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Supplementary information

Atg9 is a lipid scramblase that mediates autophagosomal membrane expansion

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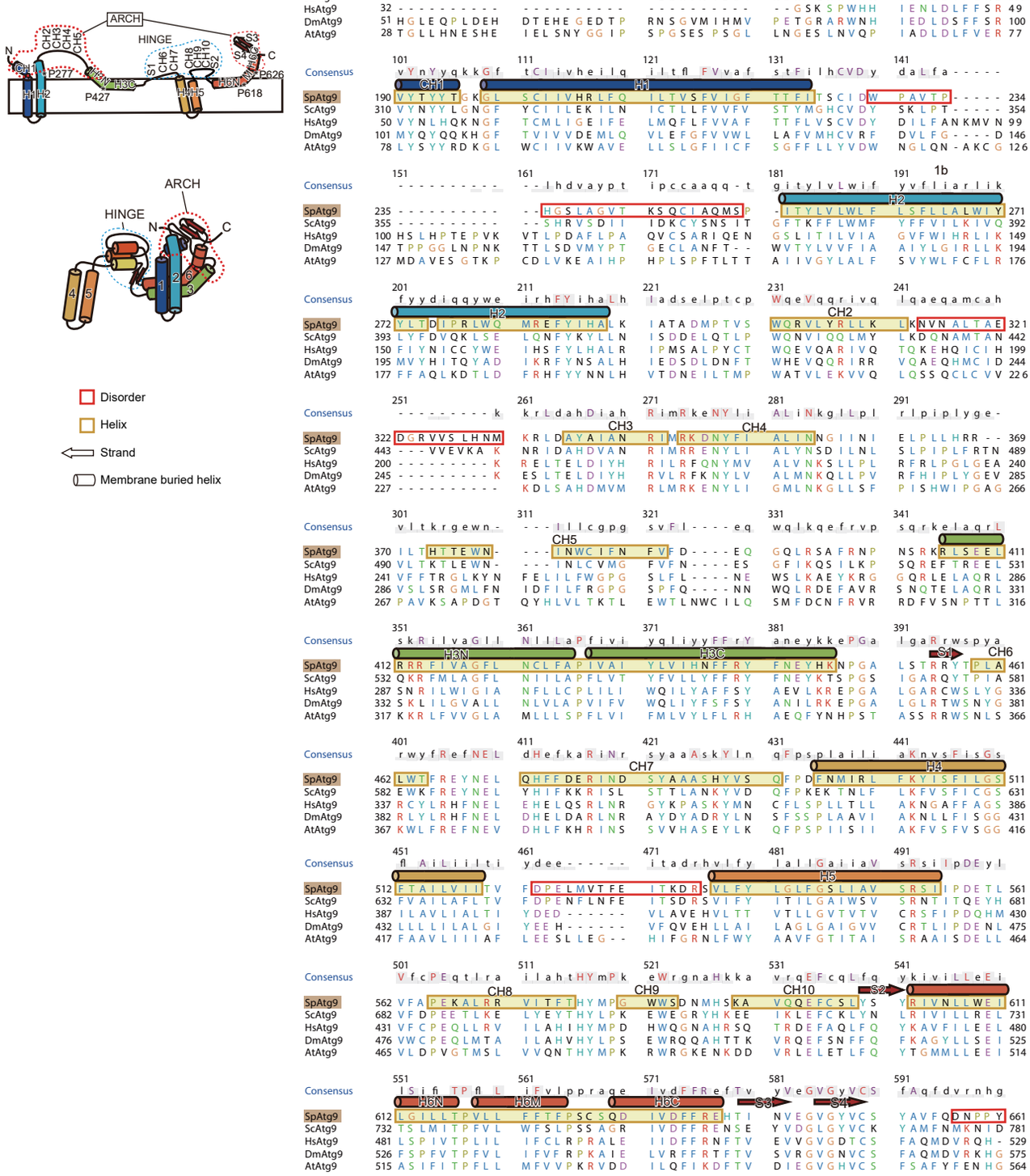


Supplementary Fig. 1. Sequence alignment of Atg9 homologs. Sp, Sc, Hs, Dm, and At indicate *S. pombe*, *S. cerevisiae*, *H. sapiens*, *D. melanogaster*, and *A. thaliana*, respectively. Secondary structure based on the cryo-EM structure of SpAtg9 is shown above the sequence.

