The COPII subunit Lst1/SEC24C acts with an autophagy receptor
to target endoplasmic reticulum for degradation

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Abstract

The COPII-cargo adaptor complex, Lst1-Sec23, selectively sorts proteins into vesicles that bud from the endoplasmic reticulum (ER) and traffic to the Golgi. Improperly folded proteins are prevented from exiting the ER and are degraded. ER-phagy is an autophagic degradation pathway that utilizes ER-resident receptors. Here we report an unexpected role for Lst1-Sec23 in ER-phagy that is independent from its function in secretion. Upregulation of the stress inducible ER-phagy receptor, Atg40, induces the association of Lst1-Sec23 with Atg40 at distinct ER subdomains to package ER into autophagosomes. We also show that Lst1-mediated ER-phagy plays a vital role in maintaining cellular homeostasis by preventing the accumulation of aggregate-prone proteins in the ER. Lst1 function is conserved as its mammalian homologue, SEC24C, is also required for ER-phagy.
Introduction

Vesicle transport cargo adaptors sort and concentrate cargo into transport vesicles at specific stages of membrane traffic (1, 2). At the ER, the Sec24 subunit of the major COPII-cargo adaptor complex, Sec24-Sec23, binds to the cytosolic domain of ER transmembrane proteins and packages them into vesicles that traffic to the Golgi (1). Yeast also has two Sec24 paralogs, Iss1/Sfb2 (56% identity) and Lst1/Sfb3 (23% identity), that form secretory cargo adaptor complexes with Sec23, while mammals have four SEC24 isoforms. The mammalian SEC24 homologues (SEC24A and SEC24B) are 50% identical, but only share 20% identity with the Lst1 homologues (SEC24C and SEC24D) that are also 50% identical to each other (3).

To prevent improperly folded proteins from entering the secretory pathway, the unfolded protein response (UPR) pathway works in conjunction with ER-associated degradation (ERAD) to recognize terminally misfolded proteins and retrotranslocate them into the cytosol for degradation by the proteasome (4). ERAD, however, is unable to clear all aberrant proteins from the ER (5). Macroautophagy (herein called autophagy) of the ER, or ER-phagy, could be part of a global ER stress response that restores cellular homeostasis when ERAD and/or the UPR fail to respond. There are two major types of autophagy pathways, bulk and selective (6). During starvation, bulk autophagy uses autophagosomes to scavenge cytoplasmic components for nutrients. To rapidly increase autophagosome number, COPII vesicles are rerouted to serve as a membrane source for bulk autophagy (6, 7). Selective pathways, instead, employ autophagy receptors to package cargo into autophagosomes. These cargoes include damaged or superfluous organelles, protein aggregates and pathogens (8).

Although ER-phagy was initially described in 2005 (9), it was not until the first ER-phagy receptors were identified that the process was thought to be selective (10, 11). ER-phagy
occurs at discrete foci on the ER (12), however, the autophagy receptors that load ER into autophagosomes are spread throughout the contiguous network of the ER. Cytosolic machinery that identifies and binds to the ER-phagy receptors may determine where autophagy sites will form. Here we report that when ER-phagy is induced, the Lst1-Sec23 complex associates with the ER-phagy receptor, Atg40, to degrade cortical ER in yeast. In the absence of Atg40 or Lst1, ER sequestration into autophagosomes is impaired. Lst1 function in ER-phagy is conserved as its homologue, SEC24C, is required for the degradation of ER sheets and tubules in mammalian cells. Our studies have revealed a new and unexpected role for a COPII cargo adaptor complex in targeting ER domains for ER-phagy.
Results

Lst1 is required for ER-phagy, but not bulk autophagy

ER-phagy is typically induced in yeast by treating cells with rapamycin, a TOR kinase inhibitor that mimics nutrient starvation (9, 10). Rapamycin also upregulates the expression of Atg40, which increases ER-phagy activity (9, 10). Atg40, a reticulon-like ER membrane protein, is required for cortical ER degradation, but inefficiently degrades Hmg1 and other nuclear markers. Nuclear ER turnover is instead predominantly facilitated by Atg39, a nucleophagy receptor (10).

