Supplemental Information

Supplemental material

SNARE proteins are required for macroautophagy

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. Atg8/LC3–PE Is not Sufficient to Drive Membrane Hemifusion under Physiological Conditions.

(A) Tethering measured by turbidity assay. Full spectrum analysis of reactions as in Figure 1A, run with or without all reactants reveals a coupling-dependent turbidity signal. The spectrophotometer was first blanked against a no Atg3 reaction sample.

(B) 30% PE liposomes do not support Atg8-mediated fusion. At least five independent experiments are shown for each protein concentration and at least two independent preparations of liposomes and expression/purifications of protein were tested. Even the highest concentrations of Atg8 were insufficient to drive fusion of 30% liposomes. The inherent fusogenicity of unreacted negative controls in the presence of 55% PE was at least three-fold higher than the highest rates observed in any experiment with lower PE, regardless of Atg8 concentration. The highest rate of fusion observed is plotted for each experiment. Blue dots are full reaction conditions, red dots are negative controls lacking Atg7. The highest rate rather than initial rate is plotted because each experiment typically displayed a 5 min delay in the onset of fusion. Rate is more meaningful than endpoint because the most robust experimental conditions reach a plateau in as little as 20 min.

(C) To mimic the enzyme-dependent ubiquitin-like coupling of a PE to the C terminus of Atg8, we introduced a C-terminal cysteine residue in place of the normally lipid-associated C-terminal glycine to generate $Atg8^{G116C}$. Liposomes were prepared with 5 mole % of a maleimide-associated lipid, which spontaneously reacts with the cysteine when $Atg8^{G116C}$ is introduced. In this way, coupling of Atg8 to the liposomes is essentially independent of molar PE

concentration. Quenching of the reactive group with β-mercaptoethanol (β-Me) serves as a negative control.

(D) Purified recombinant LC3 with a C-terminal cysteine (LC3-C) or glycine (LC3-WT) were conjugated *in vitro* in the presence of the indicated concentrations of PE and maleimide PE (malPE).

(E) A dequenching fusion reaction was carried out with the indicated version of LC3 conjugated to liposomes containing various concentrations of PE. A sample with only labeled liposomes (labeled alone), which is not able to undergo dequenching, is a negative control. Error bars correspond to standard deviation (SD) from 3 independent assays.

Figure S2, related to Figure 2. The Exocytic Q/t-SNAREs but not v-SNARES Are Required for Autophagy.

(A) Complementation of autophagy activity at the NPT in the *sso1*Δ*/2*^{ts} and *sec9-4* strains. The empty vector with the *CUP1* promoter (pCu416), or the plasmid pCuHASec9(416) or pCuSso1(416) was transformed together with pGFP-Atg8(414) into *sso1*Δ*/2*^{ts} (H603) or *sec9-4* (JGY243) cells. Transformants were cultured and examined by immunoblotting as described in Figure 2A.

(B) Autophagic activity in the *snc1*Δ *snc2*Δ strain. Cell lysates from wild-type (WT; TN124), *atg1*Δ (UNY5) and *snc1*Δ *snc2*Δ (UNY130) strains were used to measure Pho8Δ60 activity. Cells were cultured in SMD medium until OD_{600} = 0.8 and then incubated in SD-N for 2 h. Error bar, SD from three independent experiments.

(C) Mutations in Sso1/2 affect the vesicle formation step. Cultures of *pep4*Δ (JGY248), *atg1*Δ (TYY164), and *sso1*Δ*/2*^{ts} (H603) cells were pre-incubated at 34°C for 30 min and then subjected

to a protease protection assay as described in the Supplemental Experimental Procedures. In the wild-type (*pep4*Δ) cells, prApe1 was protected from cleavage by proteinase K alone and was only digested in the presence of detergent. The protease protection pattern in the *sso1*Δ*/2*^{ts} strain was similar to that seen in *atg1*Δ cells. T, total; S5, 5,000 x g supernatant fraction; P5, 5,000 x g pellet fraction; TX, Triton X-100; PK, proteinase K. The cytosolic marker protein Pgk1 was only detected in the total and supernatant fractions, indicating efficient spheroplast lysis.

Figure S3, related to Figure 3. The Recruitment of Atg9-3xGFP to the PAS Is Defective in the *sec9-4* Mutant.

(A) Wild-type (Atg9-3xGFP RFP-Ape1; UNY145) or *sec9-4* (Atg9-3xGFP RFP-Ape1 *sec9*-4; UNY132) cells were grown in rich medium to mid-log phase, shifted to NPT for 0.5 h to inactivate Sec9, and incubated in nitrogen-starvation medium for another 0.5 h. The cells were fixed and subjected to fluorescence microscopy. Sixteen Z-section images were captured and projected to visualize all the puncta present throughout the cells. (A) Representative projected images. Arrowheads denote the position of RFP-Ape1 at the PAS, and double arrowheads indicate overlaps of Atg9-3xGFP and RFP-Ape1.