Figure 2e

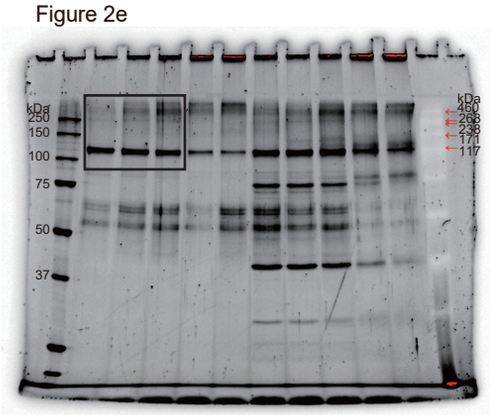
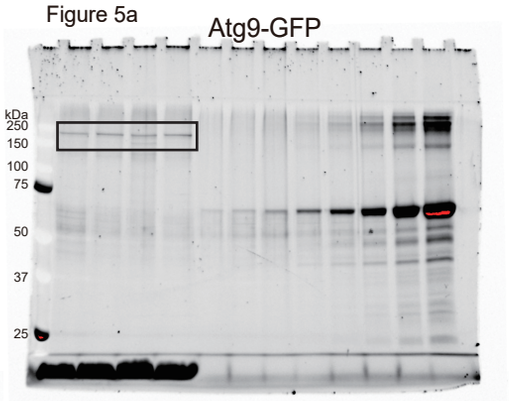
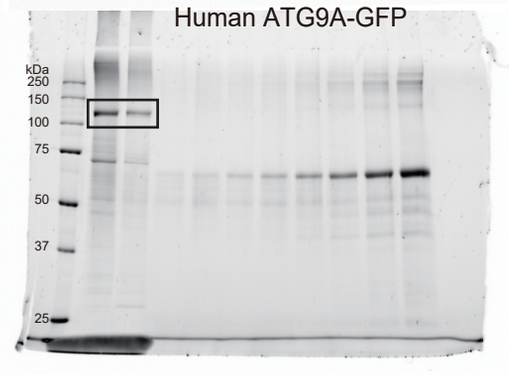


