Cdc48/p97 and Shp1/p47 regulate autophagosome biogenesis in concert with ubiquitin-like Atg8

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Introduction

Macrouthagy sequesters superfluous cytosol and organelles into double-membraned autophagosomes, which finally fuse with lysosomes for degradation. Despite identification of >30 Atg (autophagy) genes, the molecular mechanism of autophagosome biogenesis is poorly understood (Mizushima et al., 2008; Farré et al., 2009; Nakatogawa et al., 2009). All proposed models predict elongation and final closure or cisternal assembly of double-membraned precursors during autophagosome formation (Axe et al., 2008; Longatti and Tooze, 2009). Previous studies assumed that the autophagic machinery mediates the required membrane fusions independent from SNAREs (Reggiori et al., 2004) and identified the ubiquitin-like protein Atg8 as a key component, especially for elongation of the forming autophagosome (Nakatogawa et al., 2007; Xie et al., 2008). Atg8 is coupled by a ubiquitin-like conjugation system to phosphatidylethanolamine (PE; Ichimura et al., 2000). In vitro Atg8-PE induced liposome tethering and hemifusion (Nakatogawa et al., 2007). However, it remained open how Atg8 mediates membrane fusion and why macroautophagy needs a ubiquitin-like protein.

Autophagosome biogenesis is morphologically reminiscent of nuclear envelope expansion and postmitotic Golgi reassembly. The mammalian AAA-ATPase p97/VCP is a multifunctional enzyme in the ubiquitin–proteasome pathway; for example, in ER-associated degradation (ERAD) and ubiquitin fusion degradation (for reviews see Jentsch and Rumpf, 2007; Meyer and Popp, 2008). Some experiments suggested that p97 only handles ubiquitinated proteins (Dalal et al., 2004); others...
reported a direct function in membrane fusion (for reviews see Jentsch and Rumpf, 2007; Meyer and Popp, 2008). p97 mediates multiple functions by interacting with numerous ubiquitin-binding adaptors. During Golgi regrowth, p97 binds via p47 to an unknown monoubiquitinated fusion regulator, which prevents untimely SNARE pairing. As an ATPase, p97 then segregates the ubiquitin conjugate from the SNARE to allow fusion. Finally, deubiquitination by the cysteine protease VCP135 is essential (Kondo et al., 1997; Uchiyama et al., 2002; Wang et al., 2004). In this paper, we identify Cdc48 and Shp1/Ubx1, the yeast homologues of p97 and p47, as novel components of autophagosome biogenesis in Saccharomyces cerevisiae. We found no requirement of ubiquitination or the proteasome system for macroautophagy but show interaction of Atg8, which is dependent on an FK motif in its non–ubiquitin-like N-terminal helical domain (NHD), with Shp1. Based on our data, we speculate that S. cerevisiae autophagosome formation uses a protein complex analogous to that mediating mammalian nuclear envelope growth and Golgi reassembly with the distinction that Atg8 replaces ubiquitin. The cysteine protease Atg4 would then be equivalent to VCP135. Our model would explain why efficient macroautophagy requires the ubiquitin-fold Atg8- and Atg4-dependent delipidation of Atg8-PE.

Results and discussion

Cdc48 and its cofactor Shp1/Ubx1 are essential for macroautophagy and micronucleophagy

Cdc48 is essential for viability; we thus used temperature-sensitive cdc48-3 mutant cells (Latterich et al., 1995). We measured macroautophagy with a standard assay (Meiling-Wesse et al., 2002; Cheong and Klionsky, 2008). In addition to elongation of growing autophagosome membranes, Atg8 is involved in cargo recognition. Accordingly, macroautophagy selectively targets part of GFP-Atg8 to vacuoles, where degradation yields proteolysis-resistant GFP. Increasing GFP levels in immunoblots therefore reflects the macroautophagic rate. At the permissive temperature, starved cdc48-3 cells showed normal macroautophagy, and shift to nonpermissive 38°C severely blocked macroautophagy (Fig. 1, a and b). Cellular survival was unaffected at 38°C. To exclude strain-dependent effects, we repeated the experiment in another genetic background (unpublished data). At 23 or 38°C, no free GFP appeared in autophagy-deficient atg1Δ cells (Fig. 1, a and b).

