Selective Microautophagy of Proteasomes is Initiated by ESCRT-0 and Is Promoted by Proteasome Ubiquitylation

Running title: Selective proteasome microautophagy

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30 **Summary statement**

31 ESCRT-0 selectively targets aberrant proteasomes for microautophagy by recognition of
32 proteasome ubiquitylation status to fine-tune proteasome quality control under low glucose
33 conditions.

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Abstract

The proteasome is central to proteolysis by the ubiquitin-proteasome system under normal growth conditions but is itself degraded through macroautophagy under nutrient stress. A recently described AMPK (AMP-activated protein kinase)-regulated ESCRT (endosomal sorting complex required for transport)-dependent microautophagy pathway also regulates proteasome trafficking and degradation in low glucose conditions in yeast. Aberrant proteasomes are more prone to microautophagy, suggesting the ESCRT system fine-tunes proteasome quality control under low glucose stress. Here we uncover additional features of the selective microautophagy of proteasomes. Genetic or pharmacological induction of aberrant proteasomes is associated with increased mono- or oligo-ubiquitylation of proteasome components, which appear to be recognized by ESCRT-0. AMPK controls this pathway in part by regulating the trafficking of ESCRT-0 to the vacuole surface, which also leads to degradation of the Vps27 subunit of ESCRT-0. The Rsp5 ubiquitin ligase contributes to proteasome subunit ubiquitylation, and multiple ubiquitin-binding elements in Vps27 are involved in their recognition. We propose that ESCRT-0 at the vacuole surface recognizes ubiquitylated proteasomes and initiates their microautophagic elimination during glucose depletion.

Introduction

The ubiquitin-proteasome system (UPS) is a highly conserved proteolytic system that selectively degrades cellular proteins (Lecker et al., 2006). This pathway involves a cascade of reversible enzymatic reactions that covalently conjugates ubiquitin to substrates and targets them to the proteasome for degradation. Deubiquitylating enzymes (DUBs) remove ubiquitin from the substrates for recycling before the substrates are degraded by the proteasome into small peptides (Tomko and Hochstrasser, 2013). The 26S proteasome is composed of the 20S core particle (CP) and the 19S regulatory particle (RP), and the RP in turn comprises base and lid subcomplexes (Budenholzer et al., 2017). Dysfunction of the UPS has been implicated in the pathogenesis of many human diseases, including various cancers and neurodegenerative disorders (Thibautaud and Smith, 2019).

Proteasomes are dynamic structures that change when cells are in distinct nutrient environments. Proteasomes are concentrated in the nucleus in yeast and most mammalian cells under rich nutrient conditions (Enenkel, 2014) but relocate to the cytoplasm and form membraneless proteasome storage granules (PSGs) under carbon starvation (Laporte et al., 2008). PSG formation occurs along with reorganization of many other cellular components into granules and foci in response to stress conditions; as with many other cellular granules, PSGs dissipate rapidly once favorable conditions are resumed, with proteasomes reentering the nucleus (Narayanaswamy et al., 2009; Sagot and Laporte, 2019).

Certain types of starvation can also lead to proteasomes being degraded by macroautophagy, likely in a nonselective manner (Li and Hochstrasser, 2020; Marshall et al., 2016; Marshall and Vierstra, 2018; Nemec et al., 2017; Waite et al., 2016). In macroautophagy, portions of the cytoplasm are engulfed by a double-bilayer membrane structure that matures into a closed vesicle called the autophagosome. The autophagosome then fuses with the lysosome (yeast vacuole) membrane, releasing a single large membrane vesicle, the autophagic body (AB), to the lysosome interior. The AB membrane and contents are then broken down by lysosomal hydrolases.

Recently, we have identified an AMPK (AMP-activated protein kinase)-regulated ESCRT (endosomal sorting complex required for transport)-dependent microautophagy pathway of proteasome degradation that occurs concomitantly with PSG formation under low glucose conditions (Li et al., 2019). The ESCRT pathway is a widely deployed

mechanism for topologically similar membrane remodeling events such as multivesicular body (MVB) formation, enveloped virus budding, and cell scission (Hurley, 2015). Interestingly, aberrant proteasomes are more likely to be removed by ESCRT-dependent microautophagy than to be stored reversibly in PSGs under these conditions. These findings suggest an important role for AMPK signaling and ESCRT-mediated membrane remodeling in proteasome quality control when glucose levels are low (Li and Hochstrasser, 2020; Segev, 2020).

AMPK and ESCRTs are highly conserved protein complexes participating in diverse cellular processes (Coccetti et al., 2018; Vietri et al., 2020). AMPK is a heterotrimeric complex, composed of one catalytic α subunit and two regulatory subunits, β and γ (Ghillebert, 2011). The kinase is a master regulator of cellular energy homeostasis and is activated by glucose limitation and other stress conditions. In cells, AMPK is regulated by phosphatases and upstream kinases, as well as by autoinhibition of the α subunit (Coccetti et al., 2018). Its activity is altered in many disease states, such as inflammation, diabetes and cancer, and a variety of compounds have been developed to regulate AMPK activity in human disease treatment (Carling, 2017).

The ESCRT factors are part of at least five distinct complexes, ESCRT-0, I, II, III and Vps4 (Schmidt and Teis, 2012). These complexes mediate a series of membrane binding and remodeling steps, starting with target protein recognition and enrichment by ESCRT-0, I and II and leading to membrane remodeling by ESCRT-III and Vps4 (Schmidt and Teis, 2012; Vietri et al., 2020). These steps can occur at different cellular membranes. To understand the roles of ESCRTs in determining the selectivity of proteasome microautophagy, we focused on Vps27, which along with Hse1, forms the *Saccharomyces cerevisiae* ESCRT-0 complex (Bilodeau et al., 2002). Vps27 is regarded as a “gateway protein” for cargo protein recognition and downstream ESCRT complex assembly (Schmidt and Teis, 2012).

In the current study, we identify a mechanistic role for ESCRT-0 in selective proteasome microautophagy. Specifically, proteasomes in cells subjected to low glucose stress are preferentially modified by low molecular weight (LMW) ubiquitylation of proteasome subunits. Multiple ubiquitylating enzymes contribute to this pathway; we could show that the essential Rsp5 ubiquitin ligase is involved and that the N-lobe noncovalent

ubiquitin-binding site in Rsp5 catalytic domain contributes as well. Proteasomes ubiquitylated in this way can be recognized by the ubiquitin-binding domains (UBDs) in the Vps27 subunit of ESCRT-0 and are subsequently cleared through ESCRT-dependent microautophagy. Our data suggest a model in which AMPK functions upstream in the ESCRT-dependent microautophagy pathway under low glucose conditions by regulating Vps27 subcellular trafficking and vacuolar degradation.

Results

AMPK promotes Vps27 vacuolar trafficking and degradation in low glucose conditions

To understand how AMPK is involved in ESCRT-dependent microautophagy, we examined steady state levels of Vps27 in various mutants under low glucose (0.025% C) and glucose-free (–C) conditions. Vps27 is known to relocate from endosomes to the vacuole membrane when cultured yeast cells deplete the glucose supplied in the medium (diauxic shift), and this ESCRT-0 subunit is necessary for microautophagy (Oku et al., 2017). To determine if Vps27 is also degraded in the vacuole during this process, we inactivated vacuolar proteases by deleting the *PEP4* and *PRB1* genes (Hecht et al., 2014). As shown in Fig. 1A (lane 8 vs. lane 1), disappearance of Vps27 was greatly decreased in this double mutant. Moreover, AMPK was also essential for Vps27 degradation since deletion of either the Snf1 or Snf4 subunits led to strong Vps27 accumulation (Fig. 1A, lanes 2, 3).

Interestingly, Atg8 and Vps4 made quantitatively distinct contributions to Vps27 degradation in low glucose medium (top), but their loss had similar effects under glucose-free conditions (bottom) (Fig. 1A, lanes 4, 5). Specifically, Vps27 depletion was only weakly impaired in the *atg8Δ* strain under the former conditions, whereas *vps4Δ* cells had a greater defect. Atg8 is essential for macroautophagy but is dispensable for microautophagy; under low glucose conditions, defects in microautophagy due to loss of the Vps4 ESCRT factor are much more pronounced (Li et al., 2019). These results suggest

that Vps27 degradation occurs primarily via the ESCRT-dependent microautophagy pathway under low glucose conditions.

To investigate the requirement for other ESCRT components in Vps27 degradation, we checked Vps27 steady-state levels in ESCRT-I (Vps28), -II (Vps25), and -III (Snf7) mutants. Vps27 degradation was inhibited in *snf7Δ*, but not in *vps28Δ* or *vps25Δ* cells, suggesting that Vps27 degradation does not go through the canonical MVB pathway, which requires all the major ESCRT factors, including ESCRT-I and ESCRT-II (Fig. 1B). We also tested if degradation of Vps27 depended on proteasome activity under these conditions by treating cells with the MG132 inhibitor. The results in Fig. 1B indicated that proteasomes were not involved in Vps27 depletion.

To visualize whether AMPK mutations affect Vps27 subcellular trafficking, we fused a yeast codon-optimized enhanced green fluorescent protein (yEGFP) to the N-terminus of Vps27 in the context of the normal *VPS27* promoter and terminator, and chromosomally integrated the *yEGFP-VPS27* fragment into the *URA3* locus of *vps27Δ* cells. When wild-type (WT) cells were grown in low glucose for one or four days, the yEGFP-Vps27 fusion protein trafficked to the vacuole where it was degraded but left an intact yEGFP cleavage fragment (Fig. 1E); this fragment could also be visualized in the vacuole by yEGFP fluorescence (Fig. 1C). GFP is known to be resistant to vacuolar protease digestion (Klionsky et al., 2021). By contrast, in AMPK mutant cells under the same conditions, yEGFP-Vps27 failed to reach the vacuole and instead concentrated in cytoplasmic granules; these granules underwent what appeared to be rapid docking and separation events (Fig. 1C-1E; highlighted with dashed square in Movie 1, which depicts a *snf1Δ* cell after one day in low glucose). Therefore, under glucose-limiting conditions, AMPK is required for the trafficking of Vps27 to the vacuole where it would normally be degraded.

Given the punctate cytoplasmic localization of Vps27 in AMPK mutants (Fig. 1C), we examined whether Vps27 cytoplasmic granules colocalized with proteasomal foci under glucose starvation. Interestingly, Vps27 puncta frequently colocalized with proteasomal foci in AMPK mutant cells after four days in low glucose, but not under glucose-free conditions (Fig. S1). Hence, Vps27 might function directly in proteasome

quality control by selecting proteasomes for ESCRT-dependent microautophagy in low glucose.