As COPII coat subunits participate in membrane budding events at the ER (1), we asked if coat subunits play a role in packaging ER domains into autophagosomes during ER-phagy. ER-phagy is induced by treating cells for 12-24 h with rapamycin, which precluded us from analyzing temperature-sensitive mutants harboring COPII mutations as they die within hours at their restrictive temperature. The lst1Δ, iss1Δ and sec24-3A mutants, however, do not significantly impair growth or secretion (13, 14). In sec24-3A cells, bulk autophagy is defective as a consequence of a failure to phosphorylate Sec24 at T324, T325, and T328 (14). Sec24 phosphorylation at these sites, which are conserved in Iss1, enables COPII coated vesicles to bind the core autophagy machinery (14). Interestingly, we found a defect in the degradation of the ER protein Sec61 in lst1Δ, but not in the sec24-3A iss1Δ and iss1Δ mutants (Fig. 1A-D; Fig. S1A-C). One of the kinases that phosphorylates Sec24 during bulk autophagy, Hrr25 (14), is dispensable for ER-phagy (Fig. S1D-E). Additionally, Sec24 phosphorylation is dispensable for pexophagy (Fig. S2A-B) and mitophagy (Fig. S2D-E). The degradation of Rtn1, which marks ER tubules, the edges of sheets and the nuclear ER in a small fraction of cells (15), was also defective in the lst1Δ mutant (Fig. 1E-F; Fig. S3A-B). Similarly, a third ER protein, Per33, was degraded less efficiently in lst1Δ cells (Fig. S3C-F). Like Sec61, Per33 resides on the nuclear
and cortical ER (16). In addition to rapamycin, Lst1-mediated ER degradation could also be induced by growth to the stationary phase (Fig. S4A-C).

To ask if Lst1 functions in bulk autophagy, we measured the activation of vacuolar alkaline phosphatase (Pho8Δ60) after starvation (Fig. S4D), as well as the cleavage of GFP-Atg8 in the vacuole (Fig. S4E-F) 24 h after rapamycin treatment (17). When bulk autophagy is induced, GFP-Atg8 and Pho8Δ60 are delivered to the vacuole, where Pho8Δ60 is activated by proteolytic cleavage. No defect in bulk autophagy was detected in lst1Δ cells by either assay. Additionally, Lst1 was not needed for pexophagy (Fig. S2B-C), mitophagy (Fig. S2E-F) or the biosynthetic cytosol to vacuole targeting (Cvt) pathway (Fig. S2G), which also uses autophagy machinery (17). Therefore, Lst1 and its parologue Sec24, appear to function in different autophagy pathways. While phosphorylation of the Sec24 membrane distal surface is required for bulk autophagy (14), it is dispensable for ER-phagy. In contrast, Lst1 acts exclusively in ER-phagy.

Lst1 is primarily known for the formation of larger COPII coated vesicles and the trafficking of GPI anchored proteins (1). Unlike Sec24, Lst1 cannot package the ER-Golgi fusion machinery (i.e., SNAREs) into COPII vesicles. Consequently, vesicles containing Lst1, but not Sec24, are unable to fuse with the Golgi (18). When we measured ER-phagy in two lst1 mutants (LST1-B1 and LST1-B2) that disrupt the packaging of GPI anchored proteins into COPII vesicles (19), no ER-phagy defect was observed (Fig. S5A-D). Thus, the role of Lst1 on the secretory pathway can be uncoupled from its contribution to ER-phagy. Together, these results establish a previously unanticipated role in autophagy for this conserved coat protein.
**Lst1 acts in concert with Atg40**

Interestingly, Sec61-GFP positive ER cisternae and foci accumulated in rapamycin-treated \( atg40\Delta \) and \( lst1\Delta \) mutants (Fig. S6A-B), and the ER degradation defect in the \( lst1\Delta atg40\Delta \) double mutant was not significantly different than either single knockout (Fig. S6C-F). These findings imply that Lst1 and Atg40 function on the same pathway. As elevated levels of autophagy receptors increase ER-phagy and the number of ER-containing autophagosomes (10, 11), ER-phagy receptors were proposed to play a role in packaging ER into autophagosomes (11). To address if Lst1 works in conjunction with Atg40 to perform this function, we induced Atg40 expression with rapamycin (10) and asked if this promotes an association with Lst1. To colocalize Lst1-3xGFP with Atg40-2xmCherry, we focused on Atg40 puncta that lie within the cell interior as previous studies indicated these puncta are accessible to the autophagy machinery (16). Sec24-GFP and Sec13-GFP, a component of the COPII coat scaffold complex (Sec13-Sec31) (1), were examined as controls. Although all COPII coat subunits mark ER exit sites (ERES) in nutrient-rich growth conditions (20), only Lst1-3xGFP and its partner Sec23-GFP showed increased colocalization with Atg40-2xmCherry puncta (Fig. 2A-C; Fig. S7A-C). Unlike Atg40, Lst1 and Sec23 expression did not increase with rapamycin treatment (Fig. S7D). The number of Lst1-3xGFP and Sec23-GFP puncta were also unchanged (Fig. S7E). While previous studies showed \( CUP1\)-driven Atg40 overexpression only increased ER-phagy in the presence of rapamycin (10), overexpression was sufficient to drive Atg40 colocalization with Lst1 (Fig. S8A-B). Rapamycin also induced the expression of the nucleophagy receptor, Atg39 (10), but did not induce the colocalization of Lst1 with Atg39 (Fig. S8C-D). Furthermore, Lst1 did not contribute to the degradation of the nuclear ER marker Hmg1 (Fig. S8E-H).
Selective autophagy receptors bind to the ubiquitin-like protein, Atg8, at sites of autophagosome formation (8). Consistent with the proposal that Lst1 functions with Atg40 to target ER domains for degradation, rapamycin induced Atg8-Lst1 colocalization in wild-type (WT), but not atg40Δ cells (Fig. 2D; Fig. S9A). In contrast, Atg8 showed increased colocalization with two COPII coat subunits that are required for bulk autophagy, Sec24 and Sec13 (21), in WT and atg40Δ cells (Fig. 2D; Fig. S9A). Because Sec23 is a binding partner for both Lst1 and Sec24, the colocalization of Atg8 with Sec23 was only partially dependent on Atg40 (Fig. 2D; Fig. S9A).