(B) The quantification of the percentage of cells showing colocalization between Atg9-3xGFP and RFP-Ape1. Error bars, SEM from three independent experiments; n = 218 for the wild type, and n = 156 for the mutant. DIC, differential interference contrast. Scale bar, 2.5 μm.

(C-F) The colocalization between RFP-Ape1 and GFP-Atg11 (C and D) or GFP-Atg1 (E and F) is not affected in the *sso1*Δ*/2*^{ts} mutant. Wild-type (UNY146) or *sso1*Δ*/2*^{ts} (UNY147) cells were transformed with plasmids bearing GFP-Atg11 or GFP-Atg1. The cells were grown in rich medium to mid-log phase and shifted to NPT for 0.5 h to inactivate Sso2, after which RFP-Ape1

and GFP-Atg11 localization was examined by fluorescence microscopy. For examining the localization of RFP-Ape1 and GFP-Atg1, cells were cultured as mentioned above; however, prior to visualization by fluorescence microscopy, incubation was continued for another 0.5 h in nitrogen-starvation medium. (C and E) representative images showing the colocalization between RFP-Ape1 and GFP-Atg11 or GFP-Atg1, respectively; and (D and F) the corresponding quantification of colocalization. The error bars represent standard error of the mean (SEM) from three independent experiments. For the graph shown in D, n = 146 for the wild type, and n = 139 for the mutant; and for the graph shown in F, n = 124 for the wild type and n = 158 for the mutant. For images shown in C and E, scale bar, 5 μm.

Figure S4, related to Figure 4. The *sso1/Δ2*^{ts} Mutant Is Defective in Atg8 Localization.

Wild-type (*pep4*Δ; JGY248) or *sso1*Δ*/2*^{ts} *pep4*Δ (UNY142) cells were transformed with a plasmid expressing GFP-Atg8 (p*CUP1*-GFP-ATG8(416)). Cells were grown in SMD-Ura until mid-log phase at PT and shifted for 30 min to NPT in the same medium to inactivate *sso2*. In order to facilitate the formation of autophagosomes, the cells were washed in SD-N and incubation was continued for 1.5 h at NPT. Cells were processed for immuno-electron microscopy, and labeled with anti-YFP followed by immunogold. (A and B) In agreement with previously published data (Kirisako et al., 1999), in wild-type cells we detected GFP-Atg8 in autophagic bodies (black arrow), and on phagophores (white arrow).

(C and D) In *sso1*Δ*/2*^{ts} *pep4*Δ *vps4*Δ cells autophagic bodies were never observed in the vacuole; white arrowheads mark dispersed GFP-Atg8, and the white arrow represents a phagophore membrane decorated with GFP-Atg8. Scale bar, 250 nm.

Figure S5, related to Figure 5. Components of the Exocyst Complex, but not Genes Necessary for Endosomal Function, Are Required for Autophagy.

(A) Wild-type (WT), *sec3-2*, *sec8-9*, *sec15-1* or *atg1*Δ cells transformed with a plasmid expressing the endogenous *ATG8* promoter-driven GFP-Atg8, were cultured in rich medium to mid-log phase at 24°C. The cultures were shifted to 37°C to for 30 min to inactivate the *sec* mutations, and then starved at the same temperature for 2 h. For recovery (R), the cells were shifted back to 24°C, and incubation was continued for another 2 h. Samples were collected before (+) and after (–) starvation, or after recovery. TCA precipitated cell lysates were resolved by SDS-PAGE, and GFP-Atg8 processing was analyzed. Pgk1 was used as a loading control.

(B) Wild-type (WT), *rvs167*Δ, *end3*Δ or *atg1*Δ cells in the BY4742 strain background were transformed with a plasmid expressing the endogenous *ATG8* promoter-driven GFP-Atg8. The cells were grown in rich medium to mid-log phase, and then shifted to nitrogen-starvation medium for the indicated time points. Approximately 1 OD_{600} unit of cells was collected by centrifugation, TCA precipitated, washed with acetone and resolved by SDS-PAGE. GFP-Atg8 processing was analyzed by immunoblotting with anti-YFP antibody and anti-Pgk1 (as a loading control) antiserum. The positions of full-length GFP-Atg8 and free GFP are indicated.

Figure S6, related to Figure 6. **Tlg2 cannot** D**rive in vitro Proteoliposome Fusion with Sso1 and Sec9.**

(A) A Tlg2-Sso1-Sec9 complex does not promote liposome fusion in vitro. A kinetic fusion graph representing the percent of maximum fluorescence during a two-hour time course. An in vitro fusion assay was carried out as described in Supplemental Experimental Procedures.

(B) The same reaction was carried out as in (A) with the addition of 3.5 nmol of a peptide corresponding to the C terminus of Snc2 (Paumet et al., 2001). Similar results were obtained with Sso1-His_6.