Cdc48/p97 is expected to extract proteins from protein complexes or membranes during membrane fusions and other processes (for reviews see Jentsch and Rumpf, 2007; Meyer and Popp, 2008). To mediate its divergent roles, it associates with numerous substrate-recruiting and -processing cofactors (Jentsch and Rumpf, 2007; Schuberth and Buchberger, 2008). The Ubx domain proteins are Cdc48/p97 regulators involved in substrate recruitment (Schuberth et al., 2004). S. cerevisiae has seven Ubx proteins, with Shp1/Ubx1 being the mammalian p47 homologue. The GFP-Atg8 degradation assay showed block of starvation-induced macroautophagy in shp1Δ cells but not in cells lacking any other Ubx protein (Fig. 1 c). As a second assay for nonselective macroautophagy, we expressed 3-phosphoglycerate kinase (Pgk1) fused to GFP (Pgk1-GFP) and followed with immunoblot generation of GFP by proteolysis. The lack of GFP in atg1Δ cells confirmed autophagy dependence of GFP formation. shp1Δ cells were defective in the macroautophagic breakdown of this cytosolic marker (Fig. S1 a). During starvation with the proteinase B inhibitor PMSF, autophagic bodies accumulate in the vacuoles of wild-type, but not of autophagy-deficient, cells. Light microscopy showed that shp1Δ cells failed to accumulate autophagic bodies in the vacuole, further supporting a defect in autophagosome formation or their vacuolar fusion (Fig. S1 b).

We next assessed the requirement of Cdc48 and Shp1 for selective autophagy. Piecemeal microautophagy of the nucleus (PMN) requires the core Atg proteins (Krick et al., 2008). It occurs at nucleus–vacuole junctions formed by the interaction of Vac8, Nvj1, Tsc13, and Osh1 (Roberts et al., 2003). The microautophagic rate can be monitored in immunoblots after generation of proteolysis-resistant GFP through vacuolar breakdown of GFP-Osh1 (Krick et al., 2008). cdc48-3 cells at the nonpermissive temperature and shp1Δ cells, but not other ubx mutants, showed defective PMN (Fig. 1, d and e). The cytoplasm to vacuole targeting (Cvt) pathway, as a selective macroautophagy pathway, delivers proaminopeptidase I to the vacuole under nonstarvation conditions. In shp1Δ and cdc48-3 cells, mature aminopeptidase I formed even at nonpermissive temperature (Fig. 1, a and d; and Fig. S1, a and c).

Functionality of the Cvt pathway in shp1Δ and cdc48-3 cells seems surprising at first glance, because, as shown subsequently, Atg8 interacts with Shp1. However, for unknown reasons, the requirement of Atg8 differs between the Cvt pathway and macroautophagy. In Atg8-deficient cells the Cvt pathway is blocked, whereas few aberrantly small autophagosomes still form during starvation induction of macroautophagy (Abeliovich et al., 2000; Chang and Huang, 2007). Indeed, Atg8 is crucial for control of autophagosomal size (Nakatogawa et al., 2007; Xie et al., 2008). Because few autophagosomes are sufficient for proaminopeptidase I transport (Suzuki et al., 2002), we anticipate either that Cdc48 and Shp1 predominantly affect elongation of autophagosomes or that few aberrant autophagosomes even form without their action.