Next, we asked whether ER organization plays a role in the colocalization of Lst1 with Atg40. Lnp1, a transmembrane ER protein that resides at the three-way junctions, stabilizes ER network rearrangements (22). In the absence of Lnp1, Atg40 puncta fail to access the autophagy machinery (16). As a consequence, ER is not packaged into autophagosomes in lnp1Δ cells (16). We found that Lst1-3xGFP failed to colocalize with Atg40-2xmCherry (Fig. 2E, Fig. S9B; also see Pearson’s coefficient in Fig. S9C) in lnp1Δ cells. Additionally, even though Lst1-3xGFP and RFP-Atg8 puncta appeared unaltered in the lnp1Δ mutant, Lst1 also failed to colocalize with Atg8 (Fig. 2F; Fig. S9D). We propose that Atg8-Lst1 colocalizing puncta (usually one per cell) represent active ER-phagy sites stabilized by Lnp1. Although Atg40 puncta tend to accumulate at the cell periphery and fail to reside in the cell interior in Lnp1-depleted cells (16), the distribution of Atg40 puncta is unperturbed in lst1Δ cells (Fig. S9E).

The localization studies described above suggested that Lst1 and Atg40 interact with each other during ER-phagy. Lst1 could interact with Atg40 via its cytosolic domain (10), however, because the Atg40 cytoplasmic domain was unstable, we could not directly test this possibility. We were also unable to express full length Atg40 from bacteria. Therefore, to address this
question, bindings were performed with lysates prepared from untreated and rapamycin-treated cells (Atg40-3xFLAG). Atg40-3xFLAG from both lysates bound to purified bacterially produced GST-Lst1, but not to GST-Sec24, GST-Sec13, or GST-Sec23 (Fig. 3A; Fig. S10A-C). GST-Lst1 also failed to bind to two other ER proteins, Ufe1 and Yip1 (Fig. S10D). These findings indicate that Lst1, but not Sec24 or other COPII coat subunits, binds to Atg40. Sec13-Sec31 could fail to associate with Atg40 because the binding of Lst1-Sec23 to Atg40 sterically hinders its interaction. Supporting the proposal that the interaction of Atg40 with Lst1 is needed for ER-phagy, the rapamycin-induced degradation of Lst1-GFP required Atg40, and Atg40-GFP degradation required Lst1 (Fig. S11A-H).

To count ER-containing autophagosome numbers in the lst1Δ mutant, cells depleted of the vacuolar protease Pep4 were examined by conventional electron microscopy (EM) (Fig. 3B-D). While autophagic bodies were absent in the atg14Δ mutant that blocks autophagosome formation (Fig. 3B-D), the number of autophagosomes formed in the lst1Δ mutant was the same as in WT (Fig. 3D). In contrast, a significant reduction in autophagic bodies that contained ER tubules and sheets was observed in the lst1Δ mutant (Fig. 3B-C). Consistent with the proposal that Lst1 works with Atg40 to package cortical ER into autophagosomes, the lst1Δ defect was comparable to that seen in atg40Δ cells (Fig. 3B-C).

