AMPK regulates ESCRT-dependent microautophagy of proteasomes concomitant with proteasome storage granule assembly during glucose starvation

Short title: AMPK and ESCRT-dependent proteasome microautophagy

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Abstract

The ubiquitin-proteasome system regulates numerous cellular processes and is central to protein homeostasis. In proliferating yeast and many mammalian cells, proteasomes are highly enriched in the nucleus. In carbon-starved yeast, proteasomes migrate to the cytoplasm and collect in phase-separated proteasome storage granules (PSGs). PSGs dissolve and proteasomes return to the nucleus within minutes of glucose refeeding. The mechanisms by which cells regulate proteasome homeostasis under these conditions remain largely unknown. Here we show that AMP-activated protein kinase (AMPK) together with endosomal sorting complexes required for transport (ESCRTs) drive a glucose starvation-dependent microautophagy pathway that preferentially sorts aberrant proteasomes into the vacuole, thereby biasing accumulation of functional proteasomes in PSGs. The proteasome core particle (CP) and regulatory particle (RP) are regulated differently. Without AMPK, the insoluble protein deposit (IPOD) serves as an alternative site that specifically sequesters CP aggregates. Our findings reveal a novel AMPK-controlled ESCRT-mediated microautophagy mechanism in the regulation of proteasome trafficking and homeostasis under carbon starvation.
Introduction

The ubiquitin-proteasome system (UPS) is a conserved proteolytic system responsible for the highly selective degradation of cellular proteins. Conjugation of ubiquitin to substrates targets them to the proteasome for degradation [1, 2]. The 26S proteasome comprises a 20S core particle (CP) with a 19S regulatory particle (RP) on one or both ends of the CP [1]. In the CP, four stacked rings are assembled from different β-subunits (β1-β7) and α-subunits (α1-α7). The RP is assembled from two multisubunit subcomplexes termed the base and lid [1]. The RP is responsible for substrate binding, deubiquitylation, unfolding, and translocation [1, 3-5].

The UPS is responsible for about 80-90% of cellular proteolysis under normal growth conditions, and therefore, alterations in proteasome activity have a major impact on protein homeostasis (“proteostasis”) [6, 7]. For instance, the age-related decline of proteostasis can be compensated by increasing proteasome activity, while cancer cell growth can be limited by inhibiting proteasome activity [8-10]. One way to regulate the availability of proteasomes is by formation of Proteasome Storage Granules (PSGs), which are phase-separated cytoplasmic structures that collect proteasomes during specific stresses. PSGs most likely serve as an adaptive mechanism to regulate proteasome activity [11, 12]. Multiple factors have been reported to be relevant to the assembly and disassembly of PSGs, including carbon starvation [13], intracellular pH [14], protein N-α-acetylation [11], the activity of Blm10 [15], and the integrity of certain proteasome lid subunits [12, 16, 17]. Nevertheless, relatively little is known about how proteasome nucleocytoplasmic trafficking and homeostasis is regulated, particularly under carbon starvation conditions.

Autophagy is another major means of degrading cellular components, which protects cells from damaged proteins and organelles and promotes cell survival under various stresses, such as
starvation, oxidative stress, protein aggregation, and ER stress. The cellular components of macroautophagy have been extensively studied in yeast and assigned roles in selective or nonselective macroautophagy or both [18]. All forms of macroautophagy share a common set of core autophagy (Atg) proteins that are involved in autophagosome initiation and formation [19]. Other Atg factors are required for specific types of selective macroautophagy [18]. Atg17 is required only for nonselective macroautophagy [20].

In comparison to macroautophagy, microautophagy is poorly understood [21]. Recently, endosomal sorting complexes required for transport (ESCRT)- and clathrin-dependent microautophagy has been described in yeast undergoing diauxic shift; the substrate protein followed in this study was the transmembrane vacuolar protein Vph1 [22]. The ESCRT machinery is an ancient system for membrane remodeling and scission; in eukaryotes it includes five distinct subcomplexes ESCRT-0, I, II, III, and the AAA ATPase Vps4 [23]. The ESCRT pathway drives diverse cellular processes, such as multivesicular body (MVB) formation, nuclear pore complex (NPC) quality control, virus budding, viral replication-complex assembly, macroautophagy, and microautophagy [23, 24]. How the ESCRT machinery is activated and regulated in microautophagy, however, is unclear.