Figure 5a





Ext Data Fig. 1c

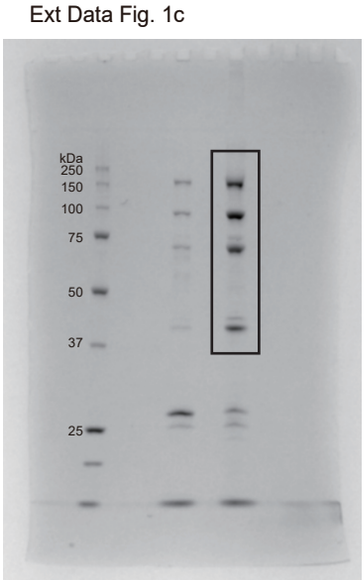


Figure 3c

anti-Atg9

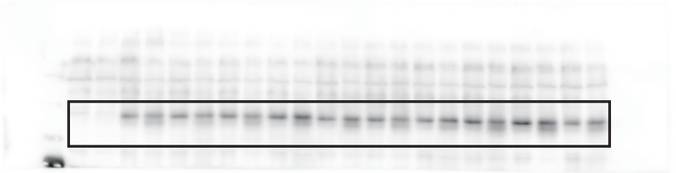


Figure 3c

anti-Ape1

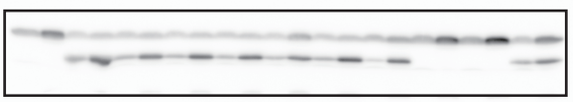
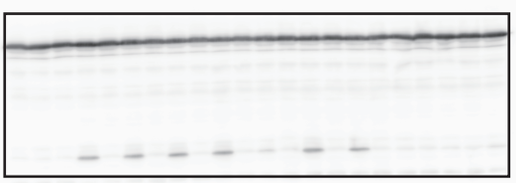
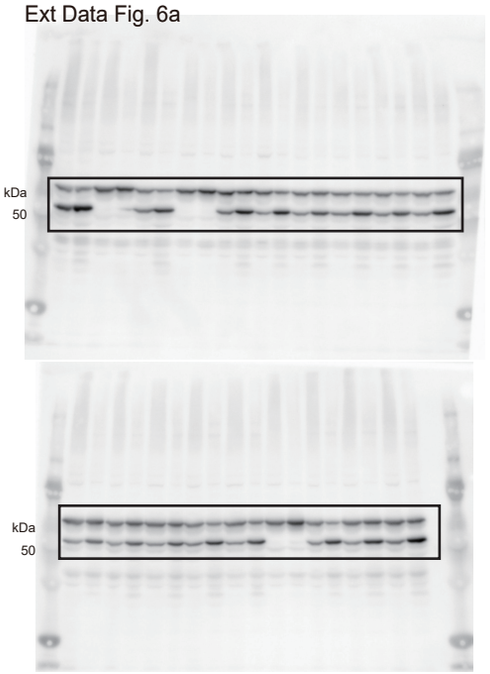


Figure 3c

anti-GFP



Ext Data Fig. 6a



Supplementary Fig. 2. Uncropped gel and blot images.

Supplementary Note 1

Yeast strains and media

The yeast strains used in this study are listed in Supplementary Table 1. Gene disruption and tagging of fluorescent proteins were performed by a PCR-based method[57]. Cells carrying plasmids derived from pRS316[58] were cultured at 30℃ in SD+CA+AT medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (YNB w/o a.a. and a.s.), 0.5% ammonium sulfate, 2% glucose, 0.5% casamino acids, 0.002% adenine sulfate, and 0.002% tryptophan). Autophagy was induced by addition of 200 ng/ml rapamycin or incubation in SD-N nitrogen starvation medium (0.17% YNB w/o aa and as and 2% glucose). For preparation of SCA culture media for large-scale production of Atg9 proteins, 150 x salt stock solution (potassium phosphate monobasic 150 g/L, magnesium sulfate 75 g/L, sodium chloride 15 g/L) and vitamin pre-mixed powder (biotin 2 µg/L, calcium pantothenate 400 µg/L, folic acid 2 µg/L, myo-inositol 2000 µg/L, niacin 400 µg/L, p-aminobenzoic acid 200 µg/L, pyridoxine hydrochloride 400 µg/L, riboflavin 200 µg/L, thiamine hydrochloride 400 µg/L, boric acid 500 µg/L, copper(II) sulfate 40 µg/L, potassium iodide 100 µg/L, iron(III) chloride 200 µg/L, manganese(II) sulfate 400 µg/L, sodium molybdate 200 µg/L, zinc sulfate 400 µg/L, calcium chloride 100 mg/L) were prepared. The SCA culture media was prepared at a final concentration of 1 x salt, hicasamino acids DAIGO 5 g/L, vitamin pre-mixed powder 106 mg/L, ammonium sulfate 5 g/L, L-tryptophan 0.02 g/L, and adenine sulfate 0.02 g/L, all from Nacalai Tesque Inc., Kyoto, Japan, except for boric acid (Wako Pure Chemical, Osaka, Japan) and hicasamino acids DAIGO (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan). $SDCA_{0.2}$ $AS_{0.2}$ -URA, -TRP selection plates were prepared as follows: 0.17% (w/v) YNB w/o a.a. and a.s., 0.1% (w/v) ammonium sulfate, 0.1% (w/v) casamino acids, 2% (w/v) glucose, adenine sulfate 0.02 g/L, 2% (w/v) ager and 2.5 µg/mL phloxine B.