**ER stress upregulates Atg40**

In mammalian cells, the putative ER-phagy receptor, FAM134B, targets ERAD-insensitive substrates to autophagy (5). How the ER communicates with the autophagy machinery when ERAD does not respond or is overwhelmed is unknown.
The Z variant of human alpha-1 antitrypsin (ATZ) is an ERAD substrate that is also targeted to a non-autophagosome-mediated lysosomal pathway in mammalian cell culture (23). When ATZ is highly overexpressed in yeast under the inducible galactose promoter (GAL1), however, the excess ATZ not degraded by ERAD is directed to the vacuole via two pathways, vacuolar protein sorting and autophagy (24). Blocking the latter pathway leads to the aggregation of ATZ in the ER (24). Currently it is unknown if this autophagosome-mediated pathway is ER-phagy. To ask whether ATZ aggregates in the absence of Atg40 and Lst1, microsomal membrane fractions prepared from WT, atg14Δ, atg40Δ and lst1Δ cells were analyzed on sucrose gradients. Soluble ATZ was primarily found at the top of the WT gradient, while ATZ from mutant lysates was largely in the pellet (Fig. 4A-C; Fig. S12A-B). Aggregation appeared to be due to a block in ER-phagy because significantly less ATZ was observed in the pellet when lysates from a mutant (lst1-B1) that disrupts membrane traffic (19), and not ER-phagy (Fig. S5), were examined (Fig. S12C). Aggregation was also specific to misfolded ATZ, as appreciably less wild-type M variant alpha-1 antitrypsin (ATM) was observed in pellet fractions of lst1Δ or atg40Δ cells (Fig. 4D). Consistent with this observation, a portion of the ATM (but not ATZ) was secreted (Fig. S12D). Together, these data indicate that only mutant alpha-1 antitrypsin aggregates in the ER in the absence of ER-phagy. Interestingly, the loss of the pancreatic ER-phagy receptor, CCPG1, also leads to the accumulation of aggregated proteins in the ER (12).

Previous studies showed that multiple COPII coat subunits, including Sec24 and Lst1, are targets of the UPR (25). To ask if Atg40 is also a UPR target, we overexpressed ATZ in the presence or the absence of Ire1, an ER transmembrane serine/threonine protein kinase that triggers the UPR (26). We found that ATZ induction enhanced Atg40 expression in WT cells (Fig. 5A-B, compare with Fig. S12E). Additionally, Atg40 expression and ATZ accumulation
were further enhanced in \textit{ire1}Δ mutant cells, which block UPR activation and augment ER stress (4) (Fig. 5A-B). In contrast, overexpressed ATM did not increase Atg40 expression in the \textit{ire1}Δ mutant (Fig. S12F). To determine whether the loss of Atg40 induces the UPR, we examined pathway induction by flow cytometry in cells carrying an integrated UPR-regulated GFP reporter. While the \textit{atg39}Δ (Fig. 5C), and \textit{sec24-3A iss1}Δ mutants behaved like WT (Fig. S12G), the level of UPR induction in the \textit{atg40}Δ and \textit{lst1}Δ mutants was the same as in an ERAD-deficient \textit{hrd1}Δ strain (Fig. 5C) (4). Therefore, although Atg40 is not a UPR target, the UPR responds to the loss of ER-phagy.

As Atg40 expression is not under the control of the UPR, we next sought to address how it is regulated. Since ER stress is known to modulate the expression of core autophagy machinery (27), we considered the possibility that autophagy transcriptional regulators control Atg40 expression. Pho23, which associates with the histone deacetylase large complex (Rpd3L), is a transcriptional repressor of autophagy that modulates autophagosome abundance via Atg9 (28). Interestingly, Atg40, but not the UPR targets, Kar2 and Lst1, were upregulated in the \textit{rpds3}Δ and \textit{pho23}Δ strains (Fig. 5D-G). Atg40 and Atg9 mRNA levels also dramatically increased in these mutants (Fig. 5H), indicating that the Atg40 ER-phagy receptor is a target of the same transcriptional regulators that modulate core autophagy machinery (Atg9).

**Mammalian SEC24C is required for ER-phagy**

It was recently reported that SEC24A and SEC24B, but not SEC24C and SEC24D, are needed for bulk autophagy (29) To ask if any of the SEC24 isoforms are required for ER-phagy in mammalian cells, all four were knocked-down by siRNA (Fig. S13A), and the delivery of two ER-phagy receptors, FAM134B and RTN3, to lysosomes was monitored in Torin2 (TOR
inhibitor2) treated cells. We chose to examine these two mammalian ER-phagy receptors because Atg40 has a domain structure that is similar to FAM134-B, yet localizes to the tubular ER like RTN3 (11, 30). 3xFLAG-FAM134B was resistant to degradation in SEC24C-depleted cells, but not cells depleted of SEC24A, SEC24B or SEC24D (Fig. 6A; Fig. S13B). Moreover, the delivery of 3xFLAG-FAM134B to lysosomes (LAMP1) required SEC24C and ULK1, a component of the autophagosome biogenesis machinery (Fig. 6B-C; Fig. S13C). The delivery of RTN3 to lysosomes was also SEC24C and ULK1-dependent (Fig. 6D-E; Fig. S14). Therefore, in mammals as in yeast, TOR inhibition stimulates the delivery of ER to lysosomes via Lst1/SEC24C-mediated ER-phagy.
Discussion