Proteasome homeostasis involves a balance between proteasome assembly and degradation. Normally, proteasomes are very stable entities within the cell [25], but their degradation by autophagy is induced under certain conditions. Proteasome degradation by macroautophagy (“proteaphagy”) occurs in response to nitrogen starvation and has been described in yeast, plants, and mammals [26-30]. A recent study reported that PSGs protect proteasomes from autophagic degradation during carbon starvation [31], suggesting an physiological connection between PSGs
and autophagy. It is unknown how the partitioning of proteasomes between assembling PSGs in the cytoplasm and proteolysis in the vacuole is regulated.

AMP-activated protein kinase (AMPK) is a highly conserved regulator of energy homeostasis in eukaryotes. The AMPK heterotrimeric complex is composed of a catalytic $\alpha$ subunit (called Snf1 in *S. cerevisiae*) and two regulatory subunits: a $\beta$ subunit (Sip1, Sip2, or Gal83) and a $\gamma$ subunit (Snf4) [32]. The AMPK pathway is activated when energy stores are depleted, which modulates the switch from fermentation to respiration in yeast [32, 33]. Moreover, AMPK coordinates a wide range of cellular processes, such as cell growth, autophagy, metabolism, cell polarity, and cytoskeletal dynamics [34].

In this study, we demonstrate that AMPK, the ESCRT machinery, and the insoluble protein deposit (IPOD) function together in the regulation of proteasome trafficking and degradation under glucose starvation. We show that cells utilize AMPK- and ESCRT-dependent microautophagy to clear aberrant proteasomes through vacuolar proteolysis under these conditions and thus safeguard reversible PSG assembly and the maintenance of functional proteasomes during glucose starvation. Our cytological data suggest very high levels of microautophagy can occur under these conditions. We find that proteasomes dissociate into CP and RP complexes that are regulated through distinct trafficking mechanisms during glucose starvation. Furthermore, irreversible CP aggregates accumulate if AMPK is inactivated and cells are carbon starved. In such mutants, irreversible CP aggregates concentrate in the IPOD compartment. These findings reveal the convergence of multiple cellular pathways that maintain proteasome homeostasis during changes in nutrient availability.

Results
A high-content screen for defects in PSG dynamics identifies AMPK and ESCRT proteins

To identify factors that are involved in PSG dissipation and nuclear reimport of proteasomes upon glucose refeeding, we screened a yeast gene deletion library [35] using automated mating approaches [36] in which a strain with an integrated \textit{RPN5-GFP} allele (encoding a GFP tag at the C-terminus of a lid subunit) was crossed into the deletion library and the desired meiotic segregants were imaged on a high-throughput fluorescence microscopy platform [37]. The screen identified more than 200 potential hits, with clear enrichment of two conserved cellular machineries: AMPK and the ESCRT machinery. The hits included two subunits of the AMPK heterotrimeric complex (Snf1 and Snf4) and multiple constituents of the ESCRT pathway (ESCRT-0 [Vps27], ESCRT-II [Vps25], ESCRT-III [Did2, Vps2/Did4, Vps24], and the AAA ATPase Vps4).

To validate the candidates from the AMPK and ESCRT complexes, we added an mCherry (mC) tag at the C-terminus of three individual proteasome subunits: Pre1-mC (a CP subunit, \(\beta_4\)), Rpn2-mC (a base subunit), and Rpn5-mC (a lid subunit), and we then introduced the corresponding genes into yeast strains also expressing a nuclear envelope marker, Nup49-GFP, but lacking individual candidate genes. In wild-type (WT) cells, all of these tagged proteasomes localized to the cytoplasm and assembled into bright, round PSGs with dark cytoplasmic backgrounds under glucose starvation (Fig 1A). The PSGs rapidly dissolved and proteasomes were reimported into the nucleus in ~90% of cells within 20 min after 2% glucose addition (Fig 1B). These observations are consistent with the original report on PSG formation and dissipation in yeast cells [13].

By contrast, cells with mutations in the ESCRT machinery displayed a mixture of diffuse cytoplasmic proteasomes and proteasome granules under glucose starvation conditions (Fig 1A and S1A Fig). Upon glucose refeeding, the mutants also showed defective proteasome granule
dissipation and nuclear reimport of proteasomes, as predicted from the original screen (Fig 1B and S1B Fig). These findings suggest that the ESCRT machinery is involved in PSG assembly during glucose starvation and that normal PSG assembly is a prerequisite for efficient PSG dissipation and nuclear reimport of proteasomes upon glucose restoration.