Figure S7, related to Figure 7. Analysis of the N-Ethylmaleimide-Sensitive Fusion Protein (NSF) Sec18, and the R/v-SNAREs Sec22 and Ykt6 in the Cvt and Autophagy Pathways.

(A and B) The Cvt pathway is defective in *sec18-1* and *sec22-1* mutants. The *sec18-1*, *sec22-1* and corresponding wild-type strains were examined by pulse-chase at the NPT (32°C and 37°C for *sec18-1* and *sec22-1*, respectively) and immunoprecipitated with anti-Ape1 antiserum.

(C) The anterograde movement of Atg9 is affected in the *sec22-1* mutant. Representative projected images showing the transport of Atg9 after knocking out *ATG1* in the wild-type (UNY171) or *sec22-1* (UNY165) cells expressing Atg9-3xGFP and RFP-Ape1. DIC, differential interference contrast. Scale bar, 2.5 μm.

(D and E) Representative images showing the colocalization between Atg9-3xGFP and RFP-Sec22 or RFP-YKT6, respectively, in strain W303-1B.

Table S1, strains used in this study, related to Experimental Procedures

Strain	Descriptive Name	Genotype	Source or reference
BY4742	WT	*MATα his3Δ leu2Δ lys2Δ ura3Δ*	Invitrogen
atg1Δ	*atg1Δ*	BY4742 *atg1Δ::Kan*	Invitrogen
end3Δ	*end3Δ*	BY4742 *end3Δ::Kan*	Invitrogen
H603	*sso1/2^{ts}*	W303-1A *sso1Δ::HIS3 sso2-1*	(Jantti et al., 2002)
JGY191	WT	BY4742 *ATG-3xGFP::URA3 RFP-APE1::LEU2*	This study
JGY236	*sec9-4*	BY4742 *sec9-4 pho13Δ::ble pho8Δ60::URA3*	This study
JGY247	*sso1/2^{ts}*	H603 *pho13Δ::ble pho8Δ60::URA3*	This study
JGY248	*pep4Δ*	W303-1B *pep4Δ::LEU2*	This study
KWY76	*tlg2Δ*	BY4742 *pho8::pho8Δ60 pho13Δ::LEU2 tlg2Δ::HIS3*	This study
*rvs*167Δ	*rvs*167Δ	BY4742 *rvs*167Δ*::Kan*	Invitrogen
sec1-1	*sec1-1*	BY4742 *sec1-1*	This study
sec9-4	*sec9-4*	BY4742 *sec9-4*	This study
sec17-1	*sec17-1*	BY4742 *sec17-1*	This study
sec18-1	*sec18-1*	BY4742 *sec18-1*	This study
sec22-1	*sec22-1*	BY4742 *sec22-1*	This study
sec22-3	*sec22-3*	BY4742 *sec22-3*	This study
TN124	WT	*MATα leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13Δ::LEU2*	(Noda et al., 1995)
TYY164	*atg1Δ*	W303-1B *pho13Δ::Kan pho8Δ60::HIS3 atg1Δ::URA3*	(Yorimitsu et al., 2007)
UNY5	*atg1Δ*	TN124 *atg1Δ::Kan*	This study
UNY130	*snc1Δ snc2Δ*	TN124 *snc1Δ::Kan snc2Δ::ble*	This study
UNY132	*Sec9-4* Atg9-3xGFP RFP-Ape1	BY4742 *sec9-4 RFP-APE1::LEU2 ATG9-3x-GFP::URA3*	This study
UNY138	*sso1/2^{ts}* Atg9-3xGFP RFP-Ape1	W303-1B *sso1Δ::HIS3 sso2-1ATG9-3xGFP::URA3 RFP-APE1::LEU2*	This study
UNY140	*sso1/2^{ts} atg1Δ* Atg9-3xGFP RFP-Ape1	W303-1A *sso1Δ::HIS3 sso2-1ATG9-3xGFP::URA3 RFP-APE1::LEU2 atg1Δ::Kan*	This study
UNY141	*sso1/2^{ts} pep4Δ*	W303-1B *sso1Δ::HIS3 sso2-1 pep4Δ::LEU2*	This study
UNY142	*sso1/2^{ts} pep4Δ vps4Δ*	W303-1B *sso1Δ::HIS3 sso2-1*	This study

