Supplemental Figure Legends

Figure S1. Structurally annotated sequence alignment of Atg17. Abbreviations used: SACE: *Saccharomyces cerevisiae*, KLTH: *Kluyveromyces thermotolerans*, and KLLA*: Kluyveromyces lactis.* Related to Figure 1.

Figure S2. Structurally annotated sequence alignment of Atg31 (A) and Atg 29 (B). The placement of helix α2 and α3 in Atg29 is approximated based on secondary structure prediction and the register of α1, as the electron density for α2 and α3 lacked side-chain definition. Abbreviations used: SACE: *Saccharomyces cerevisiae*, KLTH: *Kluyveromyces thermotolerans*, and KLLA*: Kluyveromyces lactis.* Related to Figure 1.

Figure S3. Anomalous difference map contoured at 4 σ, illustrating the two additional Met residues (L87M and L110M) that were mutated in Atg31 to verify the register of the sequence. Related to Figure 1.

Figure S4. Pho8Δ60 assay to monitor autophagy was performed in the absence (white) and presence (grey) of rapamycin treatment for 6 hours. S. cerevisiae Atg31 residues 175-196 correspond to the K. lactis Atg31 residues 123-145 studied in the biochemical truncation experiment. Samples were normalized to the activity of Atg31 in rapamycin treated cells. Related to Figure 2.

Table S1. Crystallographic Data Collection and Refinement Statistics

	SeMet-1	**SeMet-2**
Space Group	$P2_1$	$P2_1$
Cell Dimensions a, b, c (Å) α, β, γ (deg)	 146.2, 64.1, 184.9 90, 110.6, 90	 144.4, 64.2, 184.2 90, 110.8, 90
Resolution	50 – 4.0 Å	50 – 3.05Å
R Merge	0.321(0.438)	0.084 (0.217)
I/σ(I)	21.5 (6)	14.8 (3.6)
Completeness (%)	87.7 (65.6)	77.3 (19.4)*
Redundancy	3.1 (2.7)	5.9 (3.2)
Structure Refinement		
Resolution Range		50- 3.05Å
Reflections used		60710
R_{work} /R_{free}		30.6/33.4
Rms Deviations		
Bond lengths (Å)		0.021
Bond Angles (deg)		1.757

Anisotropic data were cut off at 3.3 Å in b and 3.05 Å in both a* and c*. Data are 99% complete to 3.8 Å.

Related to Figure 1.

Table S2. Light scattering table

	Expected Monomer MW	Expected Dimer MW	Experimental MW
Atg17-Atg31-Atg29	8.0×10^4	1.6×10^5	1.8×10^5
Atg17Δ1- Atg31-Atg29	7.0×10^4	1.4×10^5	9.0×10^4
Atg17Δ2-Atg31-Atg29	6.9×10^4	1.4×10^5	8.8×10^4
Atg17Δ3-Atg31-Atg29	6.8×10^4	1.4×10^4	8.7×10^4
EAT domain	3.1×10^4	6.2×10^4	6.8×10^4

Related to Figure 3.

Table S3. SAXS measurements.

Sample	Concentration	R_g (Å)	D_{max} (Å)	R_c (Å)
1	0.53 mg ml^{-1}	100.6	310	16.6
2	0.84 mg ml^{-1}	102.9	310	16.2
3	2.3 mg ml^{-1}	101.3	310	16.2
Dimer 1		102.7	343.0	13.3
Dimer 2		61.0	309.4	21.4
Dimer 3		60.8	268.2	21.9

Related to Figure 3.

Table S4. Yeast Strains Used in this Study

Strain	Descriptive name	Genotype	Source or Ref.
HAY1135	*GFP-ATG8*	*MATa leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 atg8::GFP-ATG8*	R. Youle
MJR9	*atg17Δ pho8Δ60*	TN124 *atg17Δ::KAN*	This study
MJR10	*atg11Δ atg17Δ GFP-ATG8*	HAY1135 *atg11Δ::TRP1 atg17Δ::NAT*	This study
MJR13	*atg31Δ pho8Δ60*	TN124 *atg31Δ::TRP1*	This study
TN124	*pho8Δ60*	*MATa leu2-3, 112 trp1 ura3-52 pho8::pho8Δ 60 pho13Δ::LEU2*	Scott et al., 1996
YSC1021-554580	*atg17Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 atg17Δ::KAN*	Open Biosystems

Related to Figure 5.