Ubiquitin-binding domains of Vps27 regulate its trafficking and degradation

Vps27 contains multiple well-studied functional domains, including the VHS (Vps27, Hrs, and STAM) UBD and two UIMs (ubiquitin-interacting motifs) (Bilodeau et al., 2002) (Fig. 2A). It also has several proline-threonine-alanine-proline-like (PTAPL) motifs for interaction with Vps23 and ESCRT-I complex recruitment (Bilodeau et al., 2003; Katzmann et al., 2003); a FYVE (Fab-1, YOTB, Vps27, and EEA1) domain that binds phosphatidylinositol-3'-monophosphate (PI3P)-rich membranes (Gaullier et al., 1998); and a C-terminal clathrin-binding domain (Bilodeau et al., 2003). We tested whether simultaneous mutation of the Vps27 UBDs, vps27-VHS-UIM (Ren and Hurley, 2010), affected vacuolar degradation of Vps27 in low glucose. Notably, few apparent yEGFP cleavage products were detected, yet yEGFP-vps27-VHS-UIM loss was still apparent (Fig. 2B). This suggested that vps27-VHS-UIM degradation might involve a pathway other than autophagy. Indeed, disappearance of the mutant protein, unlike the WT version, was inhibited in MG132-treated *pdr5Δ* cells, and this occurred regardless of whether Vps27 was fused to yEGFP (Fig. 2B, E).

Membrane association and vacuolar trafficking of yEGFP-vps27-VHS-UIM also appeared to be disrupted (Fig. 2C, D). In cells grown in rich medium, WT yEGFP-Vps27 concentrated in small foci or granules, which are assumed to be endosomes (Hatakeyama et al., 2019; Katzmann et al., 2003), whereas the mutant derivative displayed a more diffuse pattern, which correlates with cargo protein-sorting defects (Ren and Hurley, 2010). In low glucose, WT yEGFP-Vps27 did not accumulate appreciably within cytoplasmic granules even in the presence of MG132; proteasome inactivation slightly reduced vps27-VHS-UIM granule staining (Fig. 2C, D). These results confirmed that the Vps27 UBDs, and presumably ubiquitin binding, are needed for efficient Vps27 recruitment to endosomes and thus proper cargo sorting, as previously reported (Ren and Hurley, 2010). They also suggest that these domains modulate the interaction of Vps27 with proteasomes during carbon limitation.

Vps27, as well as components of all the other ESCRT complexes, are essential for proteasome microautophagy during low-glucose stress (Li et al., 2019). This can be followed by the fragmentation of proteasome subunits into GFP-sized and larger fragments; these more complex fragmentation patterns are specifically seen when microautophagy is active, although the basis of this is unclear (Li et al., 2019). They nevertheless provide a useful signature for microautophagy-mediated proteasome degradation and likely other substrates such as Vps27 itself, e.g., Fig. 2B.

It is striking that despite the sharp drop in overall Vps27 levels during carbon limitation, Vps27-dependent proteasome microautophagy, as measured by subunit fragmentation, continued (Fig. 2E). In the *vps27-VHS-UIM* mutant, proteasome fragmentation was modestly reduced under these conditions, while as noted above, the *vps27-VHS-UIM* protein itself was stabilized. Inhibition of proteasome activity generally enhances detection of fragmented proteasome subunits (Li et al., 2019), so we determined if MG132 still affected the degree of subunit fragmentation in the *vps27-VHS-UIM* mutant. As shown in Fig. 2E, in glucose-starved proteasome-inhibited cells, a moderate reduction in proteasome subunit fragmentation was seen compared to similarly inhibited WT cells. At the same time, PSG formation, in particular that of RP-containing PSGs, was significantly reduced in MG132-treated *vps27-VHS-UIM* cells accompanied by enhanced nuclear proteasomes (Figs. 2F, S2). From these results, we hypothesize that proteasomes, particularly aberrant or inactive ones, are recognized by Vps27 for microautophagy during glucose starvation and that this requires the Vps27 UBDs for full efficacy.

Proteasome subunit fragmentation is ubiquitin dependent

To begin testing this hypothesis, we checked whether ubiquitin affected proteasome subunit degradation, since ubiquitylation plays an important role in cargo protein recognition by ESCRTs (Shields and Piper, 2011). Doa4 is a DUB enzyme that participates in the ESCRT pathway by removing ubiquitin from ubiquitylated cargos (Amerik et al., 2000), while deleting *DOA4* disrupts ubiquitin homeostasis and causes ubiquitin depletion from cells (Swaminathan et al., 1999). The microautophagy-specific proteasome subunit fragmentation pattern was lost in the *doa4Δ* mutant, while generation of free GFP from GFP-tagged subunits increased (Fig. 3A), presumably due to concomitant macroautophagy

(Li et al., 2019). The WT fragmentation pattern was restored by ectopically expressing ubiquitin (Fig. 3A), suggesting microautophagy of proteasomes is ubiquitin dependent. As expected, Doa4 DUB activity is required for subunit fragmentation as the Doa4 catalytically dead mutant *doa4-C571S* (Papa and Hochstrasser, 1993) failed to rescue the defect (Fig. 3B).

Mutations of ESCRT components interact genetically with *doa4Δ* and suppress the ubiquitin depletion phenotype in cells lacking active Doa4 (Swaminathan et al., 1999). The microautophagy-specific proteasome fragmentation pattern was restored in *doa4Δ vps27Δ* and *doa4Δ vps4Δ* cells to the levels of the *vps27Δ* and *vps4Δ* single deletions (Fig. 3C); this partial suppression was expected because these ESCRT components are themselves necessary for normal proteasome fragmentation in low glucose conditions (Li et al., 2019). AMPK is also necessary for proteasome microautophagy (Li et al., 2019), but loss of AMPK, unlike loss of the ESCRT factors, did not suppress the fragmentation defects of *doa4Δ* cells, although it did suppress the hyperaccumulation of free GFP (Fig. S3). This could be due to the more penetrant effect on proteasome fragmentation of AMPK (Li et al., 2019). Overall, these results suggest that ubiquitin is important for ESCRT-mediated proteasome sorting and degradation by microautophagy.

LMW ubiquitylation of proteasomes is observed in ESCRT mutants

Using a nanobody to GFP, we directly examined proteasome ubiquitylation by co-immunoprecipitation (co-IP) from extracts of cells expressing GFP-tagged proteasomes. Interestingly, we observed a distinct low molecular weight (LMW) ubiquitylation pattern of proteasome subunits in ESCRT mutants along with a reduction in high molecular weight (HMW) conjugates; the latter are likely to be noncovalently bound ubiquitylated proteasome substrates (Figs. 4, S4A). A broad range of LMW ubiquitylated species also accumulated in total cell lysates of ESCRT mutants; however, we attributed this to the known accumulation of Lys63-linked ubiquitin chains conjugated to cargo proteins that is caused by the block in the MVB pathway in these ESCRT mutants (Lauwers et al., 2009). Importantly, no ubiquitin-K63 chain conjugates were detected in the proteasome co-IPs (Fig. S4B); consistent with this, in the IP samples, we detected a small number of ubiquitin-ubiquitin linkages by mass spectrometry (MS) and all involved K48. Proteasome

ubiquitylation was also found to be higher following large-scale affinity purifications from ESCRT mutants expressing a 3xFLAG-tagged Rpn11 RP subunit (Fig. S5). Proteasomes that were salt washed (Marshall et al., 2016) retained the LMW ubiquitylated species (Fig. S4C). Finally, besides ubiquitin, proteasome subunits and proteasome-associated proteins such as RP assembly chaperones were the dominant proteins identified by MS analysis from the immunoprecipitated proteasomes from WT and ESCRT mutants (Fig. S6, Table S1), also arguing against ubiquitylated substrates bound to proteasomes being major contributors to the LMW ubiquitylation species in ESCRT mutants. Overall, these results support the idea that the LMW ubiquitylated species are primarily ubiquitin-modified proteasome subunits that accumulate in ESCRT mutants under low glucose conditions.

Aberrant proteasomes are subject to LMW ubiquitylation and cleared through ESCRT-0

To test the hypothesis that aberrant or incompletely assembled proteasomes are preferred substrates for ubiquitylation, we performed proteasome ubiquitylation analyses in proteasome mutants (*pre9Δ* and *sem1Δ*) that bear high amounts of aberrant proteasomes (Tomko Jr and Hochstrasser, 2014; Velichutina et al., 2004) as well as in MG132-treated *pdr5Δ* cells containing catalytically inhibited proteasomes. As predicted, LMW ubiquitylated proteasome species were enriched in *pre9Δ* and MG132-treated *pdr5Δ* cells (Fig. 5A). This was not the case for *sem1Δ* cells; this mutation limits 26S proteasome egress from the nucleus and prevents PSG accumulation in low glucose (Li et al., 2019), suggesting proteasome subunit ubiquitylation occurs after nuclear export. Conversely, ubiquitylation of proteasomes was almost abolished in *doa4Δ* cells, which have very little free ubiquitin (Fig. 5A), in line with the role of ubiquitin in proteasome fragmentation (Fig. 3). These results are consistent with aberrant proteasomes being more strongly modified by LMW ubiquitylation.

Next, we tested whether ESCRT-0 subunit Vps27 interacted with proteasomes. Given that endogenous Vps27 was also significantly degraded by microautophagy in low glucose (Fig. 1) and that ESCRT-0-proteasome interaction is likely transient, we made constructs for constitutive overexpression of HA-tagged Vps27 and a mutant vsp27-VHS-UIM-PTAPL construct expressed from the strong *GPD* promoter to facilitate detection of

Vps27-proteasome interaction. The vps27-VHS-UIM-PTAPL mutant protein (Fig. 2A) was designed to reduce or eliminate the ubiquitin-binding ability by mutating the Vps27 UBDs and simultaneously disturbing its association with downstream ubiquitin-binding ESCRT complexes (Bilodeau et al., 2002; Bilodeau et al., 2003; Katzmann et al., 2003). Multiple UBDs have been identified in ESCRT components, including Hse1 and Vps27 in ESCRT-0, Vps23 and Mvb12 in ESCRT-I, and Vps36 in ESCRT-II (Schmidt and Teis, 2012). The Vps27 PTAPL motifs interact with Vps23 for ESCRT-I recruitment, thereby increasing sorting efficiency of ubiquitylated cargo proteins (Bilodeau et al., 2003; Katzmann et al., 2003). With *GPD*-driven expression, HA-Vps27 and HA-vps27-VHS-UIM-PTAPL could be detected in cell lysates from *vps27Δ* cells grown under low glucose conditions (Fig. 5B). No ATP was added to the IP lysis buffer, which favored CP-RP dissociation so that any preference of Vps27 for binding CP or RP could be determined.