Here we report a new role for the COPII-cargo adaptor complex, Lst1-Sec23, and show that it functions with the ER-phagy receptor, Atg40, to specify ER domains for ER-phagy. By analogy to its role as a cargo adaptor in vesicle traffic (19), Lst1-Sec23 may sort ER domains into autophagosomes via an interaction with the cytoplasmic domain of Atg40 (Fig. S15). Several lines of evidence highlight the key events mediated by Lst1 on this pathway. First, the Lst1-Sec23 complex associates with Atg40 when its expression is upregulated by the TOR inhibitor rapamycin. Second, in accord with the proposal that the colocalization of Lst1 with Atg40 is needed for ER-phagy, Lst1 failed to colocalize with Atg40 and Atg8 in the lnplΔ mutant, which disrupts ER network dynamics and the incorporation of ER into autophagosomes (16). Third, consistent with a role in ER packaging, Lst1 is required for the degradation of Atg40, and the number of ER-containing autophagosomes is significantly reduced in the lst1Δ mutant.

To date, six ER-phagy receptors have been identified in mammalian cells (11, 12, 30-34). While some autophagy receptors respond to starvation, others act in response to ER stress. Here we show that Atg40 responds to both types of cellular stress. It is upregulated by both rapamycin (10), and the overexpression of an aggregate-prone misfolded secretory protein (ATZ). In the latter case, the induction of Lst1-mediated ER-phagy reduced the level of aggregated ATZ in the ER (Fig. S15). Consistent with the observation that a TOR inhibitor upregulates Atg40 (10), we found that Atg40 expression is modulated by autophagy transcriptional regulators. As recent studies have shown that ER stress inhibits TOR (35), TOR-dependent autophagy transcriptional regulators may also modulate the upregulation of ER-phagy receptors for the purpose of reducing ER protein aggregation.
Our findings imply that a specific subcomplex of the COPII coat, that contains Lst1-Sec23, binds to Atg40 to package ER domains into autophagosomes during ER-phagy (Fig. S15). ER-phagy occurs at distinct sites on the ER that we named **ERPHS** (**ER-phagy** sites). The absence of Sec24 from these sites distinguishes them from the **ERES** (**ER** exit sites) that bud conventional COPII vesicles (Fig. S15). Because previous studies in yeast did not reveal a significant accumulation of cytosolic ER fragments during ER-phagy, it was suggested that ER subdomains are engulfed into autophagosomes on the ER network, or in close proximity to the network (9) (Fig. S15). ER fragments appear to be packaged into autophagosomes before they completely uncoat, as we found Lst1-GFP gets degraded in the vacuole in an Atg40-dependent fashion (Fig. S15). Since the Lst1-Sec23 complex cannot package SNAREs, fragments that bud from the ER should not fuse with the Golgi prior to their incorporation into autophagosomes.

The formation of **ERPHS** depended on the ER protein Lnp1, which is needed for stable three-way ER junctions (Fig. S15) (22). Consistent with this observation, the mammalian ER-phagy receptor, TEX264, was recently shown to be incorporated into autophagosomes at these junctions (33, 34). The localization of LNP1 to ER junctions depends on ATLASTIN, which is also required for ER-phagy (15, 36, 37). Interestingly, ATLASTIN 3 (ATL3) was recently reported to be an ER-phagy receptor (32). Mutations in ATLASTIN and LNP1 have been linked to hereditary spastic paraplegias (HSP) and HSP-like neuropathies (38, 39). The ER-phagy receptor FAM134B, which is required for both ER-phagy and autophagosome-independent ER-to-lysosome degradation (23), has also been linked to neuronal function (11).

Additionally, we found that the mammalian Lst1 homologue, SEC24C, is required for the delivery of ER sheets (FAM134B) and tubules (RTN3) to lysosomes for degradation. This observation is in accord with recent data showing that the interactome for the ER-phagy receptor,
RTN3, includes SEC24C but not the other SEC24 isoforms (30). Although professional secretory cells are not sensitive to SEC24C depletion, its loss in postmitotic neurons leads to microcephaly and perinatal death (40). While SEC24C and SEC24D are ~50% identical, their functions only partially overlap in the brain (40), and we did not detect a significant role for SEC24D in ER-phagy. As our experiments and the RTN3 interactome studies both used U2OS cells, it remains formally possible that other cell types may require SEC24D for ER-phagy. Together, these findings suggest that ER-phagy plays a crucial role in maintaining neuronal homeostasis.

In summary, we propose that in yeast and mammals, COPII cargo adaptors package different cargoes (secretory proteins or ER domains) into membranes and target these cargoes to distinct pathways. Our findings imply that **ERES**, which bud canonical COPII vesicles on the secretory pathway, are distinct from the **ERPHS** used in autophagy. The secretory and ER-phagy pathways also appear to be regulated by different stress response pathways.
Figure Legends

Figure 1. Lst1 is required for ER-phagy.