		*pep4*Δ::*LEU2 vps4*Δ::*ble*	
UNY145	WT Atg9-3xGFP RFP-Ape1	W303-1B *ATG9-3xGFP::URA3 RFP-APE1::LEU2*	This study
UNY146	WT RFP-Ape1	W303-1B *RFP-APE1::LEU2*	This study
UNY147	*sso1/2*^{ts} RFP-Ape1	W303-1B *sso1*Δ*::HIS3 sso2-1 RFP-APE1::LEU2*	This study
UNY148	WT *pep4*Δ *vps4*Δ	W303-1B *pep4*Δ::*LEU2 vps4*Δ::*ble*	This study
UNY149	WT Atg9-3xGFP RFP-Ape1 *atg1*Δ	W303-1B *ATG9-3xGFP::URA3 RFP-APE1::LEU2 atg1*Δ*::Kan*	This study
UNY151	WT *pep4*Δ	W303-1B *pep4*Δ::*LEU2 ATG9-GFP::URA3*	This study
UNY159	*tlg2*Δ Atg9-3xGFP RFP-Ape1	BY4742 *tlg2*Δ::*Kan ATG9-3xGFP::URA3 RFP-APE1*::*LEU2*	This study
UNY162	*vam3*^{ts}	BY4742 *TRP*Δ::*ble VAM3*^{ts} ::*TRP*	This study
UNY165	*sec22-1 atg1*Δ Atg9-3xGFP RFP-Ape1	*sec22-1* ATG9-3XGFP::*URA3 RFP-APE1*:: *LEU2 atg1*Δ*::HIS3*	This study
UNY168	GST-Tlg2	W303-1B pGAL-*GST-TLG2::HIS3*	This study
UNY170	*tlg2*Δ *atg1*Δ Atg9-3xGFP RFP-Ape1	BY4742 *tlg2*Δ::*ble ATG9-3xGFP*::*URA3 RFP-APE1*::*LEU2 atg1*Δ::*Kan*	This study
UNY171	WT Atg9-3xGFP RFP-Ape1 *atg1*Δ	BY4742 *ATG9-3xGFP*::*URA3 RFP-APE1*::*LEU2 atg1*Δ::*Kan*	This study
UNY172	GST-Sec9	W303-1B pGAL-*GST::SEC9::HIS3*	This study
UNY180	GST-Ufe1	W303-1B pGAL-*GST::UFE1::Kan*	This study
W303-1A	WT	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Wallis et al., 1989)
W303-1B	WT	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Wallis et al., 1989)
ykt6^{ts}	*ykt6*^{ts}	BY4742 *ykt6*	This study
YTS158	WT	BY4742 *pho8::pho8*Δ*60 pho13*Δ::*kan*	(He et al., 2006)
ZFY202	WT	W303-1B *pho13*Δ *pho8*Δ*60::HIS3*	(Yang et al., 2010)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

To generate pCuSso1(416), the open reading frame (ORF) of *SSO1* was amplified by PCR from yeast genomic DNA and cloned into the EcoRI/ClaI sites of pCu416. For cloning pCuHA-Sec9, the ORF of *SEC9* was amplified by PCR from yeast genomic DNA. The sequence for the HA tag was incorporated into the forward primer. The PCR product was digested with XmaI and XhoI and cloned into pCu416 digested with the same enzymes. PA-Sso1 (pCuPA-Sso1(414)) was generated by cloning the *SSO1* ORF as an EcoRI-XhoI fragment in frame with PA in the pRS414-CuPA vector (Kim et al., 2002). PA-Sec22 (pCuPA-Sec22(414)), PA-Ykt6 (pCuPA-Ykt6(414)) and PA-Gos1 (pCuPA-Ykt6(414)) were generated by cloning the *SEC22, YKT6* and *GOS1* ORFS, respectively, as EcoRI-SalI fragments in frame with PA in the pRS414-CuPA vector (Kim et al., 2002). RFP-Sso1 (pCuRFP-Sso1(414)), RFP-Sec22 (pCuRFP-Sso1(414)), RFP-Ykt6 (pCuRFP-Sso1(414)), were constructed by cloning the *SSO1, SEC22* and *YKT6* ORFs, respectively, in the pRS414-CuRFP vector. The pRS316 GFP-APG1 plasmid was a kind gift from Dr. Yoshinori Ohsumi (Tokyo Institute of Technology, Japan). Plasmids expressing GFP-Atg8 (pGFP-AUT7(416)) (Huang et al., 2000); GFP-Atg11 (pGFP-ATG11(416)) and pRFPApe1 (pRFP-Ape1(305)) (He et al., 2006); and Atg9-3xGFP (pAtg9-3xGFP(306)) (Monastyrska et al., 2008) have been described previously.

Media

The following media were used in this study: YPD (1% yeast extract, 2% peptone, and 2% glucose), YPG (1% yeast extract, 2% peptone, and 2% galactose), SMD (2% glucose and 0.67% yeast nitrogen base without amino acids, supplemented with vitamins and appropriate amino

acids), or SMG (2% galactose and 0.67% yeast nitrogen base without amino acids, supplemented with vitamins and appropriate amino acids). Nitrogen starvation experiments were performed in synthetic medium lacking nitrogen: SD-N; 0.17% yeast nitrogen base without amino acids, ammonium sulfate, vitamins, and 2% glucose or SG-N 0.17% yeast nitrogen base without amino acids, ammonium sulfate, vitamins, and 2% galactose.