Vps27 interacted with the proteasome RP complex, as demonstrated by its co-IP by anti-GFP antibodies when RP subunits Rpn5 or Rpn2 were tagged with GFP; binding was not detected with the CP, using CP subunit Pre10-GFP for co-IP (Fig. 5B). Surprisingly, the vps27-VHS-UIM-PTAPL mutant co-precipitated more RP complex than did WT Vps27, but there was also more of the mutant ESCRT subunit in the total lysates (Fig. 5B). This suggests that Vps27 can likely interact with the RP in ways that are independent of RP ubiquitylation and also that vps27-VHS-UIM-PTAPL may be less susceptible to degradation, possibly by stalling microautophagy. Consistent with this interpretation, the difference between WT and mutant Vps27 binding to the RP complex was similar in *vps4Δ* cells (Fig. 5C), which cannot carry out microautophagy (Li et al., 2019).

Vps27 interacts with Hse1 to form the ESCRT-0 complex (Bilodeau et al., 2003; Ren and Hurley, 2010). Hse1 is unable to bind ubiquitin *in vitro* in the absence of Vps27 (Bilodeau et al., 2002). We checked whether overproduced Hse1 was sufficient to interact with proteasomes *in vivo*. Using *GPD*-driven Hse1-HA expression in *hse1Δ* cells, we did not detect any interactions with proteasomes, nor did levels of LMW ubiquitylated proteasome species change (Fig. S7). However, when both Vps27 and Hse1 were overproduced, Hse1 co-precipitated with the RP along with Vps27 (Fig. 5D), suggesting that the ESCRT-0 complex binds the RP via its Vps27 subunit.

We predicted that a Vps27 mutant, such as *vps27-VHS-UI-M-PTAPL* that disturbed the ubiquitin-binding ability of ESCRT components, should accumulate (aberrant) proteasomes that are modified by LMW ubiquitylation under glucose-limited conditions. To reconstitute endogenous levels of Vps27, we created a set of strains with either WT *VPS27* or different *vps27* mutant alleles (*vps27-VHS-UI-M*, *-PTAPL*, *-VHS-UI-M-PTAPL*) integrated at the chromosomal *URA3* locus; the alleles were under the control of the natural *VPS27* promoter and terminator in *vps27Δ* cells that also expressed GFP-tagged proteasome subunits (Pre10 or Rpn5). Integration of the empty vector served as a negative control. Based on proteasome ubiquitylation analysis, LMW ubiquitylated proteasome species accumulated in the *vps27-VHS-UI-M-PTAPL* and *vps27-VHS-UI-M* strains but not in *VPS27* or *vps27-PTAPL* cells (Fig. 5E). This suggests that proteasomes modified by LMW ubiquitylation accumulate when these ubiquitylated forms cannot be recognized by ESCRT-0.

It should be noted that these mutants will also block other ESCRT-0-dependent mechanisms such as the endosomal-MVB pathway (Bilodeau et al., 2003; Katzmann et al., 2003; Ren and Hurley, 2010). A distinctive ladder of ubiquitylated species accumulated in the mutants, as seen in the total lysates in Fig. 5E. These may be K63-linked ubiquitin oligomers that are important in the MVB pathway but appear to play little or no role in ubiquitylation of proteasomes (Fig. S4B). Because of their abundance, a fraction may nevertheless bind to proteasomes under the conditions used for the experiment in Fig. 5E.

Ubiquitin-ESCRT binding is essential for ILV formation and proteasome microautophagy

We next examined whether the above Vps27 mutants also affected proteasome trafficking and degradation in the vacuole. We used the same set of yeast strains (Fig. 5E) and checked proteasome fragmentation (as a biochemical signature of microautophagy) and trafficking. Agreeing well with the data from *vps27-VHS-UI-M pdr5Δ* cells (Fig. 2E), a minor proteasome fragmentation defect was observed in *vps27-VHS-UI-M* cells (Fig. 6A). As predicted from proteasome ubiquitylation (Fig. 5E), *vps27-PTAPL* cells did not have any defects in proteasome fragmentation; by contrast, a much stronger defect in proteasome fragmentation was detected in *vps27-VHS-UI-M-PTAPL* cells, particular after four days of

glucose deprivation (Fig. 6A), indicating the *vps27-VHS-UIM-PTAPL* mutations disrupted ESCRT-dependent microautophagy of proteasomes to the same extent as *vps27Δ* cells.

As noted above, Vps23 (ESCRT-I) directly associates with Vps27 by binding its PTAPL motifs (Bilodeau et al., 2003; Katzmman et al., 2003). We created a *vps27-VHS-UIM vps23Δ* strain, which retains the PTAPL elements in Vps27, and determined whether this mutant mimicked the proteasome microautophagy defect of *vps27-VHS-UIM-PTAPL VPS23* cells. Interestingly, an additive defect in proteasome fragmentation was observed in *vps27-VHS-UIM vps23Δ* as compared to the individual *vps23Δ* and *vps27-VHS-UIM-PTAPL* mutants, especially in low glucose conditions, at early times (Fig. 6A, left panel, e.g. lane 7 vs. lanes 5, 6 in the set of Rpn2-GFP strains), suggesting that these ESCRT components function collaboratively to recognize proteasomes for microautophagy.

Cytological analyses by fluorescence and immunogold electron microscopy further demonstrated equivalent defects in proteasome trafficking and degradation in *vps27-VHS-UIM-PTAPL* and *vps27Δ* cells. Rather than forming spherical and bright PSGs as in *VPS27* (WT) cells, irregular and faint proteasome puncta were observed in the cytoplasm, particularly at the perivacuolar region of *vps27-VHS-UIM-PTAPL* and EV (*vps27Δ*) cells (Fig. 6B), suggesting an accumulation of aberrant proteasomes in the glucose-limited cells. To preserve autophagic body (AB) and intraluminal vesicle (ILV) structures generated by macroautophagy and microautophagy, respectively, under low glucose stress, the chromosomally integrated *VPS27* and *vps27-VHS-UIM-PTAPL* alleles (at the *URA3* locus) were placed in a *vps27Δ pep4Δ prb1Δ* background. Although ILV formation in the vacuole was previously observed with a Vps27-Hse1 complex lacking any intact UIMs (Bilodeau et al., 2002), ILV formation was blocked and only ABs were observed in *vps27-VHS-UIM-PTAPL* and *vps27Δ* (EV) cells, while both ILVs and ABs were observed in *VPS27* cells (Fig. 6C, D). This phenotype is reminiscent of the microautophagy defect in *vps4Δ* cells (Li et al., 2019). Proteasome distribution in the vacuole was followed by anti-CP antibodies labeled with gold beads (black arrows in Fig. 6C). A comparable proteasome distribution pattern was found in *vps27-VHS-UIM-PTAPL* and *vps27Δ* (EV) cells (Fig. 6E, F). The results suggest that the ubiquitin-binding ability of ESCRTs is essential for ILV formation and thus proteasome microautophagy.

Rsp5 ubiquitin ligase is involved in proteasome ubiquitylation

Given the intriguing ubiquitylation pattern of proteasomes in ESCRT mutants, we sought to find the ubiquitin ligase(s) responsible for their ubiquitylation. Rsp5 is the principal ligase involved in cargo protein ubiquitylation in the ESCRT-dependent endosomal/MVB pathway of protein targeting to the vacuole and is also known to interact with ESCRT-0 (MacDonald et al., 2020). Rsp5 belongs to the NEDD4 family of HECT (homologous to E6AP C-terminus) ligases (Shah and Kumar, 2021). NEDD4 ligases share an N-terminal lipid-interacting C2 domain, 1-4 central WW protein interaction motifs (Chen and Sudol, 1995), and a large C-terminal catalytic HECT domain; the HECT domain bears an N-lobe with a noncovalent ubiquitin-binding site and a C-lobe with a covalent (catalytic) ubiquitin-binding site (Finley et al., 2012; Shah and Kumar, 2021).

We tested whether Rsp5 contributed to proteasome ubiquitylation under low glucose conditions. Rsp5 is essential for cell viability in yeast, so we performed the analysis in a temperature sensitive (*ts*) mutant, *rsp5-1*, which has an L733S mutation that causes dysfunction of Rsp5 at non-permissive temperatures (Wang et al., 1999). Proteasome ubiquitylation was inhibited in *rsp5-1* at elevated temperature (30°C) but was comparable to WT at room temperature (RT, ~24°C) (Fig. 7A). The defects in proteasome ubiquitylation correlated with reduced proteasome subunit fragmentation in *rsp5-1* (Fig. S8). Fragmentation of RP subunits Rpn5 and Rpn2 was more sensitive to defective Rsp5 compared to the Pre10 CP subunit, suggesting the RP is the main ubiquitylation target for initiating proteasome microautophagy.

To verify the importance of Rsp5 for proteasome ubiquitylation, we made a set of Rsp5-expressing constructs on CEN plasmids carrying *RSP5*, *rsp5-L733S*, and *rsp5-F618A* to disable the HECT domain noncovalent ubiquitin-binding site (French et al., 2009) and placed them in an *rsp5Δ* yeast background. As expected, plasmid-borne *rsp5-L733S* caused defects in proteasome ubiquitylation comparable to those seen in *rsp5-1* cells at 30°C (Fig. 7A, B). The *rsp5-F618A* mutant also displayed defects in proteasome ubiquitylation (Fig. 7B), indicating that noncovalent ubiquitin binding by the Rsp5 HECT domain is important for proteasome ubiquitylation. Together, these results implicate the Rsp5 ligase in proteasome ubiquitylation in cells subject to low glucose stress.

Discussion

Previously, we had identified a novel proteasome degradation pathway in low glucose conditions involving AMPK-regulated ESCRT-dependent microautophagy (Li et al., 2019). In this study, we sought to understand more about AMPK regulation and the mechanistic role of ESCRTs in proteasome microautophagy. In low carbon conditions, we find that AMPK promotes Vps27 protein turnover and its trafficking to the vacuole (Fig. 1). Vps27 is essential for ILV generation in vacuoles during microautophagy (Fig. 6C, D). Genetic or pharmacological induction of aberrant or inactive proteasomes leads to increased LMW ubiquitylation of these particles in cells under low glucose stress. Vps27 (ESCRT-0) appears to play an essential role in recognizing proteasome ubiquitylation and eliminating aberrant proteasomes through microautophagy (Figs. 4-6). The Rsp5 ubiquitin ligase contributes to proteasome ubiquitylation in this proteasome quality control pathway (Fig. 7A, B). Our results are summarized in the working model in Fig. 7C.