Macroautophagy does not depend on the ubiquitin–proteasome system

To our knowledge, no precise molecular function was assigned to Shp1; only slight effects on proteasomal degradation of ubiquitinated proteins were reported (Schuberth et al., 2004). We analyzed whether the role of Cdc48 and Shp1 in macroautophagy requires the ubiquitin–proteasome system and respective Cdc48 cofactors. Ufd1 is a crucial substrate-recruiting Cdc48 cofactor (Ye et al., 2001; Jentsch and Rumpf, 2007). In agreement with mutually exclusive binding of Shp1 and Ufd1 to Cdc48, no macroautophagy defect was detectable in ufd1-1 mutants (Fig. 2 a). Macroautophagy was also normal in cells lacking the substrate-processing cofactors Ufd2 and Ufd3 (Fig. 2 a), the ubiquitin ligase Ufd4, the proteasome regulator Ufd5 (Fig. 2 a), the ERAD component Der1 (Fig. 2 c), and the proteasome-deficient prel-1 prel-2
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Macroautophagy and PMN require Cdc48 and Shp1. (a) GFP levels from GFP-Atg8 degradation reflect the autophagic rate. S. cerevisiae cells grown stationary at 23°C were starved at 23 or 38°C and analyzed in immunoblots with antibodies to GFP (top), proaminopeptidase I (middle), and Pgk1 as a loading control (bottom). (b) Quantification of GFP levels, mean and SD, from at least three experiments. (c) Immunoblot measurement of macroautophagy in ubx mutants at 30°C. (d) GFP levels from breakdown of the PMN marker GFP-Osh1 reflect the PMN rate. Cells were treated as in panel a. (e) Measurement of the PMN rate, as in panel d, in ubx mutants at 30°C.

cells (Fig. 2 b). Mammalian VCIP135 is distantly related to yeast Otu1, another Cdc48 substrate-processing cofactor. otu1Δ cells showed normal macroautophagy (Fig. 2 c). These data argue against the need for the ubiquitin–proteasome system in the macroautophagic function of Cdc48 and Shp1. Indeed, overexpression of mutated ubiquitin K48A, which is unable to form polyubiquitin via lysine-48 that is recognized by the proteasome (Chau et al., 1989; Sloper-Mould et al., 2001), did not inhibit macroautophagy (Fig. 2 d). Overexpression of mutated ubiquitin I44A suppressed Golgi reassembly, which is consistent with the proposed extraction of a monoubiquitinated fusion regulator from the membrane by the p97–p47 complex (Wang et al., 2004). However, overexpression of ubiquitin I44A did not affect macroautophagy (Fig. 2 d). To further analyze the requirement of ubiquitination, we used cells lacking the deubiquitinating enzyme Doa4. In doa4Δ cells, processes requiring monoubiquitination,
requires Ypt7. Accordingly, the part of GFP-Atg8 enclosed in autophagosomes is protease protected in ypt7Δ, but not in wild-type, cells because of the rapid vacuolar fusion of autophagosomes (Fig. 3 d). The absence of protease-protected GFP-Atg8 in starved shp1Δ cells indicated defective autophagosome biogenesis or closure (Fig. 3 d). Many S. cerevisiae Atg proteins colocalize at the pre-autophagosomal structure (PAS), the site of autophagosome biogenesis. However, strong cytosolic staining masked detection of Cdc48 and Shp1 at the PAS in direct and indirect fluorescence microscopy. Because Shp1 is dispensable for proaminopeptidase I maturation, it may function in elongation of the isolation membrane, a role proposed for Atg8 (Nakatogawa et al., 2007; Xie et al., 2008). We thus examined whether Shp1 affects localization or lipidation of Atg8. Upon starvation, 42% of shp1Δ and 32% of wild-type cells showed GFP-Atg8–positive PAS punctae (Fig. 3 e), indicating normal Atg8 PAS recruitment. Also, Atg8-PE was formed in shp1Δ cells, and compared with wild type, the Atg8 level was slightly increased, most likely as a result of the autophagic defect (Fig. 3 f).

**Shp1 interacts with Atg8 and Cdc48**

We used the split-ubiquitin system to test interaction of Atg8 with Shp1. We fused the baits with the Nui (N-terminal ubiquitin half) such as the multivesicular bodies pathway, are also affected. doa4Δ cells in two genetic backgrounds showed efficient macroautophagy (Fig. 2 e). In sum, macroautophagy requires Cdc48 and Shp1 independent of ubiquitination and proteasomal degradation.

