (green) and Lamp1 (magenta). Many of the GFP-LC3–positive structures did not overlap with the Lamp1 labeling (arrows). Bar, 10 μm.

(b) Conventional ultrathin section EM. Autophagosomes surrounded by two parallel membranes (arrows) and containing intact cytoplasmic structures were frequently observed. Bar, 0.5 μm.

(c) PtdIns(3)P (small gold, 6 nm) and GFP-LC3 (large gold, 10 nm) were doubly labeled by QF-FRL. Autophagosomes were observed as convex (i, ii, iii) and concave (iii, iv) structures. Most PtdIns(3)P labeling in the EF (marked with red circles) was found near the PF–EF boundary. Bar, 0.2 μm. (i) and (iii) are low magnification pictures of those shown in Fig. 3a.
**Supplementary Figure 5.**

Atg4<sup>C78A</sup>-expressing NIH3T3 cells incubated in Earle's balanced salt solution for 1 h. A gallery of QF-FRL figures showing autophagosomal membranes labeled for PtdIns(3)P. The label was observed only in PF (i.e., the cytoplasmic leaflet) of the membrane. Bars, 0.1 μm.
Supplementary Figure 6.
Defects of autophagic process in ymr1Δsjl3Δ cells.

(a) Formation of autophagic bodies. Wild-type and ymr1Δsjl3Δ cells were cultured in S(-NC) medium in the presence of 1 μM PMSF for 3 h. Far more autophagic bodies (*) were observed in wild-type cells than in ymr1Δsjl3Δ cells. Bars, 0.2 μm. The bar graph shows the number of autophagic bodies per μm² vacuole profile in four strains, indicating that significantly fewer autophagic bodies were formed in ymr1Δsjl3Δ cells than in others. The measurement was performed in more than 30 cells for each strain.

(b) Western blotting of API in wild-type, ymr1Δ, sjl3Δ, ymr1Δsjl3Δ cells cultured in YPD medium or in S(-NC) medium for 2 h or 4 h. Processing of prAPI to generate mature API was significantly suppressed in ymr1Δsjl3Δ.

(c) Western blotting of Atg8 in wild-type (WT), ymr1Δ, sjl3Δ, ymr1Δsjl3Δ, and atg5Δ cells cultured in YPD medium or starved in S(-NC) medium for 2 h. Atg8 conjugated to phosphatidylethanolamine (PE) increased significantly in ymr1Δsjl3Δ cells after starvation.
Supplementary Figure 7.
Autophagosomes with aberrant constrictions and creases in ymr1Δsjl3A cells cultured in S(-NC) medium for 4 h. Despite the abnormal morphology, the autophagosomes were observed as IMP-deficient double-layered membranes and labeled positively for PtdIns(3)P in both membrane leaflets. Bars, 0.2 μm.
Supplementary Figure 8.
Full scan data of Western blotting.