Exactly how AMPK regulates ESCRT-dependent microautophagy has yet to be determined. Previous studies have shown that TORC1 (Target of Rapamycin Complex 1) phosphorylates and antagonizes the function of Vps27 in microautophagy (Hatakeyama et al., 2019), while AMPK negatively regulates TORC1 activity through phosphorylation and aggregation of its Kog1 subunit, which results in disassembly of the TORC1 complex during glucose starvation (Hughes Hallett et al., 2015). The AMPK and TORC1 signaling pathways have interlinked and generally antagonistic effects (González et al., 2020). For ESCRT-dependent microautophagy in low glucose conditions, AMPK may inhibit TORC1 and thus relieve the suppressive effects of TORC1 on this process. It is also possible that AMPK competes with TORC1 and functions directly on ESCRT components for microautophagy initiation or activation, which would be reminiscent of the role of AMPK in macroautophagy (Kim et al., 2011).

Ubiquitylation of proteasomes could fine-tune their function in various ways, such as protein-proteasome interaction, proteasome activity, and cellular localization (Kors et al., 2019). At least 12 yeast proteasome subunits are known to be ubiquitylated *in vivo* and over 19 ubiquitylation sites have been detected (Hirano et al., 2015; Starita et al., 2012; Swaney et al., 2013). Only a small set of growth conditions and methods for proteasome purification have been reported in these studies, so the real numbers are likely to be

substantially higher. Previous studies on possible functions of proteasome ubiquitylation are limited as well. In human prostate cancer cells, ubiquitylation of the CP subunit $\alpha 2$ favors the binding of delta-aminolevulinic acid dehydratase (ALAD); ALAD binds in place of 19S RP and functions as an endogenous proteasome inhibitor (Schmitt et al., 2016). RP ubiquitylation can also regulate proteasome activity. For example, polyubiquitylation of Rpn13 interferes with proteasome binding and degradation of ubiquitin-conjugated substrates in human cell lines (Besche et al., 2014), while monoubiquitylation of Rpn10 can limit the capacity of the UIM of Rpn10 to interact with substrates (Isasa et al., 2010). In our study, it is intriguing that aberrant proteasomes are preferably subject to LMW ubiquitylation (Fig. 5A), which appears to serve as a sorting signal for ESCRT-dependent clearance by microautophagy. It is unknown whether aberrant proteasomes are targeted by ESCRT-0 after their incorporation into PSGs or by bypassing PSGs altogether.

Ubiquitylation also plays a key role in proteasome macroautophagy (Marshall et al., 2016). The exact nutrient stress conditions help determine the distinct routes of proteasome autophagy in cells, and different proteasome ubiquitylation patterns may allow the diversification of proteasome autophagy routes, including the selective removal of aberrant or inactive proteasomes. Nitrogen starvation normally triggers nonselective proteasome macroautophagy that does not appear to involve extensive proteasome ubiquitylation (Marshall et al., 2016). By contrast, low glucose levels can simultaneously induce proteasome macroautophagy and microautophagy, and the latter appears to favor the removal of aberrant or inactive proteasomes that are either directly modified by LMW ubiquitylation or tightly bound to proteins that are modified in this way.

Chemical inactivation of proteasomes in yeast cells grown under nonstarvation conditions triggers a selective proteasome macroautophagy pathway accompanied by extensive ubiquitylation of the inactivated proteasomes (Marshall et al., 2016). Although proteasome ubiquitylation is a common feature in determining the selectivity of proteasome microautophagy and macroautophagy, the molecular components involved in these two selective proteasome autophagy pathways are different. For instance, the Cue5 autophagy receptor and the Hsp42 chaperone are engaged in the recognition of inactivated proteasomes for selective macroautophagy (Marshall et al., 2016) but are dispensable for selective proteasome microautophagy (JL and MH, unpublished data). On the other hand,

the ESCRT-0 complex is involved in the recognition of aberrant proteasomes accumulated under low glucose conditions; its role in selective proteasome macroautophagy has not been examined. Insofar as ESCRTs are required in nonselective proteasome macroautophagy upon nitrogen starvation, we would predict ESCRTs are also involved in selective proteasome macroautophagy but likely in a different way than in microautophagy, such as autophagosome closure (Zhou et al., 2019).

Multiple components of the ESCRT-0, -I, and -II complexes contain UBDs, which provides structural plasticity for a wide variety of ubiquitylated cargo recognition and sorting mechanisms (Schmidt and Teis, 2012; Shields et al., 2009; Shields and Piper, 2011). In addition, having multiple UBDs can boost the avidity for ubiquitylated cargos as individual ESCRT UBDs have low affinity for ubiquitin; this might also allow recognition of very large cargo protein complexes with complicated ubiquitylation patterns (Piper et al., 2014), such as the LMW ubiquitylation of proteasomes we report here (Figs. 4, 5).

We still detected Vps27-proteasome RP interaction when ubiquitin binding by Vps27 or ESCRT-0 should have been eliminated, as in the *vps27-VHS-UIM-PTAPL* mutant (Fig. 5B-E). This suggests that ESCRT-0 can also interact with proteasomes in ways that are independent of RP ubiquitylation. It is possible that alternative binding partners may participate in these ESCRT-0-proteasome interactions. Nevertheless, the *vps27-VHS-UIM-PTAPL* mutation blocked ILV formation in the vacuole lumen to a degree comparable to the deletion mutant of *VPS27* (Fig. 6). This implies that ubiquitin binding by ESCRT-0 is essential for microautophagy under carbon-limiting conditions. We note that the *vps27-PTAPL* mutant, which is defective for ESCRT-I and ESCRT-II binding, did not cause any major defects in proteasome degradation (Figs 5E, 6A), suggesting ESCRT-0 has additional sites for recruiting these downstream complexes, in line with previous findings (Shields and Piper, 2011).

In conclusion, we provide evidence here that ESCRT-0 functions in recognizing aberrant proteasomes through a proteasome ubiquitylation signature that makes these proteasomes favored targets for microautophagic elimination under low glucose stress, thereby ensuring that normal proteasomes, sequestered in PSGs, are readily available once a new source of glucose has been secured to allow a return to growth.

Materials and Methods

Yeast strains and cell growth

Yeast genetic manipulations were conducted using standard protocols (Dunham et al., 2015). Yeast strains used in this study are listed in Table S2. Unless specified in the figure legends, yeast cells were grown overnight at 30°C with vigorous agitation in synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 0.002% adenine, 0.004% tryptophan, 0.002% uracil, and 2% glucose); tryptophan and uracil single or double dropout media were used for plasmid selection. Cells were then back-diluted in fresh SC medium and grown to mid-exponential phase. The cells were pelleted and rinsed once with sterile water, followed by different treatments. For glucose starvation, cells were resuspended in SC medium containing 0.025% glucose or lacking glucose and cultured for ~1 day or/and ~4 days at 30°C, as specified in the figures. For experiments with the proteasome inhibitor MG132, all strains included the *pdr5Δ* allele to allow efficient intracellular accumulation of the drug (Collins et al., 2010). Cells were grown in SC medium as above, and mid-log cells were resuspended in SC medium containing 0.025% glucose and DMSO or 50 μM MG132 (Santa Cruz Biotechnology, catalog # sc-201270, lot # H1219) dissolved in DMSO and cultured for ~1 day at 30°C.

Plasmid construction

Genes of interest were amplified from yeast genomic DNA and cloned into the following plasmids: pRS316 (Sikorski and Hieter, 1989), p424GPD, or p426GPD (Mumberg et al., 1995) through restriction enzyme digestion and T4 DNA ligase-mediated ligation (NEB). The resulting plasmids were verified by enzymatic digestion and DNA sequencing. Quikchange site-directed mutagenesis (Agilent) was performed to generate the *doa4-C571S* and *rsp5-L733S* and *rsp5-F618A* mutants. Plasmids used in this study are listed in Table S3.

Fluorescence microscopy

For epifluorescence microscopy, yeast cells were visualized on an Axioskop microscope (Carl Zeiss) equipped with a Plan-Apochromat 100×/1.40 oil DIC objective

lens, a CCD camera (AxioCam MRm; Carl Zeiss), and a HBO100W/2 light source. Images were taken using AxioVision software with an auto exposure setup and processed using Adobe Photoshop CC software.

For time-lapse video recordings, yeast cells were viewed on an LSM 880 Airyscan NLO/FCS confocal microscope with an Alpha Plan-Apochromat 100×/1.46 NA oil objective lens. Excitation was performed with an argon laser at 488 nm, and emission was collected in the range of 493-556 nm for GFP imaging. Time-lapse images were acquired using Airyscan 32-channel ultra-sensitive area detector and the video was processed with ZEN software.

Immunogold labeling electron microscopy

Yeast cells were fixed with 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde in PBS for 30 min at 30°C followed by further fixation in 4% PFA for 1 h at 4°C. The fixed cells were rinsed once with PBS and resuspended in 10% gelatin. The solidified blocks were trimmed and placed in 2.3 M sucrose on a rotor overnight at 4°C, and then transferred to aluminum pins and frozen rapidly in liquid nitrogen. The frozen blocks were cut into 60 nm thick sections on a Leica Cryo-EM UC6 UltraCut using the Tokuyasu method (Tokuyasu, 1973). The cell sections were placed on carbon/Formvar-coated grids and floated in a dish of PBS for immunolabeling. Grids were placed section side down on drops of 0.1 M ammonium chloride to quench untreated aldehyde groups, then blocked for nonspecific binding on 1% fish skin gelatin in PBS. Single labeled grids were incubated with a primary rabbit anti-20S antibody (Enzo Life Sciences, catalog # BML-PW9355, lot # 05101719) at 1:200 dilution, and Protein A-gold beads (10 nm; Utrecht Medical Center) were used for secondary staining. The grids were rinsed in PBS, fixed with 1% glutaraldehyde for 5 min, rinsed again and stained with a mixture of 0.5% uranyl acetate to methylcellulose. Grids were viewed under a transmission electron microscope (FEI Tecnai G2 Spirit BioTWIN) at 80 kV. Images were taken using a SIS Morada 11-megapixel CCD camera and iTEM (Olympus) software. Acquired images were processed using Adobe Photoshop CC software.

Protein extraction and Western blotting

Total proteins were extracted using the method of Kushnirov (2000), and Western blotting was performed as described previously with minor modifications (Li et al., 2016). The equivalent of one OD₆₀₀ of cells were pelleted and washed once with sterile water. Cells were incubated in 400 µl 0.1 M NaOH for 5 min at room temperature (RT) and were then pelleted and resuspended in 100 µl SDS sample buffer (10% glycerol, 2% SDS, 0.1 M DTT, 62.5 mM Tris-HCl pH 6.8, 4% 2-mercaptoethanol, 0.008% bromophenol blue) and heated at 100°C for 5 min. Cell debris was removed by centrifugation. Equal volumes of the supernatants were loaded onto 10% (v/v) SDS-PAGE gels, followed by the transfer of proteins to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, catalog # IPVH00010, lot # R0HB88840).

