Figures and figure supplements

Two distinct mechanisms target the autophagy-related E3 complex to the pre-autophagosomal structure

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Figure 1. Atg12- and PI3P-dependent mechanisms cooperatively act to recruit the Atg16 complex to the PAS. Cells expressing Atg5-GFP and Atg17-mCherry were treated with rapamycin for 90 min, and analyzed by

Figure 1 continued on next page
Figure 1 continued

fluorescence microscopy. DIC, Differential interference contrast microscopy. Bars, 5 μm. The ratios of Atg17-mCherry puncta positive for Atg5-GFP to total Atg17-mCherry puncta were calculated, and the mean values are shown with standard deviations (n = 3). **p<0.01; ***p<0.001 (unpaired two-tailed Student’s t-test).

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Figure 2. The Atg16 complex interacts with the Atg1 complex. (A–C, E) Yeast cells expressing Atg5-FLAG (A, C–E) or Atg16-FLAG (B) from each chromosomal locus were treated with rapamycin for 2 hr, and subjected to immunoprecipitation using anti-FLAG antibody. The immunoprecipitates continued on next page.
were analyzed by immunoblotting using antibodies against FLAG (A, B), Atg12 (C, E), Atg17 (A–C, E), and Atg1 (C). (D) atg13Δ cells expressing wild-type Atg13, the F375A mutant, or the F430A mutant from centromeric plasmids were treated with rapamycin for 2 hr, subjected to immunoprecipitation using anti-FLAG antibody, and the immunoprecipitates were analyzed by immunoblotting using antibodies against Atg12, Atg13 and Atg17. (F) Yeast cells were treated with or without rapamycin for 2 hr, and coimmunoprecipitation of Atg17 with Atg5-FLAG was examined as described in Figure 2C. (G) Coimmunoprecipitation of Atg17 with Atg5-FLAG was analyzed in cells expressing wild-type Atg1 or the D211A mutant from the original chromosomal locus as described in Figure 2C.

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Figure 2—figure supplement 1. Proteomic analysis to identify proteins bound to the Atg16 complex. (A) Yeast cells expressing Atg5-FLAG were converted to spheroplasts, treated with rapamycin for 2 hr, solubilized with 1% DDM, and subjected to immunoprecipitation using anti-FLAG antibody. Immunoprecipitates were analyzed by SDS-PAGE, followed by SYPRO Ruby staining. (B) Immunoprecipitates analyzed in (A) were subjected to mass spectrometry, and the MASCOT scores for Atg proteins identified are shown. Proteins with gray background are components of the Atg1 complex. (C) Immunoprecipitates of Atg5-FLAG were prepared as described in (A), and analyzed by immunoblotting using antibodies against Atg12, Atg1, Atg13, Atg17, Atg29 and Atg31.

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Figure 2—figure supplement 2. Atg17 interacts with Atg12. (A–C) AH109 cells expressing Atg proteins fused with the Gal4 activation domain (GAD) or Gal4 DNA-binding domain (GBD) as indicated were grown at 30°C for 3 days.
on SC agar plates lacking leucine and tryptophan for the maintenance of the GAD and GBD plasmids, respectively (-LW), and additionally either histidine (-LWH) or adenine (-LWA) for the assessment of interactions. Cells carrying pGBD-ATG8 and pGAD-ATG19 serve as a positive control. pGAD-C1 and pGBD-C1 were used as vector controls. (D) atg1Δ atg13Δ atg29Δ atg31Δ cells expressing both ATG17 and ATG12-GFP with the ADH1 promoter (P_{ADH1}) were treated with rapamycin for 2 hr, subjected to immunoprecipitation using GFP-binding protein-conjugated beads, and the immunoprecipitates were analyzed by immunoblotting using antibodies against Atg12 and Atg17.

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Figure 3. The interaction of the Atg16 complex with the Atg1 complex is involved in the PAS targeting of the Atg16 complex. (A) atg12Δ cells expressing wild-type Atg12 or Atg12^N56 from centromeric plasmids were treated...
Figure 3 continued

with rapamycin for 2 hr, and examined for coimmunoprecipitation of Atg17 with Atg5-FLAG as described in Figure 2C. The upper and middle panels were immunoblots obtained using antibodies against Atg5 and FLAG, respectively. Asterisk, non-specific bands. (B) Yeast cells were treated with rapamycin for 2 hr, and the PAS localization of Atg5-GFP was assessed by fluorescence microscopy as described in Figure 1. **p<0.01; ***p<0.001 (unpaired two-tailed Student’s t-test). (C) atg12Δ and atg12Δ atg21Δ cells expressing wild-type Atg12 or Atg12ΔN56 from centromeric plasmids were grown in nutrient-rich medium (open bars) and then starved in SD-N medium for 4 hr (closed bars), and their autophagic activities were evaluated by ALP assay. The mean values are shown with standard deviations (n = 3). *p<0.05; **p<0.01 (unpaired two-tailed Student’s t-test).

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Figure 3—figure supplement 1. The N-terminal region of Atg12 is not required for the E3 activity of the Atg12-Atg5 conjugate. Recombinant proteins and liposomes (20 mol% DOPE, 70 mol% POPC, and 10 mol% yeast PI) were prepared as described previously (Nakatogawa et al., 2007; Sakoh-Nakatogawa et al., 2013). Atg8 (5 μM), Atg7 (1 μM), Atg3 (1 μM), ATP (1 mM), and liposomes (350 μM lipids) were incubated with wild-type Atg12-Atg5 or Atg12ΔN70-Atg5 (0.2 μM) at 30°C for the indicated time periods, and analyzed by urea-SDS-PAGE and subsequent CBB staining. Protein bands of Atg12-Atg5 are not visible in this analysis due to its low concentration.

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Figure 4. The Atg16 complex recruited via the association with the Atg1 complex facilitates Atg8 lipidation and PAS scaffold assembly. (A) Yeast cells were incubated in nitrogen starvation medium and examined for the production of Atg8-PE by urea-SDS-PAGE and immunoblotting using anti-Atg8.
antibodies (see Materials and methods). The ratio of Atg8-PE to total Atg8 was calculated, and the mean values are shown with standard deviations (n = 3). *p<0.05; **p<0.01 (unpaired two-tailed Student’s t-test). Pgk1 serves as a loading control. (B and C) Yeast cells expressing Atg17-GFP were treated with rapamycin for 90 min (B) or 2 hr (C), and observed under a fluorescence microscope. The proportion of cells containing Atg17-GFP puncta to total cells was calculated, and the mean values are shown with standard deviations (n = 3). **p<0.01 (unpaired two-tailed Student’s t-test).

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Figure 5. Model for the PAS recruitment of the Atg16 complex. The Atg16 complex is recruited to the PAS through two different pathways (Atg12-dependent targeting and PI3P-dependent targeting). Upon autophagy induction (starvation or TORC1 inactivation), the Atg1 complex is assembled, and multiple copies of Atg1 complexes further form a higher order assembly. During the process, the Atg16 complex associates with Atg1 complexes via the N-terminal region of Atg12, promoting PAS scaffold assembly. The Atg16 complex recruited at this stage also facilitates lipidation of Atg8 at a later stage in PAS organization (dashed arrow). As reported previously, following the recruitment of PI3K complex I and the production of PI3P by this complex, the Atg16 complex localizes to the PAS via the interaction with the PI3P-binding protein Atg21 to stimulate Atg8 lipidation.

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