Supplemental Experimental Procedures

Protein Expression and Purification of the Atg17-Atg31-Atg29 Complex

Proteins from three different species were used in this study, from the budding yeasts *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and *Lachancea thermotolerans* because each set of proteins had unique advantages for various purposes. *S. cerevisiae* was used for initial biochemical studies. *K. lactis* was used for in vitro dimerization studies, light scattering, and liposome sedimentation experiments, and *L. thermotolerans* was used for crystallization and SAXS. The same general cloning, expression and purification protocol was used for all three species.

Saccharomyces cerevisiae, *Kluyveromyces lactis*, and *Lachancea thermotolerans* Atg17, Atg29, and Atg31 were subcloned into the multicassette vector pST39 (Tan, 2001), with a His_6 tag on either the C-terminus of Atg31 or Atg29. A mutant of *L. thermotolerans* Atg31 that contained two additional Met residues was purchased from Genescript. The ternary complex was expressed in *E. coli* BL21 DE3 cells. Cells were grown to log phase and then induced with 0.75 mM IPTG for 3 hours at 37^{o} C. The cells were lysed by either sonication or high pressure homogenization in a buffer containing 50 mM Tris pH 8.0, 500 mM NaCl and an EDTA-free complete protease inhibitor tablet (Roche). The lysate was clarified by centrifugation and then affinity purified using either Ni-NTA or Talon affinity resin. The protein was further purified by size exclusion chromatography (SEC; Superose 6) in a buffer containing 20 mM Tris pH 8.0, 200 mM NaCl, and 0.2 mM TCEP. The selenomethionyl (SeMet) protein was expressed in *E. coli* BL21(DE3) cells in minimal media supplemented with SeMet and the protein was purified as specified above.

Crystallization of Atg17-Atg31-Atg29

Native crystals of *L. thermotolerans* Atg17-Atg31-Atg29 were grown at 21^{o} C by either hanging drop vapor diffusion or microbatch. For vapor diffusion, 1-2 μl of protein (2 mg ml^{-1}) were combined with 1-2 μl of well solution containing 50 mM Tris pH 8.0, 4-10% Peg 2KMME, 10-20% ethylene glycol, and 100 mM NaCl. Microbatch crystals were grown by mixing 1-2 μl of protein with 1-2 μl of 50 mM Tris pH 8.0, 4-10% PEG 2KMME, 10-20% ethylene glycol, and 100 mM NaCl and then covering the drop with 20 μl of paraffin oil. Crystals were further improved by subsequent rounds of microseeding and grew to final dimensions of up to 0.5 x 0.3

x 0.2 mm. Selenomethionyl crystals were obtained as described above with the exception of the inclusion of 1-5 mM TCEP in the well solution. Crystals were cryo protected by increasing the concentration of ethylene glycol to 25% step-wise prior to flash freezing in liquid nitrogen.

Small angle X-ray scattering

Small angle X-ray scattering (SAXS) data were recorded on Atg17-Atg31-Atg29 at APS beamline 12ID-B at Argonne National Laboratory. Samples were prepared in 20 mM Tris pH 8.0, 200 mM NaCl, 0.2 mM TCEP and concentrated to 0.5, 0.8, and 2.3 mg ml^{-1}, snap frozen in liquid nitrogen and stored at -80^{o} C until data collection. Prior to scattering experiments the samples were thawed on ice and centrifuged at 16,000 x g for 10 min at 4^{o} C. 100 µl of sample was continuously flowed through a 1.5 mm diameter quartz capillary with a 10 µm wall during the experiment. 5 µl of sample was exposed to the X-ray beam for 2 seconds, and twenty independent scattering trials were recorded and averaged per experiment. Data were recorded on a Pilatus 2M detector at 2.1 m from the sample to provide a q range of 0.005-0.7 $Å^{-1}$. The radius of gyration of the cross section (R_c) of a rod-like particle was calculated using $qI(q) = qI(0)\exp(-\frac{1}{2}q^2R_c^2)$ where the slope of the linear portion of a plot of ln $qI(q)$ and q^2 is R_c^2 (Koch et al., 2003). PRIMUS was used for data visualization and determination of R_c (Konarev et al., 2003). The similarity of R_c values for all concentrations measured was used to confirm that no aggregation was present throughout the experiment. HYDROPRO (Ortega et al., 2011) was used to calculate the R_g for each possible dimer. Theoretical scattering curves were calculated for each possible dimer using FOXS (Schneidman-Duhovny et al., 2010). $P(r)$ functions were calculated using GNOM for the three experimental scattering curves as well as the three possible dimers (Svergun, 1992).