The membranes were incubated with the following primary antibodies: rabbit anti-Vps27 (Hatakeyama and De Virgilio, 2019) at 1:1000 dilution; JL-8 anti-GFP monoclonal antibody (TaKaRa, catalog # 632381, lot # A8034133) at 1:2,000 dilution; or an anti-Pgk1 monoclonal antibody (Abcam, catalog # ab113687, lot # GR3373682-5) at 1:10,000 dilution. Primary antibody binding was followed by anti-mouse (GE Healthcare, catalog # NXA931V, lot # 17041890) or anti-rabbit IgG (GE Healthcare, catalog # NA934V, lot # 17136627) secondary antibody conjugated to horseradish peroxidase at 1:10,000 dilution. The membranes were incubated in ECL detection reagent (Mruk and Cheng, 2011), and the ECL signals were detected using film (Thomas Scientific, catalog # 1141J52).

Immunoprecipitation and proteasome ubiquitylation analysis

Cells equivalent to 35 OD₆₀₀ units were pelleted and washed once with ice-cold sterile water. Cells were resuspended in 450 µl lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA pH 8, 200 mM NaCl, 10% glycerol, 1 mM PMSF, 10 mM NEM, Roche cOmplete protease inhibitor catalog # 11836153001). Total cell lysates were prepared by beating with acid-washed glass beads using a FastPrep-24 device at 4°C for five cycles of 30 s agitation with 1 min breaks. After addition of 600 µl lysis buffer and Triton X-100 to 0.1% final concentration in cell lysates, cellular membranes were solubilized by incubating for 30 min on ice. Crude cell lysates were cleared at 16,000 x g for 10 min at 4°C. Cleared cell lysates were incubated with 25 µl GFP-Trap agarose resin slurry (ChromoTek) for 1 h to immunoprecipitate proteasomes. The resin was washed three times with lysis buffer

containing 0.02% Triton X-100 (except that NaCl concentration was increased to 250 mM in Figure S4C) and resuspended in 50 μ l 2 x SDS sample buffer. Bound proteins were eluted by incubating the beads for 10 min at 42°C. The elutes were analyzed by Western blotting as above with the following primary antibodies: polyclonal rabbit anti-ubiquitin (Dako, catalog # Z0458, lot # 20016967) at 1:2,000 dilution; monoclonal rabbit anti-ubiquitin-K63, clone Apu3 (EMD Millipore Corp, catalog # 05-1308, lot # 3137755) at 1:1,000 dilution; or monoclonal anti-HA (Sigma, catalog # H9658, lot # 089M4796V) at 1:2,000 dilution.

Affinity purification of proteasomes

Proteasomes were affinity purified from yeast cells as described previously, with minor modifications (Li et al., 2015). Briefly, cells were harvested after growth under indicated conditions and grounded to fine powder with liquid nitrogen. About 6-7 ml of cell powders were thawed on ice, resuspended in 10 ml buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM MgCl₂, 5 mM ATP, Roche cOmplete EDTA-free protease inhibitor catalog # 11872580001), and incubated for 15 min on ice. Cell debris were pelleted at 30,000 x g for 20 min at 4°C. Total protein concentrations of the supernatants were determined using a Pierce BCA protein assay kit (Thermo Scientific, catalog # 23225, lot # SJ256254) according to the manufacturer's protocol. Supernatant equivalent to ~100 mg total protein was incubated with 200 μ l (packed) resin of anti-FLAG M2 affinity gel (Sigma, catalog # A2220, lot # SLCH0130) for 2 h on a rotator at 4°C. The proteasome-bound resin was washed twice with 12 ml buffer A for 10 min, and then incubated with 3 resin volumes of 200 μ g·ml⁻¹ 3xFLAG peptide (Sigma, catalog # F4799, lot # SLCJ4916) for 45 min to elute proteasome complexes. Proteasomes were concentrated with 100K MWCO centrifugal filters (Merk Millipore, catalog # UFC510024, lot # R9HA55989) and quantified with a BSA standard using a G:Box Chemi HR16 imager (Syngene).

Protein digestion for LC-MS/MS

Protein samples were prepared as described above in the Immunoprecipitation section and resolved on 10% SDS-PAGE gels. Gel bands were excised and fixed with two

incubations in 45% methanol and 10% acetic acid for 30 min at RT, and rinsed once with Milli Q water before the in-gel digestion procedure.

Gel bands were cut into small pieces and rinsed with 800 μ l water on a tilt-table for 10 min and then washed for 20 min with 800 μ l 50% acetonitrile (ACN)/100 mM NH_4HCO_3 (ammonium bicarbonate, ABC). The samples were reduced by incubating with 200 μ l 4.5 mM DTT in 100 mM ABC for 20 min at 37°C, then alkylated by incubating with 200 μ l 10 mM iodoacetamide in 100 mM ABC for 20 min at RT in the dark. The gels were washed for 20 min with 800 μ l 50% ACN/100 mM ABC, then washed for 20 min with 800 μ l 50% ACN/25 mM ABC. The gel pieces were briefly dried by SpeedVac, then resuspended in 200 μ l of 25 mM ABC containing 500 ng of sequencing grade trypsin (Promega, catalog # V5111) and incubated for 16 h at 37°C. Peptides in the supernatant were moved to a new Eppendorf tube, and residual peptides were extracted from the gel by the addition of 500 μ l 80% ACN/0.1% trifluoroacetic acid (TFA) and combined with the first supernatant. Peptides were dried in a SpeedVac and dissolved in 25 μ l MS loading buffer (2% ACN, 1% TFA), with 5 μ l injected for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analysis

LC-MS/MS analysis was performed on a Thermo Scientific Q Exactive Plus equipped with a Waters nanoAcquity UPLC system utilizing a binary solvent system (A: 100% water, 0.1% formic acid; B: 100% acetonitrile, 0.1% formic acid). Trapping was performed at 5 μ l·min⁻¹, 99.5% Buffer A for 3 min using a Waters ACQUITY UPLC M-Class Symmetry C18 Trap Column (100 Å, 5 μ m, 180 μ m x 20 mm, 2G, V/M). Peptides were separated at 37°C using a Waters ACQUITY UPLC M-Class Peptide BEH C18 Column (130 Å, 1.7 μ m, 75 μ m X 250 mm) and eluted at 300 nl·min⁻¹ with the following gradient: 3% buffer B at initial conditions; 5% B at 1 min; 25% B at 90 min; 50% B at 110 min; 90% B at 115 min; 90% B at 120 min; return to initial conditions at 125 min. Mass spectra was acquired in profile mode over the 300-1,700 m/z range using 1 microscan, 70,000 resolution, AGC target of 3E6, and a maximum injection time of 45 ms. Data-dependent MS/MS were acquired in centroid mode on the top 20 precursors per MS scan using 1 microscan, 17,500 resolution, AGC target of 1E5, maximum injection time of 100

ms, and an isolation window of 1.7 m/z. Precursors were fragmented by HCD activation with a collision energy of 28%. MS/MS were collected on species with an intensity threshold of 1E4, charge states 2-6, and peptide match preferred. Dynamic exclusion was set to 20 sec.

Peptide identification

Tandem mass spectra were extracted by Proteome Discoverer software (version 2.2.0.388, Thermo Scientific) and searched in-house using the Mascot algorithm (version 2.7.0, Matrix Science). The data were searched against the Swissprotein database with taxonomy restricted to *Saccharomyces cerevisiae* (7,905 sequences). Search parameters included trypsin digestion with up to 2 missed cleavages, peptide mass tolerance of 10 ppm, and MS/MS fragment tolerance of 0.02 Da. Cysteine carbamidomethylation was configured as a fixed modification. Methionine oxidation; phosphorylation of serine, threonine and tyrosine; propionamide adduct to cysteine; and GG dipeptide (ubiquitin residue) on lysine were configured as variable modifications. Normal and decoy database searches were run, with the confidence level was set to 95% ($p < 0.05$). Scaffold Q+S (version 4.11.0, Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides.

Statistical analysis

GraphPad Prism 9 was used to generate the heat map in Figure S6B and most bar graphs. Microsoft Excel 2013 was used to perform ANOVA single factor analysis and generate bar graphs in Figures 6D-6F. ImageJ was used to quantify protein band intensities in Figures 5B-5D. The number of cells counted for image quantification are described in the figure legends. Each experiment was repeated at least three times and the percentages shown in the figures represent the average of all the experiments. Error bars represent standard deviations.

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Competing interests

The authors declare that no competing interests exist.

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Fig. 1

Fig. 1. AMPK promotes vacuolar degradation of Vps27 under low glucose conditions.

(A) Anti-Vps27 immunoblot analyses in WT, AMPK α subunit mutant *snf1* Δ and γ subunit mutant *snf4* Δ , macroautophagy-defective mutant *atg8* Δ , ESCRT mutants (*vps4* Δ , *vps27* Δ), *doa4* Δ cells, and the corresponding mutant cells in the vacuolar protease-defective mutant *pep4* Δ *prb1* Δ background. Cells were harvested from cultures in SC medium containing 0.025% glucose (0.025% C as indicated in the figure panels, or as low glucose medium

hereafter) or lacking glucose (-C as indicated in the figure panels) for ~1 d at 30°C. Vps27 was degraded by vacuolar proteases under glucose starvation conditions.

(B) Anti-Vps27 immunoblot analyses in WT, *snf1Δ*, *snf4Δ*, ESCRT mutants (*vps28Δ*, *vps25Δ*, *snf7Δ*, *vps4Δ*, *vps27Δ*), *atg8Δ*, *doa4Δ*, or *pdr5Δ* cells (the last with DMSO or 50 μM MG132 treatments). Cells were harvested from cultures incubated in low glucose medium for ~1 d at 30°C. Vps27 vacuolar degradation was independent of proteasome activity and ESCRT-I (Vps28) or ESCRT-II (Vps25) components.

(C) Epifluorescence images of protein expressed from chromosomally integrated *yEGFP-VPS27* in WT and AMPK mutant cells. Cells were harvested from cultures at log phase (2% C log) or in low glucose medium for ~1 d or ~4 d at 30°C. Defective trafficking of yEGFP-Vps27 to the vacuole was observed in the mutant cells. BF, bright field. V, vacuole, indicating a representative vacuole in a cell. Scale bar, 5 μm.