Plasmid construction

The plasmids used for yeast experiments are listed in Supplementary Table 2. The NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs) was used for cloning. Mutations to generate the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. All constructs were sequenced to confirm accuracy of cloning.

Protein expression and purification

Atg9 recombinant proteins were expressed using a yeast expression system. A construct of *Saccharomyces cerevisiae* Atg9 (residues 1-997) was subcloned from yeast, and synthesized *Schizosaccharomyces pombe* Atg9 (residues 1-702) and human ATG9A (residues 1-839) plasmids were purchased from GENEWIZ. The *S. cerevisiae* diploid BJ926 *atg27*Δ/*atg27*Δ strain was transformed with pRS426-GAL1pro-Atg9-GS-PS-TS-meGFP-His_8 (GS : Glycine-Serine linker, PS : Human rhinovirus 3C protease site, TS : Twin-Strep-tag®, meGFP : A206K eGFP, His_8 : 8 x histidine tag) and cultured on SDCA(-URA) plates (SCA-URA media supplemented with 2% glucose and 1% agarose). Optimal expression and solubilization conditions were ascertained by fluorescence-based SEC[31,32].

Single colonies were cultured in 5 L SCA (-URA) containing 2% glucose at 30°C until the OD_{600} reached between 6 and 8, after which culture media were supplemented with SCA(-URA), galactose, and DMSO at respective final concentrations of 1x, 2%, and 2% to a total volume of 8 L. Fed-batch culture was performed at 23°C for 20 h. Cells were harvested by centrifugation at 7,000 x g for 12 min at 4°C (unless otherwise noted, all subsequent steps were conducted at 4°C or on ice). The pellet (wet weight ~120-150 g) was suspended in CR buffer (50 mM Tris-HCl pH 7.5, 1mM EDTA, 0.6 M sorbitol, 1 mM PMSF, 1 mM 2-mercaptoethanol) and disrupted using 0.6 mm zirconia beads and agitation at 1,500 rpm in a Shake Master NEO (Bio Medical Science, Tokyo, Japan). Following lysate centrifugation at 10,000 x g for 10 min, the supernatant was subjected to ultracentrifugation at 200,000 x g for 2 h. The membrane fraction was suspended in MR buffer (20 mM Tris-HCl pH 7.5, 0.3 M sucrose, 0.1 mM $CaCl_2$) and stored at -80°C until use, and then solubilized with S buffer (50 mM Tris-HCl pH 8.5, 150 mM NaCl, 1% 6-cyclohexyl-1-hexyl-β-D-maltoside (Cymal-6, Anatrace Inc., Maumee, OH), 0.1% cholesteryl hemisuccinate (CHS, Anatrace), 1 mM PMSF, 1 mM 2-mercaptoethanol) for 1 h on a seesaw rocker.

The solubilized solution was centrifuged at 100,000 x g for 30 min in a Beckman type 45 Ti or type 70.1 Ti rotor. 0.75 mg Avidin (Wako Pure Chemical, Osaka, Japan) and 31 units Benzonase® (Sigma-Aldrich) were added to 1 L culture media supernatant. Samples were then purified using a Strep-Tactin ® XT Superflow resin (IBA, Germany) equilibrated with binding buffer (50 mM Tris-HCl pH 8.5, 100 mM NaCl, and 0.03% n-dodecyl-β-D-maltopyranoside (DDM, Anatrace)/0.003% CHS) and eluted with elution buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.03% DDM/0.003% CHS and 50 mM biotin). After concentration using an Amicon Ultra-15 100 kDa MWCO (Merck), the sample was applied to a Superose 6 increase 10/300 GL column (GE healthcare) and

equilibrated in 20 mM Tris-HCl pH8.0, 150 mM NaCl, 0.03% DDM/0.003% CHS. The peak fraction was concentrated and frozen at -80°C until use. For preparation of GFP-free Atg9, GFP was cleaved from Atg9 using human rhinovirus 3C protease at 4°C for 16 h. The cleaved sample was passed through TALON® (Clontech) and COSMOGEL® GST-Accept resins (Nacalai Tesque) and equilibrated with 20 mM Tris-HCl pH8.0, 150 mM NaCl, 0.03% DDM/0.003% CHS to remove the GFP-His_8-tag and HRV 3C protease.