**Shp1 affects autophagosome biogenesis**

Next, we examined at which step Shp1 affects macroautophagy. The last step is intravacuolar lysis of autophagic bodies dependent on vacuolar acidification and proteinases. Light and electron microscopy showed no vacuolar accumulation of autophagic bodies in starved shp1Δ cells (Fig. 3, a–c). Fluorescence microscopy further demonstrated that, in contrast to wild-type cells, the autophagic cargo GFP-Atg8 did not reach the vacuole in shp1Δ cells (Fig. 3, a and b). GFP-Atg8–positive autophagosomes did not accumulate in the cytosol and were also not detected in shp1Δ cells by electron microscopy (unpublished data). The presence of mature carboxypeptidase Y (Fig. S1 c) in starved shp1Δ cells ruled out that disturbed vacuolar proteolysis caused the GFP-Atg8 degradation defect. Shp1 thus affects either biogenesis of autophagosomes or their vacuolar fusion. We distinguished between these possibilities in a protease protection experiment with spheroplasts hypotonically lysed under conditions that preserved the integrity of autophagosomes. Vacular fusion of autophagosomes requires Ypt7. Accordingly, the part of GFP-Atg8 enclosed in autophagosomes is protease protected in ypt7Δ, but not in wild-type, cells because of the rapid vacuolar fusion of autophagosomes (Fig. 3 d). The absence of protease-protected GFP-Atg8 in starved shp1Δ cells indicated defective autophagosome biogenesis or closure (Fig. 3 d). Many S. cerevisiae Atg proteins colocalize at the pre-autophagosomal structure (PAS), the site of autophagosome biogenesis. However, strong cytosolic staining masked detection of Cdc48 and Shp1 at the PAS in direct and indirect fluorescence microscopy. Because Shp1 is dispensable for proaminopeptidase I maturation, it may function in elongation of the isolation membrane, a role proposed for Atg8 (Nakatogawa et al., 2007; Xie et al., 2008). We thus examined whether Shp1 affects localization or lipidation of Atg8. Upon starvation, 42% of shp1Δ and 32% of wild-type cells showed GFP-Atg8–positive PAS punctae (Fig. 3 e), indicating normal Atg8 PAS recruitment. Also, Atg8-PE was formed in shp1Δ cells, and compared with wild type, the Atg8 level was slightly increased, most likely as a result of the autophagic defect (Fig. 3 f).
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it removes the C-terminal arginine from Atg8-FGR to allow lipiddation, and it delipidates Atg8-PE (Nakatogawa et al., 2009). Therefore, in atg4 cells, Atg8-FGR is unlipidated, whereas Atg8-FG is permanently lipidated. Coimmunoprecipitation of Shp1-HA chromosomally expressed in atg4 cells showed a clear interaction with GFP-Atg8-FG (Fig. 4 c). No clear precipitation of GFP-Atg8-FGR was detectable (unpublished data). This suggests that Shp1 might preferentially interact with Atg8-PE.

We confirmed this interaction in a pull down with GST-Atg8. Incubation of crude extracts of S. cerevisiae cells chromosomally expressing Shp1-HA from its own promoter resulted in strong binding of Shp1-HA to GST-Atg8 but not to GST (Fig. 4 e). Pgk1 did not bind to GST-Atg8 or GST, confirming selective interaction (unpublished data). In mass spectrometry of the

Figure 3. Shp1 affects autophagosome biogenesis.