Turbidity Assay to Detect Atg8-mediated Liposome Tethering

Atg8ΔR coupling reactions were run as above for the ubiquitin-like lipid conjugation. After 90 min at 30°C, a full spectrum absorbance measurement was made (Nanodrop 2000c spectrophotometer). For each liposome sample, the increase in total absorbance was measured by blanking the instrument with the negative control (the same liposome sample reaction minus Atg3). Increases in absorbance could be observed across essentially the whole spectrum. To plot liposome aggregation versus liposome PE surface density, the change in absorbance at 450 nm was selected, as this wavelength is far from confounding absorbances including protein and ATP.

Atg8–PE and Atg8-malPE Fusion Assays

Plasmid construction and protein expression

Atg7 was cloned into the pFastBac Baculovirus plasmid. Briefly, *ATG7* was PCR-amplified directly from *S. cerevisiae* plasmid DNA via primers encoding BamHI and EcoRI restriction sites and cloned into pGEX6p2. *ATG7* was then subcloned, via PCR amplification with primers encoding BamHI+6His tag and SalI sites into the pFastbac plasmid. The resulting sequence codes for a protein product with the following N-terminal sequence: “MHHHHHHSSERV…”

Atg3, Atg8^{G116} (Atg8ΔR), and Atg8^{G116C} were PCR amplified from isolated yeast plasmid DNA via primers encoding BamHI and EcoRI sites and then cloned directly into the pGEX-6P vectors. For Atg8, the 3' primer includes a stop codon immediately after G116 (Atg8^{G116}). For Atg8^{G116C}, the 3' primer mutates G116C and then codes for a stop codon. Site directed mutagenesis was used to mutate the one natural cysteine in Atg8 (Cys33) to a serine residue (C33S mutation), thus ensuring that the maleimide-lipid reaction described below would be specific for the C-terminal cysteine.

To induce protein expression, BL21 *E. coli* cells were transformed with the appropriate plasmid as follows: Bacterial cells and plasmids were combined and placed on ice for 30 min. The mixture was incubated at 42°C for 45 s, iced for 2 min and suspended in125 µl of SOC medium. Samples were shaken for 30-35 min at 37°C. The samples (50 µl) were then plated on carbenicillin (Sigma) plates, which were then incubated at 37°C for 16 h. A single bacterial colony was then selected, added to 2 ml of Luria Broth (LB) containing carbenicillin (50 µg/ml), and shaken at 37°C for several h. The 2 ml sample was again combined with 200 ml of LB containing carbenicillin and shaken for 16 h at 37°C. From this sample, 2 ml were extracted and added to 200 ml of LB with carbenicillin. The resulting mixture was shaken at 37°C for 16 h, then added to 4 ml of LB with carbenicillin and again shaken for ~3 h at 37°C until the OD reached between 0.6 and 0.8. Bacteria were treated with 0.5 mM isopropyl β-D(-)-thiogalactopyranoside for 3 h, centrifuged at 36,000 rpm and 4°C, then frozen at -80°C.

Atg7 was expressed by baculoviral infection of SF9 cells. Atg7 in pFastBac plasmid DNA was used to transform DH10Bac *E. coli* for transposition into the bacmid. We used blue/white colony selection to identify colonies containing the recombinant bacmid and confirmed by PCR. The recombinant bacmid was then isolated and purified (Qiagen endotoxin-

free DNA purification kit). Sf9 insect cells were transfected with purified bacmid using cellfectin II (Invitrogen) in unsupplemented Grace's Insect Media (Invitrogen). When the transfected cells demonstrated signs of late stage infection (typically around 72 h), the medium containing the free virus was collected. We repeated cycles of transfection and virus collection to amplify the viral stock. Typically, we would infect SF9 cells with viral stock having titer ranges from $1x10^6$ to $1x10^7$ pfu/ml using an MOI = 0.1. Cells were collected after 72-96 h of infection and used to purify his-tagged Atg7 (below).

Purification of Atg3

To purify yeast protein Atg3, glutathione S-transferase (GST) fusions of the desired protein were constructed as follows: Bacteria were resuspended in 50 ml Breaking Buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, and 2 mM $CaCl_2$) containing 1 complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche) and sonicated. After removal of cellular debris by centrifugation at 40,000 rpm and 4°C for 35 min, the sample supernatant fraction was incubated with 1 ml Glutathione Sepharose 4B (GE Healthcare) for 1 h at 4°C to bind GST tagged proteins. Beads were centrifuged at 900 rpm and 4°C for 5 min and the supernatant fraction was poured off. Proteins then bound to the Sepharose beads were washed with a 20-bead volume of PP Elution Buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.1 mM TCEP) and the beads were subsequently suspended in a 2-bead volume of Elution Buffer and 200 units of PreScission Protease (GE Healthcare). Cleaved protein was eluted and mixed with glycerol for a final glycerol concentration of 20%. Protein was stored at -80°C.