(D) Percentage of cells with defective trafficking of yEGFP-Vps27 to the vacuole in WT and AMPK mutant cells as analyzed in panel (C). Cells counted at log phase for WT (n=418), *snf1Δ* (551), and *snf4Δ* (440); at day 1 low glucose medium for WT (625), *snf1Δ* (408), *snf4Δ* (405); and at day 4 low glucose medium for WT (681), *snf1Δ* (394), *snf4Δ* (422). Results plotted as mean±sd. ***, P<0.001 (ANOVA single factor analysis comparing AMPK mutant to WT at indicated growth stages).

(E) Anti-GFP immunoblot analyses of free yEGFP release from yEGFP-Vps27 in WT and AMPK mutant cells. Cells were from cultures in panel (C) at log phase and low glucose medium for ~1 d at 30°C. yEGFP-Vps27 vacuolar degradation was inhibited in AMPK mutants. Pgk1 served as a loading control. Representative blots and images are shown.

Fig. 2. Mutation of Vps27 UBDs impairs Vps27 and proteasome trafficking and degradation.

(A) Schematic of Vps27 functional domains and the indicated *vps27* mutations used in this study.

(B) Anti-GFP immunoblot analyses of free yEGFP release from yEGFP-Vps27 and an ubiquitin binding-defective Vps27 mutant (yEGFP-vps27-VHS-UIM) in WT and *pdr5Δ* cells. Endogenous *VPS27* was deleted in all the strains. Cells were harvested from cultures in exponential phase or in low glucose medium for ~1 d at 30°C. Additionally, *pdr5Δ* cells were treated with DMSO or 50 μM MG132. yEGFP-vps27-VHS-UIM degradation was affected by proteasome activity in cells under low glucose conditions.

(C) Fluorescence images of yEGFP-Vps27 and yEGFP-vps27-VHS-UIIM in *pdr5Δ* cells from panel (B). Ubiquitin-binding domains (UBDs) of Vps27 contributed to Vps27 trafficking and its degradation by autophagy and proteasomes. BF, bright field. Scale bar, 5 μm.

(D) Percentage of *pdr5Δ* cells with yEGFP-Vps27 and yEGFP-vps27-VHS-UIIM foci. Cells were from cultures in panel (B). Cells counted at log phase: *VPS27* (n=483), *vps27-VHS-UIIM* (540; at day 1 low glucose medium with DMSO: *VPS27* (587), *vps27-VHS-UIIM* (599); and at day 1 low glucose medium with MG132: *VPS27* (481), *vps27-VHS-UIIM* (600). Results plotted as mean±sd. *, P<0.05 (ANOVA single factor analysis comparing MG132- to DMSO only-treated *vps27-VHS-UIIM* cells).

(E) Anti-GFP immunoblot analyses of free (yE)GFP release and proteasome fragmentation (proteolysis products in between full length GFP fusion protein and free GFP) of Pre10-GFP (a CP subunit), Rpn5-yEGFP (a lid subunit), and Rpn2-GFP (a base subunit) in *pdr5Δ* cells with *VPS27* or *vps27-VHS-UIIM* alleles controlled by the natural *VPS27* promoter and terminator and integrated at the *URA3* locus. Endogenous *VPS27* gene was deleted in all the strains. Cells were harvested from cultures grown in low glucose medium containing DMSO or 50 μM MG132 for ~1 d at 30°C. Proteasome subunit fragmentation was enhanced by MG132 treatment but inhibited by *vps27-VHS-UIIM*. Middle panel: Vacuolar degradation of untagged vps27-VHS-UIIM was inhibited in cells treated with MG132, consistent with the effect seen with yEGFP fused vps27-VHS-UIIM.

(F) Percentage of *pdr5Δ* cells with proteasomal puncta from cultures in panel (E). Cells counted from cultures treated with DMSO and expressing Pre10-GFP (402, 487 for *VPS27* and *vps27-VHS-UIIM*, respectively); Rpn5-yEGFP: (632, 517) or Rpn2-GFP (614, 412), and from cultures with treated with MG132 and expressing Pre10-GFP (444, 602); Rpn5-yEGFP (434, 327); or Rpn2-GFP (526, 785). PSG formation was inhibited by MG132 treatment, and RP-containing PSG formation was further suppressed in MG132-treated *vps27-VHS-UIIM* cells. Results plotted as mean±sd. ***, P<0.001 (ANOVA single factor analysis comparing *vps27-VHS-UIIM* to *VPS27* from cultures with MG132 treatment). Representative blots and images are shown.

Fig. 3

Fig. 3. Microautophagy-dependent proteasome subunit fragmentation is ubiquitin dependent.

(A) Anti-GFP immunoblot analyses of free (yE)GFP release and proteasome fragmentation of Pre10-GFP, Rpn5-yEGFP, and Rpn2-GFP in WT and *doa4Δ* cells carrying either pES12 (empty vector) or YEp96 for ubiquitin expression under the copper-inducible *CUP1* promoter (basal expression, no copper added). Overexpressing ubiquitin suppressed the loss of proteasome fragmentation in *doa4Δ* cells.

(B) Anti-GFP immunoblot analyses of free (yE)GFP release and proteasome fragmentation of Pre10-GFP, Rpn5-yEGFP, and Rpn2-GFP in *doa4Δ* cells expressing pRS316-based alleles: empty vector (EV), wild type *DOA4*, or catalytically inactive mutant *doa4-C571S* under the control of the *DOA4* native promoter. Doa4 DUB activity was required for microautophagy-dependent proteasome fragmentation.

(C) Anti-GFP immunoblot analyses of free (yE)GFP release and proteasome fragmentation of Pre10-GFP, Rpn5-yEGFP, and Rpn2-GFP in the indicated strains. ESCRT gene deletions in *doa4Δ* that suppress ubiquitin depletion could also rescue proteasome fragmentation in *doa4Δ* cells. Representative blots are shown.

Fig. 5. LMW ubiquitylation of aberrant proteasomes and their removal by ESCRT-0.

(A) Co-IP analyses of ubiquitylated proteasome species in WT, *doa4Δ*, proteasome mutants (*pre9Δ*, *sem1Δ*), and MG132-treated cells expressing Pre10-GFP (top panel) and Rpn5-yEGFP (bottom panel). LMW ubiquitylated proteasome species accumulated in *pre9Δ* and MG132-treated cells.

(B, C) Co-IP analyses of HA-Vps27 and HA-vps27-VHS-UIM-PTAPL with proteasomes in *vps27Δ* (B) and *vps27Δ vps4Δ* (C) cells expressing Rpn5-yEGFP, Rpn2-GFP, or Pre10-GFP. Relative abundance of full-length HA-vps27-VHS-UIM-PTAPL and HA-Vps27 was calculated by normalizing to full-length Rpn5-yEGFP in total cell lysates or co-IP; the ratio of HA-vps27 mutant to HA-Vps27 is noted in panels. Vps27 interacted with proteasome

RP but not CP under low glucose conditions, and this interaction increased when *VPS4* was deleted; vps27-VHS-UIM-PTAPL was stabilized due to stalled microautophagy. (D) co-IP analyses of HA-tagged ESCRT-0 subunits (Vps27, Hse1) and proteasomes as in panel (C). The protein abundance of ESCRT-0 including full length and intermediates or breakdown products (as indicated with brackets in the figure panels) in between HA-Vps27 or HA-vps27-VHS-UIM-PTAPL and Hse1-HA was quantified as in panel (C). (E) co-IP analyses of ubiquitylated proteasome species in *vps27Δ* cells bearing chromosomally integrated pRS306 (EV) or the indicated *VPS27* alleles. LMW ubiquitylated proteasome species accumulated in ubiquitin binding-defective Vps27 mutants. Cells were harvested from cultures grown in low glucose medium for ~1 d at 30°C. Protein band intensity was quantified with ImageJ. Representative blots are shown.

1082 Fig. 6

Fig. 6. Ubiquitin binding by ESCRT components is essential for proteasome microautophagy and intralumenal vesicle (ILV) formation.

(A) Anti-GFP immunoblot analyses of Pre10-GFP, Rpn5-yEGFP, and Rpn2-GFP in *vps27Δ* cells harboring chromosomally integrated empty vector (EV, pRS306) and the indicated *VPS27* alleles in the strains shown. Cells were harvested from cultures grown in low glucose medium for ~1 d and 4 d at 30°C. Representative blots are shown.

(B) Epifluorescence images of Pre10-GFP, Rpn5-yEGFP, and Rpn2-GFP in *vps27Δ* cells bearing the indicated chromosomally integrated *VPS27* alleles. Cells were from cultures in panel (A) after ~4 d in low glucose. Proteasome puncta were observed in EV and *vps27-VHS-UIM-PTAPL* cells, indicating accumulation of aberrant proteasomes. 4×, 4-fold enlargement of the boxed regions. Scale bar, 5 μm. Representative images are shown.

(C) Cryo-immunogold electron micrographs of proteasomes in *vps27Δ pep4Δ prb1Δ* cells expressing chromosomally integrated EV, *VPS27*, or *vps27-VHS-UIM-PTAPL*. Cells were grown in low glucose for ~4 d at 30°C. Sections were immunolabeled with anti-CP primary antibody and gold-conjugated Protein A (black arrows). Cyto, cytoplasm; Vac, vacuole; AB, autophagic body.

(D) Percentage of cell sections with the indicated intravacuolar structures (ABs, vesicles, ABs & vesicles, none) in the strains used for panel (C).

(E) Percentage of the indicated structures with gold particle staining in the cells used for panel (C).

(F) Percentage of intravacuolar gold particles staining either ABs or vesicles or the limiting vacuolar membrane in the cells used for panel (C). “n” represents cell sections counted in panels (D) and (E), and intravacuolar gold particles counted in panel (F).

1113 **Fig. 7**

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1115 **Fig. 7. Rsp5 ubiquitin ligase is involved in proteasome ubiquitylation.**

1116 (A) Co-IP analyses of ubiquitylated proteasome species in WT and *rsp5-1* mutant cells
1117 expressing Pre10-GFP or Rpn5-GFP. Cultures were grown for ~1 d at 30°C.

1118 (B) Co-IP analyses of ubiquitylated proteasome species in *rsp5Δ* cells containing the
1119 indicated *RSP5* alleles in pRS316. Cells were harvested from cultures in low glucose
1120 medium after ~1 d at 30°C. Representative blots are shown.

1121 (C) Working model of AMPK-directed selective microautophagy of proteasomes under
1122 low glucose conditions. AMPK activation in response to low glucose stress promotes
1123 Vps27 vacuolar trafficking and degradation. Vps27 (ESCRT-0) in turn sorts aberrant
1124 proteasomes associated with LMW ubiquitylation to the vacuole surface to initiate
1125 microautophagy. Proteasome RP subunits are the main targets for Rsp5.