Expression and Purification of the EAT domain

K. lactis Atg1 residues 562-831 were cloned into the pHIS2 vector (Sheffield et al., 1999) in frame with the N-terminal His_6 tag and TEV cleavage site. The EAT domain was expressed in *E. coli* BL21 DE3 cells, which were grown to log phase and induced with 0.75 mM IPTG for 3 hours at 37^{o} C. The cells were lysed by sonication in a buffer containing 50 mM Tris pH 8.0, 500 mM NaCl and an EDTA-free complete protease inhibitor tablet (Roche). The lysate was clarified by centrifugation and then affinity purified using NiNTA affinity resin. The His_6 tag

was removed by overnight digestion with TEV protease followed by SEC (Superdex 75) in a buffer containing 20 mM Tris pH 8.0, and 200 mM NaCl. The fractions containing Atg1 were pooled and then put back over NiNTA affinity resin to remove the TEV protease and any EAT domain still containing the His_6 tag.

Expression and Purification of the mini Atg1-Atg13-Atg17-Atg31-Atg29 complex

K. lactis Atg1 residues 562-831 and Atg13 350-550 were cloned into the pST39 vector (Tan, 2001) with *K. lactis* Atg17-Atg31-Atg29. The Atg1 pentamer was expressed in *E. coli* BL21 DE3 cells, which were grown to log phase and induced with 0.75 mM IPTG for 3 hours at 37^{o} C. The cells were lysed by sonication in a buffer containing 50 mM Tris pH 8.0, 500 mM NaCl and an EDTA-free complete protease inhibitor tablet (Roche). The lysate was clarified by centrifugation and then affinity purified using NiNTA affinity resin followed by SEC (Superose 6) in a buffer containing 20 mM Tris pH 8.0, and 200 mM NaCl.

Light scattering by proteins

Atg17-Atg31-Atg29 samples or EAT domain were centrifuged at 16,100 x g for 10 min at 4°C to remove any aggregation prior to light scattering experiments. 20 µl of samples at 2 mg ml^{-1} for Atg17-Atg31-Atg29 samples or at 8 mg ml^{-1} for EAT domain were injected at a flow rate of 0.5 ml min^{-1} using an Agilent 1200 series HPLC onto a WTC-030S5 size exclusion column placed in line with a Wyatt Dawn Heleos II multi-angle light scattering instrument (658nm laser) and a Wyatt Optilab rEX differential refractometer (Wyatt Technology Corporation). Experiments were carried out in standard PBS buffer, except with the addition of 0.2 mM TCEP in the case of EAT domain. Data were collected and analyzed using Astra v5.3.4.18 (Wyatt Technology Corporation) with the Zimm model for static light scattering data fitting.

Yeast Strains and Plasmids

The *S. cerevisiae* strains used in this study are listed in Table S4. The *S. cerevisiae* strain TN124 (*PHO8Δ60*) was a generous gift from D. Klionsky (Scott et al., 1996) and was modified by replacing *ATG17* with the *KAN* resistance gene from pFA6a-KanMX4 to create MJR9 (*atg17Δ PHO8Δ60*). HAY1135 (*GFP-ATG8*) was a generous gift from R. Youle and was modified by