Purification of Atg7

To purify yeast protein Atg7, SF9 cells were resuspended in 20 ml Lysis Buffer (20 mM Tris, pH 7.6, 0.5 M NaCl, 10% glycerol, 20 mM imidazole, 1 mM β-Me per liter of cells with 1 complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche) per 50 ml buffer. Cells were sonicated and, after cellular debris were removed by centrifugation at 18,000 rpm at 4°C for 1 h, the sample supernatant fraction was incubated with 1 ml 50% Ni-NTA Agarose (Qiagen) per 1 liter of original cells for 2 h at 4°C. Beads were centrifuged at 1,000 rpm and 4°C for 5 min and the supernatant fraction was poured off. The beads were then combined with a 10 bead-volume of Wash Buffer (20 mM Tris, pH 7.6, 10 mM NaCl, 20 mM imidazole, 1 mM β-Me) and centrifuged again at 1,000 rpm and 4°C for 5 min. Beads were washed a second time in the same manner, transferred to a new container and washed again. Protein was then eluted 3 times as follows: beads were incubated with 1 ml Atg7 Elution Buffer (20 mM Tris, pH 7.6, 10 mM NaCl, 500 mM imidazole, 1 mM β-Me) at room temperature for 5-10 min and centrifuged at 900 rpm at 4°C for 5 min. The supernatant fraction was collected and mixed with glycerol for a final glycerol concentration of 20%. Protein was stored at -80°C.

Purification of Atg8

Yeast protein Atg8^{G116} (Atg8ΔR) and Atg8^{G116C} were purified in the same manner as Atg3.

Liposome preparation

Liposomes were prepared essentially as described previously except for changes in lipid composition (Melia et al., 2002). In brief, to prepare liposomes with various phospholipid compositions, the following lipids were combined in appropriate ratios: POPC (1-polmiyoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), DOPE (1,2-dioleoyl-sn-glycero-3-phophoethanolamine),

PI (L-α-phosphatidylinoitol) from bovine liver, DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine), Rho-DOPE (1,2-dioleoyl-sn-glycero-3-phophoethanolamine-N-[lissamine rhodamine B sulfonyl]) and, NBD-DOPE (1,2-dioleoyl-sn-glycero-3-phophoethanolamine-N-[7-nitro-2-1,3-benzoxadiazol-4-yl]) (Avanti Polar Lipids). Stock solutions, dissolved in chloroform, were mixed in glass tubes and dried to even film under nitrogen gas. To dry samples further, each glass tube was placed under vacuum at room temperature for one h. Samples were resuspended in SN buffer (50 mM Tris-HCl, ph 8.0, 100 mM NaCl, 1 mM $MgCl_2$) to a final concentration of 5-30 mM and subjected to 7 cycles of freezing and thawing. Using the LipSoFast-Basic extruder (Avestin) and 2 polycarbonate membranes of equal pore size (50 or 100 nm) (Avanti), samples were extruded 19 times to obtain unilamellar liposome, stored at 4°C.

Maleimide liposomes: Liposomes were prepared essentially as described (McNew et al., 2000b), except for buffer composition. Maleimides react approximately 10,000 faster with sulfhydryls than with amines at neutral pH, leading to their widespread-use as sulfhydryl-specific labeling molecules. However, in the context of a liposome composed of varying and sometimes very high molar concentrations of amine-containing lipids (PE), maleimide reactivity can be somewhat quenched by the existing local pool of amines. To circumvent this problem, we prepared all malemide liposomes at the more acidic pH of 6.2 and at higher lipid concentrations (30 mM), such that final pH in the reaction volumes would still be neutral but during preparation and storage the liposomes could be maintained with reactive maleimides.

In vitro reconstitution of the Atg8–PE conjugation system

To recreate Atg8–PE conjugation *in vitro,* 2.4 mM liposomes of varied composition, 0.5-7.36 μM purified Atg7, 0.5-5.63 μM purified Atg3, and 2.5-12.5 μM purified $Atg8^{G116}$ (Atg8ΔR) were

combined in the presence of SN buffer, 1 mM ATP (Sigma), and 0.2 mM DTT (American Bioanalytical). The reaction mixture was incubated at 30°C for 90 min.

Lipidation of Atg8 with maleimide-PE

To recreate Atg8-PE conjugation *in vitro* without the use of Atg7, Atg3 or PE, purified $Atg8^{G116C}$ was buffer exchanged into 20 mM HEPES, pH 7.4, 150 mM NaCl and 0.2 mM TCEP immediately before use to remove glycerol. The protein was diluted to the indicated concentrations in the same exchange buffer and then mixed with maleimide-containing liposomes at a final lipid concentration of 1 mM. For negative controls, liposomes were preincubated with β-Me (5-20 mM) such that the final β-Me concentration in the coupling reaction was always 1-2 mM. The reaction mixture was incubated at 30°C for 90 min and visualized by SDS-PAGE (below).