(A) The translocation of Sec61-GFP to the vacuole was examined by fluorescence microscopy in WT and mutant cells 24 h after rapamycin treatment. FM4-64 was used to stain the vacuolar membrane. (B) The percent of cells with Sec61-GFP delivered to the vacuole was quantitated from 300 cells. (C) The cleavage of Sec61-GFP to GFP in WT and mutant strains was analyzed by immunoblotting using anti-GFP antibody. A representative blot is shown. (D) Three separate experiments were used to quantitate the ratio of free GFP to Sec61-GFP from the data in (C). WT was set to 100%. (E) The translocation of Rtn1-GFP to the vacuole in WT and mutant cells was detected by fluorescence microscopy 24h after rapamycin treatment. (F) The percent of cells with Rtn1-GFP delivered to the vacuole was quantitated from 300 cells. Scale bars in (A), (E), 5µm. Error bars in (B), (D), and (F) represent S.E.M., N=3; *P < 0.05, **P < 0.01, ***P < 0.001, Student’s unpaired t-test.

Figure 2. Lst1 and Sec23 colocalize with Atg40 and Atg8 in rapamycin-treated cells.

(A) Representative images of cells treated for 0 or 6 h with rapamycin. Arrowheads indicate Lst1-3xGFP that colocalize with Atg40-2xmCherry puncta. (B) Bar graph shows the percent of Lst1-3xGFP or Sec24-GFP colocalizing with Atg40-2xmCherry puncta. (C) Bar graph shows the percent of Sec23-GFP or Sec13-GFP colocalizing with Atg40-2xmCherry puncta. (D) Cells were treated for 0 or 3 h with rapamycin, and RFP-Atg8 that colocalizes with GFP tagged COPII coat subunits was quantitated. (E) Left, arrowheads indicate Lst1-3xGFP that colocalizes with Atg40-2xmCherry puncta 6 h after rapamycin treatment. Quantitation is on the right. (F) Quantitation of RFP-Atg8 that colocalize with Lst1-3xGFP in WT and inp1D cells. Scale bars in (A), (E), 5µm.
Error bars in (B-F) represent S.E.M., N=3-6; NS, not significant P ≥ 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, Student’s unpaired t-test.

**Figure 3. Lst1 binds to Atg40 and facilitates the packaging of ER into autophagosomes.**

(A) Equimolar amounts (0.1 µM) of purified recombinant GST and GST fusion proteins were incubated with 2mg of yeast lysate prepared from Atg40-3xFLAG untagged or tagged cells treated with rapamycin. (B) Representative electron micrographs of WT and pep4Δ strains treated for 12 h with rapamycin. The boxed areas in the left panel are magnified on the right. Black asterisk, an autophagic body containing an ER fragment; white asterisk, an autophagic body lacking an ER fragment; arrowhead, an ER fragment inside an autophagic body. Scale bars in the left and right panels represent 1µm and 500 nm, respectively. (C) Bar graph showing the average number of ER-containing autophagosomes. (D) Bar graph showing the average number of total autophagosomes. Error bars in (C) and (D) represent S.E.M., N=100 cells; *P < 0.05, ***P < 0.001, Student’s unpaired t-test. Symbols: N, nucleus; V, vacuole.

**Figure 4. ATZ aggregates accumulate in atg40Δ and lst1Δ mutants.**

Microsomal membrane fractions prepared from WT and mutant cells, harboring plasmids expressing either ATZ (A-D) or ATM (D), were lysed in the presence of detergent and fractionated on a sucrose gradient. Gradient fractions were blotted with antibody directed against alpha-1 antitrypsin. Soluble alpha-1 antitrypsin was at the top of the gradient and aggregates reside in the pellet (P).
**Figure 5.** Atg40 expression is regulated by the Rpd3-Pho23 complex.

(A) Atg40 expression was examined 24 h after growth in 2% galactose. Western blot analysis was performed on lysates to detect ATZ (top), Atg40-3xFLAG (middle) and Adh1 (bottom). (B) The data in (A) were normalized to Adh1, and WT (-ATZ) was set to 100%. (C) UPR induction was assayed by flow cytometry in the absence or presence of 8mM DTT. WT (-DTT) was set to 1.0. (D) Atg40, Kar2 and Lst1 levels were measured by western blot analysis in the indicated strains. (E-G) The data in (D) were normalized to Adh1 and WT was set to 100%. (H) qRT-PCR was used to assess mRNA levels. The data were normalized to actin mRNA levels, and WT was set to 1.0. Error bars in (B, C, and H) represent S.E.M., N=3; NS, not significant, P ≥ 0.05. Error bars in (E) represent S.E.M., N=8. Error bars in (F, G) represent S.E.M., N=4. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s unpaired t-test.