(a and b) Fluorescence microscopy of starved cells showed defective vacuolar uptake of GFP-Atg8. No GFP-Atg8–positive autophagosomes accumulated in the cytosol. (c) Electron microscopy of starved shp1Δ cells showed no vacuolar accumulation of autophagic bodies. (d) Lysed spheroplasts of starved cells were trypsin digested with and without detergent. Immunobolts with GFP antibodies showed proteolysis-resistant GFP-Atg8 [inside autophagosomes] in ypt7Δ but not in wild-type and shp1Δ cells. GFP-Atg8 breakdown yields GFP*. (e) Cells with a GFP-Atg8–positive PAS punctum were scored in fluorescence microscopy. The mean and SD of two experiments are shown, with >200 cells analyzed per strain. (f) To analyze Atg8-PE, extracts were separated in 6 M urea SDS-PAGE and immunoblotted with anti-Atg8.
We thus analyzed its relevance for interaction with Shp1 by incubating immobilized GST-Atg8-\(\Delta N8\) and GST-Atg8-\(\Delta N24\) with extracts from cells chromosomally expressing Shp1-HA. We observed no binding (Fig. 4 e). Putatively, the NHD might help to discriminate ubiquitin from Atg8. Comparison of the first eight amino acids of Atg8 with its mammalian homologues LC3, \(\gamma\)-aminobutyrate type A receptor-associated protein, and GATE-16 showed that amino acids 5 and 6 are strongly conserved (Fig. 4 d). We generated an Atg8-F5G/K6G mutant that was unable to bind Shp1-HA (Fig. 4 e). We also generated an Atg8-S3A/T4A mutant that still effectively bound Shp1-HA (Fig. 4 e). We thus identified the FK motif within the Atg8 NHD as essential for Shp1 binding. Nakatogawa et al. (2007) reported that an Atg8-L50A mutant in the ULD was lipidated and showed increased multimerization and liposome clustering but almost no formation of autophagic bodies. As a control, we also generated an Atg8-L50A mutant and found normal binding to Shp1-HA (Fig. 4 e), supporting the crucial role of the NHD for Shp1 binding. To demonstrate the existence of a ternary Atg8, Shp1, and Cdc48 complex, we used GST-Atg8 and extracts of pull-down eluates, Cdc48, but not Shp1, was identified (unpublished data). The binding of Shp1 to \textit{Escherichia coli}–expressed nonlipidated GST-Atg8 might reflect the binding of Shp1 to unlipidated Atg8. However, we favor an alternate explanation. Lipidation induces Atg8 oligomerization, and mutations impairing oligomerization affect liposome tethering and phagophore elongation (Nakatogawa et al., 2007). In vitro Atg8 oligomerization is enhanced at 10-\(\mu\)M concentrations. We therefore propose that the \(~20-\mu\)M concentration of GST-Atg8 on beads could mimic oligomerization and thus lipidation. Atg8 contains a C-terminal ULD and a 24 amino acid NHD that is absent in ubiquitin. Crystallography of Atg8 bound to a peptide of the cargo receptor Atg19 showed a closed conformation in which the NHD buries part of the ULD (Noda et al., 2008). During this study, the nuclear magnetic resonance solution structure of Atg8 (Schwarten et al., 2010) unraveled flexibility in the NHD. The structure with the lowest target function showed the first eight amino acids in an open conformation projected away from the ULD. Speculatively, oligomerization releases the NHD from the ubiquitin-like domain (ULD) and induces the open conformation (Nakatogawa et al., 2007). Truncation of the NHD affected autophagosomal elongation and reduced the macroautophagic rate to \(~70\%\) for Atg8-\(\Delta N8\) lacking the first helix of the NHD and to \(~60\%\) for Atg8-\(\Delta N24\) lacking the complete NHD (Nakatogawa et al., 2007; Fig. S2 b). We thus analyzed its relevance for interaction with Shp1 by incubating immobilized GST-Atg8-\(\Delta N8\) and GST-Atg8-\(\Delta N24\) with extracts from cells chromosomally expressing Shp1-HA. We observed no binding (Fig. 4 e). Putatively, the NHD might help to discriminate ubiquitin from Atg8. Comparison of the first eight amino acids of Atg8 with its mammalian homologues LC3, \(\gamma\)-aminobutyrate type A receptor-associated protein, and GATE-16 showed that amino acids 5 and 6 are strongly conserved (Fig. 4 d). We generated an Atg8-F5G/K6G mutant that was unable to bind Shp1-HA (Fig. 4 e). We also generated an Atg8-S3A/T4A mutant that still effectively bound Shp1-HA (Fig. 4 e). We thus identified the FK motif within the Atg8 NHD as essential for Shp1 binding. Nakatogawa et al. (2007) reported that an Atg8-L50A mutant in the ULD was lipidated and showed increased multimerization and liposome clustering but almost no formation of autophagic bodies. As a control, we also generated an Atg8-L50A mutant and found normal binding to Shp1 (Fig. 4 e), supporting the crucial role of the NHD for Shp1 binding. To demonstrate the existence of a ternary Atg8, Shp1, and Cdc48 complex, we used GST-Atg8 and extracts of...
cells chromosomally expressing Cdc48-GFP and Shp1-HA with native promoters (Fig. 4 f).