Fusion assay

A lipid-mixing assay was performed based on methods detailed by Struck et al. (Struck et al., 1981), using approaches we have published previously for the study of SNARE fusion proteins (Melia et al., 2002; Shen et al., 2007). Acceptor liposomes were composed of 10% PI, 0-55% DOPE, and the resulting percentage of POPC (see *Liposome preparation*). Donor liposomes were identical to acceptor liposomes apart from the additional inclusion of fluorophore-tagged lipids—NBD-DOPE and Rho-DOPE—incorporated in 1% and 1.5% quantities, respectively, to permit nearly complete NBD quenching by means of energy transfer from NBD to Rho.

For $Atg8^{G116}$ (Atg8ΔR) fusion reactions, 1 mM lipid (in a 4:1 ratio of acceptor to donor liposomes), 0.2 mM DTT, 1 mM ATP, 0.5 μM purified Atg7, 0.5 μM purified Atg3, and varying

concentrations of purified Atg8^{G116} (Atg8ΔR) were combined in the presence of SN buffer. Fluorescence was measured with a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices) at 30°C and excitation and emission wavelengths of 460 nm and 538 nm, respectively. After 90 min, n-dodecylmaltoside (Roche) was added to each reaction such that detergent was present in a final concentration of 0.4% w/v. Samples were visualized using Urea-SDS-PAGE (see Gel Electrophoresis). For Atg8^{G116C} fusion reactions, 1 mM lipid (in a 4:1 ratio of acceptor to donor liposomes), 0.2 mM TCEP and varying concentrations of purified Atg8^{G116C} were combined in 20 mM HEPES pH 7.4, 150 mM NaCl and fusion was followed as above.

LC3-malPE Fusion Assay

Lipid preparation

DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), blPI (phosphatidylinositol from bovine liver), maleimide (N-(4-(p-maleimidophenyl)butyramide)-DOPE, NBD (N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-DOPE and Rho (N-(lissamine rhodamine B sulfonyl)-DOPE were all purchased from Avanti and dissolved in chloroform. Lipids were mixed at the specific ratios (as shown in the figure legend), dried under a stream of nitrogen gas followed by 30 min of vacuum. Lipids were rehydrated with 1X reaction buffer (20 mM HEPES, pH 7.5, 100 mM NaCl) for at least 1 h at room temperature. The rehydrated lipids were subjected to three cycles of freeze thaw in liquid nitrogen and smaller liposomes were generated using a mini-extruder (Avanti) with 0.05 um pore-size filters. Unlabeled liposomes contained the following compositions: 5% maleimide-DOPE (molar % of total lipids), 50-0% DOPE, 15% PI, DOPC (to make up to 100% composition), with total lipid concentration of 3.5 mM. Fluorescently labeled liposomes contained an additional 1% NBD-

DOPE and 2% Rho-DOPE. Unlabeled and labeled liposomes were mixed at a ratio of 4:1 prior to the conjugation/fusion reaction. As a control, labeled liposomes were used in a reaction without the addition of unlabeled liposomes.

LC3 conjugation to maleimide-PE

T7-His-tagged human LC3 (beta isoform) recombinant proteins, wild type and LC3C mutant (residue G120 mutated to C), were expressed and purified from bacteria. Proteins were dialyzed against 1X reaction buffer. For LC3–PE chemical conjugation, 6 µl of 20 µM LC3 (wild-type or LC3C mutant, as specified in the individual experiments) was incubated with the indicated liposomes (2 µl of liposomes which contained 3.5 mM total lipids) in 1X reaction buffer to a final volume of 14 µl for 1 h at room temperature with continuous agitation.

Lipid fusion assay to monitor the role of LC3 in hemifusion

The conjugation reaction was stopped by the addition of 6 µl of DTT-containing buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 6.67 mM EGTA, 25 mM $MgCl_2$, 5.8 mM DTT, 33.33 mM ATP). After incubation at 30°C for an additional hour, the fluorescence of each reaction was measured at excitation/emission wavelengths of 485/535 nm respectively.

Gel Electrophoresis

SDS-Page in the presence of 6 M urea was employed to distinguish $Atg8^{G116}$ (Atg8ΔR) from the Atg8–PE conjugate as follows: samples were combined with 1X Sample Buffer (50 mM Tris, pH 6.8, 1% SDS, 2.5% β-Me, 7.5% glycerol, 0.1% bromophenol blue, 4 M urea), boiled at 90°C for 5 min and run on a 13% polyacrylamide gel that contained 10% SDS and 6 M urea in the

presence of 1X Running Buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) at 100V. Gels were stained with Coomassie Blue stain per the manufacturer's instruction (Imperial Protein Stain, Thermo Scientific).

To visualize protein reaction components from maleimide coupled $Atg8^{G116C}$, samples were subjected to SDS-PAGE performed as follows: samples were combined with 1X LDS Sample Buffer (NuPage, Invitrogen), boiled at 90°C for 5 min and run on a 12.5% Tris gel (Nupage, Invitrogen) in the presence of 1X MES SDS Running Buffer (NuPage, Invitrogen) at 200V. Gels were stained with Coomassie stain per the manufacturer's instruction (Imperial Protein Stain, Thermo Scientific).