**Figure 6.** SEC24C is required for ER-phagy.

(A) Quantitation of 3xFLAG-FAM134B degradation from Torin2 treated control siRNA or SEC24 siRNA depleted U2OS cells normalized to actin. (B and D) Representative images of siRNA depleted cells treated with Torin2 and Bafilomycin A1. Quantitation of 3x-FLAG-FAM134B (C) or RTN3 (E) in LAMP1 structures. The DMSO control for each condition was set to 1.0. The data for two different siSEC24C duplexes is shown in C. Scale bar in (B and D) is 10µm. Error bars in (A, C, E) represent S.E.M., N=3-5, 50-70 cells/experiment for FAM134B and 30 cells/experiment for RTN3; NS, not significant P ≥ 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, Student’s unpaired t-test.
References


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Supplementary Materials:

Materials and methods
Figures S1-S15
Tables S1-S2
References
Figure 1
Figure 2

A) Lst1-3xGFP, Atg40-2xmCherry, Merge

B) Graph showing GFP puncta colocalized with Atg40-2xmCherry puncta (%)

C) Graph showing GFP puncta colocalized with Atg40-2xmCherry puncta (%)

D) Graphs showing RFP-Atg8 puncta colocalized with Lst1-3xGFP puncta (%), Sec24-GFP puncta (%), Sec23-GFP puncta (%), and Sec13-GFP puncta (%)

E) Merge images of WT and lnp1Δ Lst1-3xGFP, Atg40-2xmCherry

F) Graph showing RFP-Atg8 puncta colocalized with Lst1-3xGFP puncta (%), with WT and lnp1Δ
Figure 3

(A) Western blot of lysates from different conditions. Untagged lysate, Atg40-3xFLAG lysate, and Atg40-3xFLAG were loaded on lanes 1, 2, and 3, respectively. The proteins were treated with various dilutions of GST (0.1%, 0.05%, 0.025%, GST, GST-Lst1, GST-Atg40, GST-Sec13, GST-Sec24). The bands were visualized using an antibody against Atg40.

(B) Electron micrographs of wild-type (WT), atg14Δ, atg40Δ, lst1Δ strains. Arrows indicate autophagic bodies (V) and arrows indicate vacuoles (N). Scale bar: 200 nm.

(C) Graph showing the average number of ER-containing autophagic bodies per section in WT, atg14Δ, atg40Δ, and lst1Δ strains. The data is represented as mean ± SEM. *p < 0.05, **p < 0.001 compared to the WT control.

(D) Graph showing the average number of autophagic bodies per section in WT, atg14Δ, atg40Δ, and lst1Δ strains. The data is represented as mean ± SEM. *p < 0.05, **p < 0.001 compared to the WT control.
Figure 4
Figure 5

A. Western blot analysis of Atg40-3xFLAG levels with Galactose induction. + Galactose shows increased levels of Atg40-3xFLAG compared to WT and ire1Δ. ATZ-pYES2 treatment also shows a significant increase in Atg40-3xFLAG expression.

B. Quantification of Atg40-3xFLAG level (%). + Galactose treatment significantly increases Atg40-3xFLAG level compared to WT and ire1Δ.

C. Fold UPR reporter induction with DTT treatment. WT shows minimal induction, while hr1Δ, atg39Δ, atg40Δ, and lst1Δ show increased induction.

D. Western blot analysis of Atg40-3xFLAG, Kar2, Lst1, and Adh1 with different deletions. + DTT shows increased levels of Atg40-3xFLAG, Kar2, and Lst1 in WT and rpd3Δ, but not in pho23Δ.

E. Quantification of Atg40-3xFLAG level (%). WT shows a significant increase in Atg40-3xFLAG level compared to rpd3Δ and pho23Δ.

F. Quantification of Kar2 level (%). WT shows a significant increase in Kar2 level compared to rpd3Δ and pho23Δ.

G. Quantification of Lst1 level (%). WT shows a significant increase in Lst1 level compared to rpd3Δ and pho23Δ.

H. mRNA level of ATG40 and ATG9 in WT, rpd3Δ, and pho23Δ. ATG40 shows a significant increase in mRNA level in rpd3Δ compared to WT and pho23Δ.

Figure 5
Figure 6

(A) Bar graph showing the fraction of FAM134B with different conditions.

(B) Images showing FAM134B, LAMP1, Merge, and Inset for different conditions.

(C) Graph showing the relative pixel intensity of FAM134B for different conditions.

(D) Images showing RTN3, LAMP1, Merge, and Inset for different conditions.

(E) Graph showing the relative pixel intensity of RTN3 for different conditions.

Legend:
- **: p < 0.01
- ***: p < 0.001
- NS: not significant
Figure S1

A

Bars show the percentage of WT cleavage levels over time (h) with and without rapamycin treatment.

B

Western blot analysis showing Sec61-GFP, GFP, and Adh1 under different conditions.

C

Graph showing the percent of WT cleavage for different genotypes.

D

Western blot analysis of rapamycin-treated samples for Sec61-GFP, GFP, and Adh1.