replacing *ATG17* with the nourseothricin N-acetyl-transferase gene from pAG25 (Goldstein and McCusker, 1999) and *ATG11* with TRP1 from pFA6a-klTRP1-MX4, which was a generous gift from T. R. Serio, to create MJR10 (*atg11Δ atg17Δ GFP-ATG8*). The *S. cerevisiae* strain TN124 was also modified by replacing *ATG31* with TRP1 from pFA6a-klTRP1-MX4. Constructs coding for *ATG17*, *ATG17Δ1*, *ATG17Δ2* and *ATG17Δ3* proteins under the control of the endogenous promoter were cloned from *S. cerevisiae* genomic DNA (Novagen 69240) into pJK59 (Prinz et al., 2000) to produce full length *ATG17* and truncations with a C-terminal GFP tag. *ATG17*, *ATG17Δ1*, *ATG17Δ2* and *ATG17Δ3*, together with the endogenous promoter and terminator were cloned from *S. cerevisiae* genomic DNA (Novagen 69240) into the centromeric plasmids yCPLAC111 and yCPLAC33 (Gietz and Sugino, 1988). *ATG31* and $ATG31^{\Delta 175\text{-}196}$ together with the endogenous promoter and terminator were cloned from *S. cerevisiae* genomic DNA (Novagen 69240) into the centromeric plasmid yCPLAC33 (Gietz and Sugino, 1988).

Autophagy assays

For analysis of Atg17 localization, YSC1021-554580 (Open Biosystems) was transformed with *ATG17-GFP*, *ATG17Δ1-GFP*, *ATG17Δ2-GFP*, or *ATG17Δ3-GFP* subcloned into pJK59 and grown to mid log phase in SMD (0.67% yeast nitrogen base with ammonium sulfate, 2% glucose, supplemented with the appropriate amino acids). Cells were visualized at mid-log phase except for cells treated with rapamycin. In those cases, rapamycin was added to a final concentration of 0.2 μg ml^{-1}. After rapamycin treatment, cells were incubated at 30^{o} C shaking for 3 hours and then visualized. For Atg17-GFP puncta counting, 100 cells were counted, and the final experiment is the result of three independent trials. For quantification of wild-type and truncated Atg17 expression levels, cells were harvested, resuspended in 50 mM Tris (pH 8.0), 1% SDS, 6 M urea, 1 mM EDTA, and lysed by the addition of a half volume of 425–600 mm glass beads (Sigma G8772) and vortexed vigorously at 4^{o} C. Cell lysates were subjected to western blot analysis using a GFP antibody (Santa Cruz sc9996). For the Pho8Δ60 assays, MJR9 cells containing yCPLAC33, *ATG17*-yCPLAC33, *ATG17Δ1*-yCPLAC33, *ATG17Δ2*-yCPLAC33, or *ATG17Δ3*-yCPLAC33 or MJR13 cells containing yCPLAC33, *ATG31*-yCPLAC33, or $ATG31^{\Delta 175\text{-}196}$ -yCPLAC33 were grown to mid log phase in SMD. Cells were

treated with 0.2 μg ml^{-1} rapamycin for 3 hr (MJR9) or 6 hr (MJR13) and the Pho8Δ60 alkaline phosphatase assay was performed as previously described (Klionsky, 2007). For GFP-Atg8 processing assay and microscopy MJR10 cells containing yCPLAC111, *ATG17*-yCPLAC111, *ATG17Δ1*-yCPLAC111, *ATG17Δ2*-yCPLAC111, or *ATG17Δ3*- yCPLAC111 were grown to mid log phase in SMD Cells were treated with 0.2 μg ml^{-1} rapamycin for 3 hr for GFP-Atg8 processing or 1 hour for GFP-Atg8 fluorescence microscopy. For GFP-Atg8 processing, cells were harvested, resuspended in 50 mM Tris (pH 8.0), 1% SDS, 6 M urea, 1 mM EDTA, and lysed by the addition of a half volume of 425–600 mm glass beads (Sigma G8772) and vigorous vortexing at 4^{o} C. Cell lysates were subjected to western blot analysis using an anti-GFP antibody (Santa Cruz sc9996). All microscopy was performed on a LSM780 scanning confocal microscope (Carl Zeiss Microscopy) with a 1003 oil immersion objective.