In Vitro Liposome Fusion to Test Tlg2

GST-Sso1 and GST-Sec9c were mixed in octylglucoside and reconstituted into PCPS liposomes. Snc2-His_6 and His_6-Tlg2 modified to contain a thrombin cleavage site between the H3 SNARE core domain and N-terminal regulatory domain (Paumet et al., 2005) were reconstituted into fluorescently labeled liposomes by detergent dilution and dialysis as previously described (McNew et al., 2000a). Some of the Tlg2 proteliposomes were treated with thrombin to generate H3-Tlg2p. Unlabelled t-SNARE proteoliposomes were mixed with labeled v-SNARE proteoliposomes in approximately a 7:1 ratio and incubated at 37°C in a Flouroskan II plate reader, and NBD fluorescence was determined at 2 min intervals. Detergent (dodecylmaltoside) was added at 120 min to infinitely dilute the fluorescent probes and determined maximum fluorescence.

Protein A Affinity Isolation

Cells were grown to OD_{600} = 1 in SMG selective medium at 30°C. 200 ml of cells were collected by centrifugation, washed with SG-N medium two times, and incubated with shaking in 200 ml of SG-N for 1 h. Cells were collected by centrifugation and resuspended in SP buffer (1 M sorbitol and 20 mM PIPES, pH 6.8) at a concentration of 20 OD_{600} units of cells/ml of SP buffer (Krick et al., 2010). To induce spheroplast formation, 20 units of zymolyase (Zymo Research, Orange, CA) was added to the cells, which were incubated with gentle shaking (180 RPM) for 30 min, in a 30°C water bath. Spheroplasts were collected by centrifugation, and resuspended in SP buffer at a concentration of 5 OD_{600} units/ml, and DSP (dithiobis succinimidyl propionate) (Pierce, Rockford, IL) cross-linker was added to a final concentration of 200 μg/ml, followed by incubation at room temperature for 30 min at 80 RPM on an air shaker (Snyder et al., 2000). The cross-linker was quenched by the addition of Tris-HCl (pH 8.0) to a final concentration of 0.1 M and the cells were incubated for 15 min at room temperature at 80 RPM (Ames et al., 2002). Spheroplasts were collected by centrifugation and protein A affinity purification was carried out as described previously (Cheong et al., 2008). After elution of proteins from IgG Sepharose beads, 2 μl of 14 M β-Me was added to the eluate, and incubation was continued at 37°C for 30 min to reverse cross-linking. The eluates were resolved by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon polyvinylidene difluoride (IPVH00010; Millipore, Billerica, MA) and immunoblotted with polyclonal anti-GST antibody (Invitrogen, Carlsbad, CA) or monoclonal anti-YFP antibody (Clontech).

Immuno-EM

For the immuno-EM to observe Atg9-GFP, cells were grown in rich medium or incubated under starvation conditions, at both PT and NPT. Aliquots were then chemically fixed, embedded in

12% gelatin and cryo-sectioned as described previously (Griffith and Reggiori, 2009). Cryo-sections were immuno-gold-labeled using a rabbit anti-GFP (Abcam, Cambridge, United Kingdom) or an anti-Ape1 (a kind gift of I. Sandoval and M. Mazón, Universidad Autónoma de Madrid) antiserum and PA-gold 10 nm before being stained and finally viewed under a JEOL 1010 electron microscope (JEOL, Tokyo, Japan). The observed immunological reactions were specific because cryo-sections from cells not expressing GFP or Ape1 were not labeled.

For visualizing GFP-Atg8 by immuno-EM, essentially the same protocol was followed as published previously (Yen et al., 2010) with minor modifications: Cells were frozen in a freezing device (EM CPC; Leica), and ultrathin sections were examined using an electron microscope (H-8100; Hitachi High Technologies) at 100 kV.

Other Methods

Pho8Δ60 assay, GFP-Atg8 processing, fluorescence microscopy, radioactive pulse/chase, protease protection and EM analysis have been described previously (Geng et al., 2010; Krick et al., 2010; Noda et al., 1995; Yen et al., 2010). For the GFP-Atg8 processing and Pho8Δ60 assays, cells were cultured in rich medium at 24°C to mid-log phase. For each strain, half of the culture was shifted to 34°C for 30 min, whereas the rest remained at 24°C. Cells were then starved for 2 h at the same temperature, and samples were collected before and after starvation. For recovery (R), cells starved at 34°C were shifted back to 24°C and starved for another 2 h. Immunoblotting was performed with anti-YFP antibody that recognizes GFP. For the pulse/chase analysis, cells were grown to midlog phase at 24°C in rich medium. After incubation at 34°C for 30 min, cells were labeled, subjected to a nonradioactive chase and immunoprecipitated with anti-Ape1 antiserum. For the EM analysis, cells were cultured in rich medium at 24°C until mid-

log phase. After the inactivation of Sso1 for 30 min at 34°C, the cells were shifted to nitrogen-starvation medium for 1.5 h and samples prepared for EM.

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