Preparation of antibody fragments

Mouse monoclonal antibodies against Atg9 were raised according to a previously described method[59]. Briefly, a proteoliposome antigen was prepared by reconstituting purified, functional Atg9 at high density into phospholipid vesicles consisting of a 10:1 mixture of egg phosphatidylcholine (Avanti Polar Lipids) and the adjuvant lipid A (Sigma) to facilitate an immune response. BALB/c mice were immunized with the proteoliposome antigen using three injections at two-week intervals. Antibody-producing hybridoma cell lines were generated using a conventional fusion protocol. Hybridoma clones producing antibodies recognizing conformational epitopes in Atg9 were selected by an enzyme-linked immunosorbent assay on immobilized phospholipid vesicles containing purified Atg9 (liposome ELISA), allowing positive selection of antibodies recognizing the native conformation of Atg9. Additional screening for reduced antibody binding to SDS-denatured Atg9 was used for negative selection against linear epitope-recognizing antibodies. Stable complex formation between Atg9 and each antibody clone was checked using fluorescence-detection SEC. Whole IgG molecules, collected from the large-scale culture supernatant of monoclonal hybridomas and purified using protein G affinity chromatography were digested with papain, and Fab fragments were isolated using a HiLoad 16/600 Superdex 200 gel filtration column followed by protein A affinity chromatography.

Supplementary Note 2

ImageJ Script 1

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selectWindow("Result of Atg17 Maxima");
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run("Find Maxima...", "noise=1 output=Count exclude");
selectWindow("Result of Atg17 Maxima");
run("Find Maxima...", "noise=1 output=Count exclude");
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Supplementary Table 1. Yeast strains used in this study

Name	Genotype	Ref/Figures
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ref 60
ScTK1055	BY4741 *ATG17-2×mCherry*::*hphNT1 atg9Δ*::*zeoNT3 atg1Δ*::*kanMX4*	Fig. 3h
ScTK1084	BY4741 *ATG17-2×mCherry*::*hphNT1 atg9Δ*::*zeoNT3 ATG2-mNeonGreen-natNT2 atg8Δ*::*kanMX4*	Fig. 3i
ScTK850	BY4741 *his3Δ1*::pRS303-*PGK1-EGFP-HIS3 atg9Δ*::*zeoNT3*	Fig. 3c
BJ2168	MATa leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2	Ref 61
ScTK265	BJ2168, *ATG2-2xEGFP*::*kanMX6 atg9Δ*::*natNT2*	Fig. 3f
W303-1a	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Ref 10
ScTK869	W303-1A, *ade2*::*ADE2 leu2*::*2×mCherry-ATG8-hphNT1 kanMX4*::P_{GPD}-*APE1 his3-11,15*::pRS303-P_{GPD}-*APE1 atg9Δ*::*natNT2*	Fig. 4a, b
SEY6210	MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801; GAL	Ref 62 Ext Data Fig. 6a
sm37	SEY6210 *atg9Δ*::kanMX6	Ext Data Fig. 6a
BCY123	Matα *pep4*::HIS3 *prb*::LEU2 *bar1*:HISG *lys2*::GAL1/10-GAL4 *can1 ade2 ura3 leu2–3,112 trp1*	Ref 63
YOY193	BCY123 *TRX1-ADE2::ade2Δ*	Ref 56 Fig. 1g, 5b
ScHY5288	BJ926 MATa/alpha TRP1/trp1 ura3::kanMX6/ura3::natNT2 atg27Δ::hphNT1/atg27Δ::Zeocin	For cryo-EM