Supplemental information

Fig. S1

Fig. S1. Vps27 is enriched in proteasome puncta in AMPK mutant cells under low glucose conditions.

(A) Epifluorescence images of Pre1-mC (a CP subunit) in WT and AMPK mutant (*snf1Δ* or *snf4Δ*) cells expressing chromosomally integrated *yEGFP-VPS27*. Endogenous *VPS27* was deleted. Cells were harvested from cultures grown in low glucose medium for ~4 d at 30°C. White arrows point to colocalized Vps27 and proteasome foci in AMPK mutant cells. BF, bright field. Scale bar, 5 μm. Representative images are shown.

(B) Percentage of cells with colocalized yEGFP-Vps27 and proteasomal puncta (Pre1-mC, Rpn5-mC, or Rpn2-mC) in WT or AMPK mutant cells. Cultures were grown in low glucose or glucose-free medium for ~1 d and ~4 d at 30°C. Cells counted in WT: Pre1-mC (n=535, 444 for low glucose at day 1 and day 4, respectively, and n=551, 632 for glucose-free at day 1 and day 4, respectively); Rpn5-mC (560, 538, 446, 494); Rpn2-mC (337, 598, 426,

510) Cell counts in *snf1Δ*: Pre1-mC (194, 541, 517, 392); Rpn5-mC (194, 413, 538, 462),
and Rpn2-mC (268, 381, 569, 368). Cell counts in *snf4Δ*: Pre1-mC (511, 622, 766, 364);
Rpn5-mC (503, 432, 717, 322); and Rpn2-mC (488, 506, 669, 240). Results plotted as
mean±sd..

1172 Fig. S2

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1174 Fig. S2. Effect of UBD mutations of Vps27 on inactive proteasome trafficking under
1175 low glucose conditions.

1176 Epifluorescence images of Pre10-GFP, Rpn5-yEGFP, and Rpn2-GFP in *pdr5Δ* cells
1177 expressing chromosomally integrated *VPS27* and *vps27-VHS-UIM*. Endogenous *VPS27*
1178 gene was deleted. Cultures were grown in low glucose medium containing DMSO or 50
1179 μ M MG132 for ~1 d at 30°C. The same cells were used for quantification as shown in panel
1180 Figure 2F. The *vps27-VHS-UIM* mutation further inhibited PSG formation in cells with
1181 MG132 treatment. BF, bright field. Scale bar, 5 μ m. Representative images are shown.

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Fig. S3

Fig. S3. AMPK mutations do not suppress proteasome fragmentation defects in *doa4*Δ cells.

Anti-GFP immunoblot analyses of free (yE)GFP release and proteasome fragmentation of Pre10-GFP, Rpn5-yEGFP, and Rpn2-GFP in the indicated strains. Cultures were grown in low glucose or glucose-free medium for ~4 d at 30°C. Representative blots are shown.

1209 **Fig. S4**

1210 **Fig. S4. Ubiquitin-K63 chains are not detectably conjugated to proteasomes.**

1211 (A) Co-IP analyses of ubiquitylated proteasome species in the indicated strains expressing

1212 Rpn2-GFP. LMW ubiquitylated proteasomal species accumulated in ESCRT mutants.

1213 (B) Anti-ubiquitin-K63 immunoblot analyses of the coIP samples from panel (A). The

1214 ubiquitin-K63-linked ubiquitylated species accumulated in total cell lysates of ESCRT

1215 mutants but were not detected in the isolated proteasomes.

1216 (C) Co-IP analyses of ubiquitylated proteasome species using GFP-trap agarose beads and

1217 washing in high salt (250 mM NaCl) buffer before elution. LMW ubiquitylated proteasome

1218 species were retained in ESCRT mutants. Cultures were in low glucose for ~1 d at 30°C.

1225 **Fig. S5**

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1227 **Fig. S5. Ubiquitylated proteasome species accumulate in the purified proteasomes of**
1228 **ESCRT mutants under low glucose conditions.**

1229 Anti-ubiquitin immunoblot analyses and SYPRO Ruby staining of purified proteasomes
1230 (10 μ g) of WT and ESCRT mutant (*vps27Δ*, *vps4Δ*) cells. Cells were harvested from 700-
1231 ml cultures grown in low glucose medium for $\sim$ 1 d at 30°C, and lysates were made by
1232 grinding cells in liquid nitrogen with ATP present in the buffer. Proteasomes were affinity
1233 purified through 3xFLAG tag fused on Rpn11 C-terminus and eluted with FLAG peptide.

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1246 Fig. S6

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1248 Fig. S6. Ubiquitin, proteasome subunits and proteasome-associated proteins are
1249 dominant proteins seen by LC-MS/MS of the GFP-Trap elutes from WT and ESCRT
1250 mutant cells expressing Rpn5-yEGFP.

(A) Gene ontology (GO) analysis of the protein hits from WT, *vps27Δ*, and *vps4Δ* cells, with cellular component ontology with $P \leq 0.01$. GO term analysis was conducted using the Generic Gene Ontology Term Finder online software (Princeton U.).

(B) The ratio of total spectral counts to molecular weight of individual protein hits with enriched GO cellular component term as shown in panel (A). White color represents specific proteins that were not detected in the indicated strains. A full list of protein hits is given in Table S1.

Fig. S7

Fig. S7. Overexpressed Hse1-HA by itself does not interact with proteasomes under low glucose conditions.

Co-IP analyses of Hse1 and proteasomes in *hse1Δ* cells expressing Rpn5-yEGFP, Rpn2-GFP, and Pre10-GFP, as well as Hse1-HA under the *GPD* promoter with a p424GPD backbone. Proteasome ubiquitylation was not affected in *hse1Δ* cells. Cultures were grown in low glucose for ~1 d at 30°C.

Fig. S8

Fig. S8. Proteasome RP subunit fragmentation is more sensitive to defective Rsp5 than that of a CP subunit.

Anti-GFP immunoblot analyses of free GFP release and proteasome fragmentation of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in WT and *rsp5-1* cells. Representative blots are shown.

Supplementary tables

Table S1 is in a separate Excel file

Table S1. Proteins identified by mass spectrometry analyses of co-IP samples from WT and ESCRT mutant (*vps27Δ*, *vps4Δ*) cells expressing Rpn5-yEGFP.

Note: Protein hits are sorted by total spectral counts of individual proteins in the WT sample from high to low. Peptide thresholds $\geq 95\%$ and protein thresholds $\geq 99\%$ and ≥ 2 peptides.

1351 **Table S2. Yeast strains used in this study.**

Strain	Genotype	Reference
MHY500	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i>	(Chen et al., 1993)
MHY622	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2</i>	(Papa and Hochstrasser, 1993)
MHY1269	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2</i>	(Amerik et al., 2000)
MHY2443	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1</i>	(Amerik et al., 2006)
MHY5841	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN11-6xGly-3xFLAG::kanMX</i>	(Li et al., 2015)
MHY6377	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3</i>	This study
MHY6604	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX</i>	This study
MHY6614	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX RPN5-yEGFP::HIS3</i>	This study
MHY6946	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2::kanMX</i>	This study
MHY7796	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pep4Δ::natMX prb1Δ::kanMX</i>	This study
MHY9736	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY9738	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY9749	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 prb1Δ::kanMX pep4Δ::natMX PRE10-GFP::HIS3</i>	This study
MHY9754	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-GFP::kanMX</i>	This study
MHY9811	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX</i>	This study
MHY9814	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX</i>	This study
MHY9816	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps25Δ::kanMX</i>	This study
MHY9820	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf7Δ::kanMX</i>	This study
MHY9987	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps28Δ::kanMX</i>	This study
MHY9989	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN5-yEGFP::HIS3</i>	This study
MHY9993	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 RPN5-yEGFP::HIS3</i>	This study
MHY10011	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i>	This study

	<i>snf4Δ::kanMX RPN5-yEGFP::HIS3</i>	
MHY10014	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX RPN5-yEGFP::HIS3</i>	This study
MHY10019	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 atg8Δ::hphMX</i>	This study
MHY10021	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3</i>	This study
MHY10047	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX RPN2-GFP::HIS3</i>	This study
MHY10048	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX RPN2-GFP::HIS3</i>	This study
MHY10058	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN2-GFP::HIS3</i>	This study
MHY10061	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 RPN2-GFP::HIS3</i>	This study
MHY10075	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 prb1Δ::kanMX pep4Δ::natMX RPN5-yEGFP::HIS3</i>	This study
MHY10099	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pep4Δ::natMX prb1Δ::kanMX vps4Δ::TRP1 RPN5-yEGFP::HIS3</i>	This study
MHY10147	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3</i>	This study
MHY10179	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 PRE10-GFP::HIS3</i>	This study
MHY10182	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 PRE10-GFP::HIS3</i>	This study
MHY10328	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 sem1Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY10332	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 sem1Δ::kanMX RPN5-yEGFP::HIS3</i>	This study
MHY10335	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pre9Δ::hphMX PRE10-GFP::HIS3</i>	This study
MHY10339	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pre9Δ::hphMX RPN5-yEGFP::HIS3</i>	This study
MHY10432	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 PRE10-GFP::HIS3 pRS306::URA3</i>	This study
MHY10434	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 PRE10-GFP::HIS3 pRS306-VPS27::URA3</i>	This study
MHY10436	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 PRE10-GFP::HIS3 pRS306-vps27-VHS-UIM::URA3</i>	This study
MHY10438	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN5-yEGFP::HIS3 pRS306::URA3</i>	This study
MHY10440	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN5-yEGFP::HIS3 pRS306-VPS27::URA3</i>	This study
MHY10442	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i>	This study