We next analyzed the relevance of Shp1 domains for interaction with Atg8. Shp1 contains a ubiquitin-associated domain (UBA) involved in ubiquitin binding, an SEP (Shp1, eyes-closed, p47) domain involved in p47 trimerization, and a Cdc48/p97-binding Ubx domain (Fig. 5 a). BS1 or SHP box is a second p97 binding site of p47 at the end of the SEP domain. Because of the second Cdc48 binding site, BS1 deletion of the Ubx domain alone did not block macroautophagy (Fig. 5 b). Accordingly, deletion of the SEP and UBX domain, which removes both Cdc48 binding sites, severely inhibited autophagy (Fig. 5 b). The C-terminally truncated Shp1 variants were chromosomally integrated. The N-terminally truncated Shp1 species were on plasmids with the CUP1 promoter. Deletion of the UBA domain had no obvious effect on autophagy, irrespective of induction with exogenous Cu²⁺ (Fig. 5 d, left) or, when grown in normal medium, containing traces of Cu²⁺ (Fig. 5 d, right). All truncated Shp1 variants interacted with Atg8 (Fig. 5, c and e). This suggests that Atg8 binding requires the domain between the UBA and the SEP domain. We thus expect that the Atg8-FK motive, which, as part of the NHD, is absent in ubiquitin, interacts with this Shp1 domain.

Defects in the secretory pathway affect autophagosome biogenesis (Ishihara et al., 2001; Reggiori et al., 2004). However, because Atg8 does not affect sorting via the ER and Golgi, the complex of Atg8 with Cdc48-Shp1 cannot have such an indirect effect on macroautophagy. Our data further suggest that the autophagic function of Shp1 requires neither the ubiquitin–proteasome system nor the Shp1 UBA domain. In addition, Atg8 mutants with impaired tethering and hemifusion, including Atg8-ΔN24, showed unaltered PAS localization (Nakatogawa et al., 2007), leading to the conclusion that Atg8 mediates phagophore elongation at the PAS (Nakatogawa et al., 2007). Most recently, LC3 was shown to mediate phagophore elongation, whereas the γ-aminobutyrate type A receptor-associated protein/GATE-16 subfamily most likely mediates autophagosome sealing (Weidberg et al., 2010).

In analogy to Golgi reassembly, we speculate that Atg8 may act as a fusion regulator, which must be extracted from a complex with a fusion mediator by the AAA-ATPase Cdc48 and its adaptor Shp1. In this hypothetical model, deubiquitination by the cysteine protease VCIP135 would be reflected by Atg8 delipidation by the cysteine protease Atg4, which is needed for efficient macroautophagy. Indeed, GATE-16, a mammalian Atg8 homologue, interacts with the SNARE GOS-28 (Müller et al., 2002), and a Cdc48/p97 complex of Atg8 with Cdc48-Shp1 cannot have such an indirect effect on macroautophagy. Our data further suggest that the autophagic function of Shp1 requires neither the ubiquitin–proteasome system nor the Shp1 UBA domain. In addition, Atg8 mutants with impaired tethering and hemifusion, including Atg8-ΔN24, showed unaltered PAS localization (Nakatogawa et al., 2007), leading to the conclusion that Atg8 mediates phagophore elongation at the PAS (Nakatogawa et al., 2007). Most recently, LC3 was shown to mediate phagophore elongation, whereas the γ-aminobutyrate type A receptor-associated protein/GATE-16 subfamily most likely mediates autophagosome sealing (Weidberg et al., 2010).

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(1.7% yeast nitrogen base without amino acid, ammonium sulfate, and 2% glucose). Samples were analyzed by Western blotting using a GFP anti-body and secondary horseradish peroxidase-conjugated goat anti-mouse (Dianova) with the ECL or ECL Plus detection kit (GE Healthcare) and an LAS-3000 imaging system (Fujifilm). Temperature-sensitive strains were grown at 23°C to stationary phase and shifted to SD-[N] at either 23 or 38°C. To measure the GFP-Atg8 or GFP-Osh1 breakdown in cells expressing mutated ubiquitin-essential mutants pRS315-GFP-Atg8 and pRS315-GFP-Osh1, an insert containing GTF-ATG8 under control of the ATG8 promoter was isolated from pGFP-Atg8 (Suzuki et al., 2001) by Not1–Sal1 digestion and ligated into the Nat1–Sal1 cut pRS315. GFP-Osh1 under control of the PHOS promoter was isolated from prs416-GFP-Osh1 by Sal1–Nae1 digest and ligated in Sal1–Nae1 digested pRS315. Each of the generated plasmids was cotransformed with plasmids expressing the ubiquitin variants from the GST pull down promoter (Sloper-Mould et al., 2001). For the degradation assay, stationary cells were induced in selection medium with 100 µM CuSO4 for 2 h at 30°C. Then, cells were shifted to SD-[N] medium at 30°C, and samples corresponding to 2 OD600 were taken and analyzed by immunoblotting as described.