E

Graph depicting the percent of WT cleavage for different genotypes under rapamycin treatment.

Figure S1
Figure S3

A

Rapamycin (h): 0 24 0 24 0 24

Rtn1-GFP

GFP

Adh1

B

Percent of WT cleavage

WT  atg40Δ  lst1Δ

C

Per33-GFP  FM4-64  Merge

WT

atg40Δ

lst1Δ

D

Cells with Per33-GFP in vacuole (Total cells (%))

WT  atg40Δ  lst1Δ

E

Rapamycin (h): 0 12 0 12 0 12

Per33-GFP

GFP

Adh1

F

Percent of WT cleavage

WT  atg40Δ  lst1Δ

Figure S3
A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>lst1Δ</th>
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<th>lst1Δ+LST1-B1</th>
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</table>

B

- **Figure S5**
- Graph showing cells with Sec61-GFP in vacuole/Total cells (%) for different conditions:
  - WT
  - lst1Δ
  - lst1Δ+LST1
  - lst1Δ+LST1-B1
  - lst1Δ+LST1-B2

C

- **Table showing Rapamycin (h):**
  - WT
  - atg40Δ
  - lst1Δ
  - lst1Δ+LST1
  - lst1Δ+LST1-B1
  - lst1Δ+LST1-B2

- **Western Blot Analysis:**
  - Sec61-GFP
  - GFP
  - Adh1

D

- **Figure S5 cont.**
- Graph showing Percent of WT cleavage for different conditions:
  - WT
  - atg40Δ
  - lst1Δ
  - lst1Δ+LST1
  - lst1Δ+LST1-B1
  - lst1Δ+LST1-B2
Figure S8

**A**

- Atg40-GFP
- Lst1-2xmCherry
- Merge

- endo 0 h
- OE 0 h
- OE 3 h

**B**

- Algal-GFP puncta colocalized with Lst1-2xmCherry puncta (%)

<table>
<thead>
<tr>
<th>Copper (h)</th>
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<td>Rapamycin (h)</td>
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<td>0</td>
<td>24</td>
</tr>
</tbody>
</table>

**C**

- Lst1-3xGFP
- Atg39-2xmCherry
- Merge

- 0 h
- 3 h
- 6 h

**D**

- Lst1-3xGFP puncta colocalized with Atg39-2xmCherry puncta (%)

<table>
<thead>
<tr>
<th>Rapamycin (h)</th>
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<th>6</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

**E**

- Rapamycin (h): 0 24 0 24 0 24
- Hmg1-GFP
- WT
- atg39 Δ
- lst1 Δ

**F**

- Percent of WT cleavage

<table>
<thead>
<tr>
<th>WT</th>
<th>atg39 Δ</th>
<th>lst1 Δ</th>
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<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>24</td>
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</tbody>
</table>

**G**

- Hmg1-GFP
- FM4-64
- Merge

- WT
- atg39 Δ
- lst1 Δ

**H**

- Cells with Hmg1-GFP in vacuole (Total cells (%))

<table>
<thead>
<tr>
<th>WT</th>
<th>atg39 Δ</th>
<th>lst1 Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>24</td>
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</table>
Figure S9

Sec24-GFP puncta colocalized with Atg40-2xmCherry puncta (%)

Coefficient of Lst1-3xGFP colocalized with Atg40-2xmCherry
Coefficient of Sec24-GFP colocalized with Atg40-2xmCherry

WT

atg40Δ

Rapamycin (h)

0 3 6

Sec24/Atg40

WT

Inp1Δ

Sec24-GFP puncta colocalized with Atg40-2xmCherry puncta (%)

Rapamycin (h)

0 3 6

Lst1-3xGFP

RFP-Atg8 Merge

WT

Inp1Δ

Atg40-3xGFP

WT

Inp1Δ

atg40Δ

WT

atg40Δ

Lst1-3xGFP

RFP-Atg8 Merge

WT

Inp1Δ

Atg40-GFP

WT

Inp1Δ

Puncta/cell

WT

Inp1Δ

NS

WT

lst1Δ

Puncta/cell

WT

Inp1Δ

NS

Figure S9
Figure S11

A) Lst1-3xGFP FM4-64 Merge

- WT
- atg1Δ
- atg40Δ

B) Cells with Lst1-3xGFP in vacuole/Total cells (%)

C) Rapamycin (h) 0 24 0 24 0 24

- Lst1-GFP
- GFP
- Adh1

D) Percent of WT cleavage

E) Atg40-GFP FM4-64 Merge

- WT
- atg14Δ
- lst1Δ

F) Cells with Atg40-GFP in vacuole/Total cells (%)

G) Rapamycin (h) 0 24 0 24 0 24

- Atg40-GFP
- GFP
- Adh1

H) Percent of WT cleavage
Figure S13
Figure S14