Liposome sedimentation assays

Folch fraction I (Sigma) lipids were dissolved in chloroform. Lipisomes mixtures representing the lipid content of S. cerevisiae plasma membrane, endoplasmic reticulum and golgi were made by mixing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE), L-α-phosphatidylinositol (PI), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (PS) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA) dissolved in chloroform at the following molar ratios (Zinser et al 1991, Klem et al 2009). Plasma membrane-mimetic liposomes contained 24.5% PC, 20.3% PE, 17.7% PI, 33.6% PS and 3.9% PA. The endoplasmic reticulum mixture contained 52.5% PC, 33.4% PE, 7.5% PI, and 6.6% PS. Golgi liposomes contained 40% PC, 13.5% PE, 38.5% PI, 4% PS, and 4% PA. One percent of the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethlyindodicarbocyanine (DID) was added to aid in the visualization of the lipids. The chloroform was removed by evaporation under a stream of dry nitrogen followed by overnight incubation under vacuum. Lipds were hydrated in 20 mM Tris pH 8, 200 mM NaCl for 1 hour on ice and the resulting multilamellar vesicles (MLVs) were resuspended by vigorous vortexing. To make large unilamellar vesicles (LUVs), MLVs were passed through an extruder 11 times, with the appropriate sized filter. Small unilamellar vesicles (SUVs) were prepared by sonication of the MLVs on ice until the solution appeared clear. The protein concentration used for sedimentation assays was 10 μM and the concentration of Liposomes was 2.5 mg ml^{-1}. For each sedimentation assay, 25 μl of protein was mixed with 25

µl liposomes and then incubated at room temperature for 30 minutes. The liposomes were pelleted in an ultracentrifuge (SLA55 rotor) for 1 hour at 50,000 rpm, and the presence of protein in the pellet and supernatant fractions was analyzed by SDS-PAGE.

Liposome Tethering Assays

SUVs mimicking the plasma membrane from S. cerevisiae were prepared with the addition of DSPE-Peg2000-Biotin and rhodamine-PE (Avanti Polar Lipids) in the PM-Biotin liposomes and the inclusion of the fluorescent dye DID(1,1'-dioctadecyl-3,3,3',3'-tetramethlyindodicarbocyanine) in the PM-DID liposomes. The protein concentration used for tethering assays was 10 µM and the total concentration of liposomes was 2.5 mg ml^{-1} including 1.25 mg ml^{-1} PM-DID liposomes and 1.25 mg ml^{-1} PM-Biotin liposomes. For each assay 25 µl of protein was mixed with 25 µl of the liposome mixture and then incubated at room temperature for 30 minutes. The reactions were then incubated with 50 µl of pre-equilibrated Mag-Strep resin (Novagen) at 4^{o} C for 30 minutes and washed extensively with wash buffer (20 mM Tris pH 8, 200 mM NaCl). Liposomes and protein remaining bound to the resin was analyzed by SDS-PAGE. The resulting gels were imaged with a Typhoon Scanner and the data was processed with Image Quant V. 5.1. Each sample was normalized based upon the rhodamine fluorescence to correct for the total amount of lipid bound to the resin.

SUVs mimicking the plasma membrane of S. cerevisiae were prepared as described earlier without the addition of DID. 50 µl of 2.5 mg ml^{-1} liposomes was mixed with 20 µl of 10 µM protein or 20 µl buffer (20mM Tris pH 8.0 200 mM NaCl). The mixture was incubated for 30 min at room temperature and diluted to 500 µl in 20mM Tris pH 8.0 200 mM NaCl for light scattering. Dynamic light scattering was carried out at room temperature on a Brookhaven Instruments Corporation BI-200 goniometer coupled to a BI-9000 AT autocorrelator and Lexel Model 95 argon ion laser operating at 514.5 nm. A series of autocorrelation functions were collected for 6 minutes at 90.0° with sampling times of 1.0 µs to 100 ms, a laser intensity of 300 mW and photomultiplier tube aperture of 100 µm such that count rates ranged from 0.3 to 1.0 MHz. Data analysis was carried out in real time using the Brookhaven Instruments 9KDLSW 2.12 software package.

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