Fluorescence microscopy
An Axioscope 2 with a digital AxioCam MRm camera, a 100× 1.4 NA oil Plan-fluar objective, and AxioVision software (release 4.5; Carl Zeiss, Inc.) were used. The images were processed with Photoshop CS4 (Adobe) or Canvas X (ACD Systems International, Inc.). Fluorescent pictures were taken with the GFP filter set.

Proteinase protection
According to Meling-Wesse et al. (2002), 60 OD600 of stationary cells in the BY4741 background carrying a GFP-Atg8 plasmid was starved in SD-[N] medium for 4 h at 30°C. Cells were harvested, washed once with DTT buffer (10 mM Tris-sulfate, pH 9.4, and 10 mM DTT), and resuspended as a 2-OD/ml suspension in DTT buffer. The suspension was incubated at 30°C for 15 min while shaking. Cells were then harvested and resuspended as a 10-OD/ml suspension in SP buffer (1 M sorbitol and 20 mM Pipes, pH 6.8). 1.2 mg Zymolyase 20T (Seikagaku) was added, and samples were incubated for 25 min at 30°C. The resulting spheroplasts were washed with SP buffer and hypotonically lysed using PS200 buffer (20 mM Pipes, pH 6.8, 200 mM sorbitol, and 5 mM MgCl2). After two preclaring steps, 30 µl oligo-acrylamide-treated wash buffer with DTT (20 mM Tris-sulfate, pH 9.4, and 10 mM DTT), and resuspended in the presence or absence of Triton X-100 for 30 min at 30°C. The reaction was stopped by TCA precipitation. The protein pellets were washed twice with acetone and resuspended in 50 µl of Laemmli buffer. Samples were analyzed by immunoblotting as described.

GST pull down
Atg8ΔN8 and Atg8ΔN24 were amplified with GST-ΔN8ATG8 forward, GST-ΔN8ATG8 reverse, GST-ΔN8ATG8 forward, and GST-ΔN8ATG8 reverse with pGFRG-Atg8 as a template. Oligonucleotides are listed in Table S1. Products and pGEX-4T-3 were digested with BamHI–XhoI and ligation. GST-Atg8 was mutagenized with the Quickchange II kit (Agilent Technologies) and amplified with Atg8-His forward and Atg8-His reverse and pFa6a-His6-ATG8, an insert containing GFP-ATG8 under control of the ATG8 promoter was ligated from pGFP-Atg8 (Suzuki et al., 2001) by Not1–Sal1 digestion and ligated into the 2 µg HA-Nat NT2 chromosomally integrated pCUP1-SHP1-HA XhoI, cut with Cla1–Sal1, and ligatedigated in Sal1–Nae1 digested pRS315. Each of the generated plasmids was cotransformed with plasmids expressing the ubiquitin variants from the GST pull down promoter (Sloper-Mould et al., 2001). For the degradation assay, stationary cells were induced in selection medium with 100 µM CuSO4 for 2 h at 30°C. Then, cells were shifted to SD-[N] medium at 30°C, and samples corresponding to 2 OD600 were taken and analyzed by immunoblotting as described.

Electron microscopy
Cells starved for 4 h in SD-[N] were permanganate fixed and embedded in Epon (Epple et al., 2003). An electron microscope (JEM1200EX-II; JEOL) was used.

Online supplemental material
Fig. S1 shows the defect of shp1Δ cells in unselective starvation-induced macroautophagy. Fig. S2 shows coimmunoprecipitation of Shp1-HA and GFP-Atg8 in wild-type cells and proaominopeptidase I matured in cells expressing N-terminally truncated Atg8 variants. Table S1 shows oligonucleotides used in this study. Online supplemental materials are available at http://www.jcb.org/cgi/content/full/jcb.201002075/DC1.

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