Supplementary Table 2. Plasmids used in this study

Plasmid	Detail	Ref/Figures
p9KM85+	pRS426-Gal1pro-SpAtg9^{WT}-GS-PS-TwinStrep-meGFP-His8-TAA-PGKterm	For cryo-EM
pRS316-Atg9-EGFP	pRS316-ScAtg9^{WT}-EGFP	Fig. 3h, 4
p9KM135	pRS316-ScAtg9^{Junction}-EGFP	
p9KM123	pRS316-ScAtg9^{Hexamer}-EGFP	
p9KM120	pRS316-ScAtg9^{Entrance1}-EGFP	
p9KM66	pRS316-ScAtg9^{Entrance2}-EGFP	
p9KM126	pRS316-ScAtg9^{Entrance3}-EGFP	
p9KM123	pRS316-ScAtg9^{Middle}-EGFP	
p9KM129	pRS316-ScAtg9^{Basic}-EGFP	
p9KM52	pRS316-ScAtg9^{Wall}-EGFP	
p9KM50	pRS316-ScAtg9^{Acidic}-EGFP	
pRS316-Atg9	pRS316-ScAtg9^{WT}	Fig. 3c, f, i
p9KM134	pRS316-ScAtg9^{Junction}	
p9KM122	pRS316-ScAtg9^{Hexamer}	
p9KM119	pRS316-ScAtg9^{Entrance1}	
p9KM142	pRS316-ScAtg9^{Entrance2}	
p9KM125	pRS316-ScAtg9^{Entrance3}	
p9KM141	pRS316-ScAtg9^{Middle}	
p9KM128	pRS316-ScAtg9^{Basic}	
p9KM83	pRS316-ScAtg9^{Wall}	
p9KM84	pRS316-ScAtg9^{Acidic}	
p9KM62	pRS316-ScAtg9^{WT}-EGFP-TRP1	Ext Data Fig. 6a
p9KM72	pRS316-ScAtg9^{Wall}-EGFP-TRP1	
p9KM3-3	pRS316-ScAtg9$^{Mut\ 3\text{-}3}$-EGFP-TRP1	
p9KM3-4	pRS316-ScAtg9$^{Mut\ 3\text{-}4}$-EGFP-TRP1	
p9KM3-5	pRS316-ScAtg9$^{Mut\ 3\text{-}5}$-EGFP-TRP1	
p9KM3-8	pRS316-ScAtg9$^{Mut\ 3\text{-}8}$-EGFP-TRP1	
p9KM3-10	pRS316-ScAtg9$^{Mut\ 3\text{-}10}$-EGFP-TRP1	
pRS316	(empty plasmid)	
pRS424	(empty plasmid)	Used for cloning of TRP1 gene

pYO379	pRS426-ZZ-3xTEV-ScVPS30+ScVPS34+ScVPS15-3xTEV-ZZ-ADH1t	Ref 56 Ext Data Fig. 1c
pYO411	3xTEV-ScATG14	Ref 56 Ext Data Fig. 1c
p9KM0	pRS426-Gal1pro-ScAtg9^{WT}-GS-PS-TwinStrep-meGFP-His8-TAA-PGKterm	Fig. 1g, 5b
p9KM78	pRS426-Gal1pro-ScAtg9^{Wall}-GS-PS-TwinStrep-meGFP-His8-TAA-PGKterm	Fig. 1g, 5b
p9KM130	pRS426-Gal1pro-ScAtg9^{Basic}-GS-PS-TwinStrep-meGFP-His8-TAA-PGKterm	Fig. 1g, 5b
p9KM78	pRS426-Gal1pro-ScAtg9^{Acidic}-GS-PS-TwinStrep-meGFP-His8-TAA-PGKterm	Fig. 1g, 5b
p9KM152	pRS426-Gal1pro-HsAtg9A^{WT}-GS-PS-TwinStrep-meGFP-His8-TAA-PGKterm	Fig. 1g, 5b
p9KM153	pRS426-Gal1pro-HsAtg9A^{Wall}-GS-PS-TwinStrep-meGFP-His8-TAA-PGKterm	Fig. 1g, 5b
Addgene #19011	p40^{phox} PX-EYFP	Ref 64
p9KM151	pGEX6P-EYFP-p40^{phox} PX	Ext Data Fig. 1d

Supplementary References

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