	<i>vps27Δ::LEU2 RPN5-yEGFP::HIS3</i>	
	<i>pRS306-vps27-VHS-UIM::URA3</i>	
MHY10444	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN2-GFP::HIS3 pRS306::URA3</i>	This study
MHY10446	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN2-GFP::HIS3 pRS306-VPS27::URA3</i>	This study
MHY10448	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN2-GFP::HIS3 pRS306-vps27-VHS-UIM::URA3</i>	This study
MHY10611	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 PRE10-GFP::HIS3</i>	This study
MHY10614	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 RPN2-GFP::HIS3</i>	This study
MHY10616	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::kanMX</i>	This study
MHY10631	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 RPN5-yEGFP::HIS3</i>	This study
MHY10665	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 PRE10-GFP::HIS3 pRS306-vps27-PTAPL::URA3</i>	This study
MHY10671	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN5-yEGFP::HIS3 pRS306-vps27-PTAPL::URA3</i>	This study
MHY10676	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN2-GFP::HIS3 pRS306-vps27-PTAPL::URA3</i>	This study
MHY10688	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 vps27Δ::LEU2 RPN5-yEGFP::HIS3</i>	This study
MHY10689	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 vps4Δ::TRP1 RPN2-GFP::HIS3</i>	This study
MHY10692	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 vps4Δ::TRP1 PRE10-GFP::HIS3</i>	This study
MHY10693	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 vps27Δ::LEU2 RPN2-GFP::HIS3</i>	This study
MHY10695	<i>MATα/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 vps27Δ::LEU2 PRE10-GFP::HIS3</i>	This study
MHY10696	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 vps4Δ::TRP1 RPN5-yEGFP::HIS3</i>	This study
MHY10714	<i>MATα/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 pep4Δ::natMX prb1Δ::kanMX</i>	This study
MHY10720	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 atg8Δ::hphMX pep4Δ::natMX prb1Δ::kanMX</i>	This study
MHY10737	<i>MATα/α lys2 leu2 ura3-52 his3-Δ200/his4-912δ trp1-1/TRP1 rsp5-1 PRE10-GFP::kanMX</i>	This study
MHY10738	<i>MATα lys2 leu2 ura3-52 his3-Δ200/his4-912δ trp1-1/TRP1 rsp5-1 RPN5-GFP::kanMX</i>	This study

MHY10740	<i>MATa lys2 leu2 ura3-52 his3-Δ200/his4-912δ trp1-1/TRP1 rsp5-1 RPN2-GFP::kanMX</i>	This study
MHY10973	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 pep4Δ::natMX prb1Δ::kanMX</i>	This study
MHY10993	<i>MATa/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX pep4Δ::natMX prb1Δ::kanMX</i>	This study
MHY10998	<i>MATa/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX pep4Δ::natMX prb1Δ::kanMX</i>	This study
MHY11002	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pep4Δ::natMX prb1Δ::kanMX</i>	This study
MHY11021	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11442	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 vps27Δ::LEU2 RPN2-GFP:HIS3</i>	This study
MHY11443	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 RPN2-GFP:HIS3</i>	This study
MHY11446	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 hse1Δ::hphMX vps27Δ::LEU2 RPN2-GFP:HIS3</i>	This study
MHY11447	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 hse1Δ::hphMX PRE10-GFP:HIS3</i>	This study
MHY11449	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 hse1Δ::hphMX vps27Δ::LEU2 PRE10-GFP:HIS3</i>	This study
MHY11494	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11508	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pRS306-yEGFP-vps27-VHS-UIM::URA3</i>	This study
MHY11527	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX vps27Δ::LEU2 pRS306-yEGFP- VPS27::URA3</i>	This study
MHY11530	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX vps27Δ::LEU2 pRS306-yEGFP- VPS27::URA3</i>	This study
MHY11534	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX vps27Δ::LEU2 pRS306-yEGFP-vps27-VHS-UIM::URA3</i>	This study
MHY11537	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX vps27Δ::LEU2 pRS306-yEGFP- VPS27::URA3</i>	This study
MHY11539	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2- mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11542	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE1- mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11549	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX PRE1-mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study

MHY11552	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX RPN2-mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11550	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX RPN5-mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11555	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX RPN2-mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11563	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11566	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX RPN5-mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11567	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX PRE1-mC::natMX vps27Δ::LEU2 pRS306-yEGFP-VPS27::URA3</i>	This study
MHY11610	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 RPN11-6xGly-3xFLAG::kanMX</i>	This study
MHY11618	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN11-6xGly-3xFLAG::kanMX</i>	This study
MHY11678	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 snf4Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11680	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 snf1Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11682	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4Δ::LEU2 snf1Δ::kanMX RPN5-yEGFP::HIS3</i>	This study
MHY11729	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX vps27Δ::LEU2 pRS306-VPS27::URA3 RPN5-yEGFP::HIS3</i>	This study
MHY11742	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX vps27Δ::LEU2 pRS306-vps27-VHS-UIIM::URA3 RPN5-yEGFP::HIS3</i>	This study
MHY11744	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX vps27Δ::LEU2 pRS306-vps27-VHS-UIIM::URA3 PRE10-GFP::HIS3</i>	This study
MHY11747	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX vps27Δ::LEU2 pRS306-VPS27::URA3 PRE10-GFP::HIS3</i>	This study
MHY11749	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 pdr5Δ::kanMX vps27Δ::LEU2 pRS306-VPS27::URA3 RPN2-GFP::HIS3</i>	This study
MHY11754	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps23Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11757	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps23Δ::kanMX vps27Δ::LEU2 PRE10-GFP::HIS3</i>	This study

	<i>pRS306-vps27-VHS-UIM::URA3</i>	
MHY11758	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps23Δ::kanMX RPN2-GFP::HIS3</i>	This study
MHY11761	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps23Δ::kanMX vps27Δ::LEU2 RPN2-GFP::HIS3 pRS306-vps27-VHS-UIM::URA3</i>	This study
MHY11763	<i>MATa/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps23Δ::kanMX RPN5-yEGFP::HIS3</i>	This study
MHY11765	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps23Δ::kanMX vps27Δ::LEU2 RPN5-yEGFP::HIS3 pRS306-vps27-VHS-UIM::URA3</i>	This study
MHY11766	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pep4Δ::natMX prb1Δ::kanMX pRS306::URA3</i>	This study
MHY11768	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pep4Δ::natMX prb1Δ::kanMX pRS306-VPS27::URA3</i>	This study
MHY11770	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pep4Δ::natMX prb1Δ::kanMX pRS306-vps27-VHS-UIM::URA3</i>	This study
MHY11791	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pep4Δ::natMX prb1Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11793	<i>MATa/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX pep4Δ::natMX prb1Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11795	<i>MATa/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX pep4Δ::natMX prb1Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11798	<i>MATa/α his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX pep4Δ::natMX prb1Δ::kanMX RPN5-yEGFP::HIS3</i>	This study
MHY11800	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 pep4Δ::natMX prb1Δ::kanMX RPN5-yEGFP::HIS3</i>	This study
MHY11818	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1 pep4Δ::natMX prb1Δ::kanMX PRE10-GFP::HIS3</i>	This study
MHY11922	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 PRE10-GFP::HIS3 pRS306-vps27-VHS-UIM-PTAPL::URA3</i>	This study
MHY11927	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN5-yEGFP::HIS3 pRS306-vps27-VHS-UIM-PTAPL::URA3</i>	This study
MHY11929	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2 RPN2-GFP::HIS3</i>	This study

MHY11941	<i>pRS306-vps27-VHS-UIM-PTAPL::URA3</i> <i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>rsp5Δ::hphMX pRS316-rsp5-L733S::URA3</i> <i>RPN5-yEGFP::HIS3</i>	This study
MHY11944	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>rsp5Δ::hphMX pRS316-rsp5-F618A::URA3</i> <i>RPN5-yEGFP::HIS3</i>	This study
MHY11948	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>rsp5Δ::hphMX pRS316-RSP5::URA3 RPN5-yEGFP::HIS3</i>	This study
MHY11952	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>rsp5Δ::hphMX pRS316-RSP5::URA3 PRE10-GFP::HIS3</i>	This study
MHY11954	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>rsp5Δ::hphMX pRS316-rsp5-L733S::URA3</i> <i>PRE10-GFP::HIS3</i>	This study
MHY11957	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>rsp5Δ::hphMX pRS316-rsp5-F618A::URA3</i> <i>PRE10-GFP::HIS3</i>	This study
MHY12058	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>vps4Δ::TRP1 vps27Δ::LEU2 RPN5-yEGFP::HIS3</i>	This study
MHY12066	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>vps4Δ::TRP1 vps27Δ::LEU2 PRE10-GFP::HIS3</i>	This study
MHY12101	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>hse1Δ::hphMX RPN5-yEGFP::HIS3</i>	This study
MHY12103	<i>MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1</i> <i>hse1Δ::hphMX vps27Δ::LEU2 RPN5-yEGFP::HIS3</i>	This study

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Table S3. Plasmids used in this study.

Plasmid	Description	Reference
pRS316	<i>CEN, URA3</i>	(Sikorski and Hieter, 1989)
pRS316-DOA4	<i>CEN, URA3, P_{DOA4}, T_{DOA4}, DOA4</i>	This study
pRS316-DOA4 ^{C571S}	<i>CEN, URA3, P_{DOA4}, T_{DOA4}, doa4-C571S</i>	This study
pRS316-RSP5	<i>CEN, URA3, P_{RSP5}, T_{RSP5}, RSP5</i>	This study
pRS316-RSP5 ^{L733S}	<i>CEN, URA3, P_{RSP5}, T_{RSP5}, rsp5-L733S</i>	This study
pRS316-RSP5 ^{F618A}	<i>CEN, URA3, P_{RSP5}, T_{RSP5}, rsp5-F618A</i>	This study
p424GPD	2μ, <i>TRP1, P_{GPD}, T_{CYC1}</i>	(Mumberg et al., 1995)
p424GPD-HSE1-HA	2μ, <i>TRP1, P_{GPD}, T_{CYC1}, HSE1-HA</i>	This study
p426GPD	2μ, <i>URA3, P_{GPD}, T_{CYC1}</i>	(Mumberg et al., 1995)
p426GPD-HA-VPS27	2μ, <i>URA3, P_{GPD}, T_{CYC1}, HA-VPS27</i>	This study
p426GPD-HA-vps27-VHS-UIM-PTAPL	2μ, <i>URA3, P_{GPD}, T_{CYC1}, HA-vps27-VHS-UIM-PTAPL</i>	This study
pES12	2μ, <i>TRP1, P_{CUP1}, T_{CYC1}</i>	(Hodgins et al., 1992)
YEpl96	2μ, <i>TRP1, P_{CUP1}, T_{CYC1}, Ubiquitin</i>	(Ellison and Hochstrasser, 1991)

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1399 **Movie 1 is in a separate Mov file**

1400 **Movie 1. Defective vacuolar trafficking of yEGFP-Vps27 in *snf1Δ* cells under low**
 1401 **glucose conditions.**

1402 Confocal time-lapse images of yEGFP-Vps27 showing that perivacuolar granules of
 1403 yEGFP-Vps27 accumulated in *snf1Δ* cells grown in low glucose medium for ~1 day at
 1404 30°C. The Vps27 granules were highly mobile, undergoing rapid docking and separation.
 1405 The time-lapse video comprises 30 image frames taken at 5 s intervals with 2.53 s scanning
 1406 time for each frame and played at 2 frames per second; the real time length was 145 s for
 1407 this video.