In AMPK mutant cells lacking either SNF4 or SNF1, PSGs microscopically similar to those in WT cells were observed by fluorescence microscopy (Fig 1C). This was also evident from immunogold electron microscopy. PSGs of similar size (~500 nm dia.) were observed in WT and the AMPK mutant cells that had been immunogold-labeled with anti-CP antibody (Fig 1E). However, PSG disassembly and nuclear reimport of proteasomes were defective in the AMPK mutants (Fig 1D), suggesting that PSG dissipation requires AMPK pathway signaling. Collectively, these results suggest that AMPK and the ESCRT machinery are both required for reversible PSG formation during glucose starvation and refeeding but likely for different steps.

Proteasome macroautophagy requires the ESCRT pathway but not AMPK during nitrogen starvation

Prior work showed that AMPK plays a direct role in promoting macroautophagy in response to energy deprivation [38, 39], while the ESCRT machinery has roles in multiple autophagic pathways [40]. Proteasome degradation by macroautophagy is induced by nitrogen starvation in yeast cells [26-28], and a recent study reported that PSGs protect proteasomes from autophagic degradation during carbon starvation [31]. Given these interconnections, we checked whether mutations in AMPK and ESCRT factors affected proteasome macroautophagy during nitrogen starvation by using a GFP-release immunoblot assay [28]. GFP fusion proteins are hydrolyzed by proteases within the vacuole following macroautophagy, but the GFP moiety itself
is more resistant to cleavage. We assessed GFP release from nitrogen-starved cells expressing Pre10-GFP (CP subunit α7), Rpn5-GFP, or Rpn2-GFP. Free GFP release from all three fusions was strongly inhibited in yeast strains lacking components of the ESCRT machinery, including ESCRT-0 (Vps27), ESCRT-I (Vps28 and Vp37), ESCRT-II (Vps25), ESCRT-III (Snf7), and the AAA ATPase (Vps4) when compared to that in WT cells (Fig 2A). The one exception was the comparable amount free GFP release from Pre10-GFP in \( vps4\Delta \) and WT cells. This finding suggests that CP macroautophagy is independent of Vps4, while RP macroautophagy requires the complete ESCRT pathway. This is also consistent with proteasomes disassembling into CP and RP complexes before their trafficking to the vacuole for degradation during nitrogen starvation.

By contrast, a similar amount of free GFP release from Pre10-GFP, Rpn5-GFP, and Rpn2-GFP was detected in WT, \( snf4\Delta \), and \( snf1\Delta \) cells (Fig 2A), indicating AMPK is not essential for macroautophagy of proteasomes during nitrogen starvation.

We also checked the subcellular localization of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in nitrogen-starved ESCRT and AMPK mutants by fluorescence microscopy. Proteasomes were found in the vacuole in WT, \( snf4\Delta \), and \( snf1\Delta \) cells, but they were mostly adjacent to the vacuolar membrane in ESCRT mutants with the exception that Pre10-GFP was found in the vacuole lumen in \( vps4\Delta \) cells (Fig 2B and S2A Fig). These localization data were consistent with the GFP immunoblot analysis (Fig 2A), indicating the ESCRT machinery is involved in proteasome trafficking to the vacuole during nitrogen starvation. Considering the role of ESCRT machinery in sorting cargos to the lumen of MVBs, we propose that the ESCRT pathway is involved in sorting a fraction of proteasomes to the vacuole for degradation under these conditions.

**AMPK and ESCRT linked to proteasome fragmentation during glucose starvation**
Autophagy is induced in cells when they are subjected to carbon-free starvation following pre-growth in various carbon sources with the notable exception of glucose [41]. Interestingly, autophagy is induced in cells pre-grown in glucose if they are transferred to low glucose (such as 0.05% glucose) medium rather than glucose-free medium [41]. The glucose starvation medium we used for PSG induction contained 0.025% glucose, so our protocol also likely induced autophagy. Therefore, we assessed proteasome autophagy and whether it was affected in AMPK and ESCRT mutants under our conditions. We set up two glucose starvation regimens, culturing cells in either 0.025% glucose (“0.025% C” in figures) or glucose-free medium (“-C”). Interestingly, we found that in WT cells, proteasome fragmentation, specifically that of the Pre10-GFP, Rpn5-GFP, and Rpn2-GFP subunits, was greater under low glucose compared to glucose-free conditions (compare WT lanes in top and bottom panels in Fig 3A). Protein fragments larger than free GFP accumulate during carbon starvation, unlike what is observed during nitrogen starvation. Strikingly, cleavage into these larger GFP-tagged fragments was inhibited in almost all the ESCRT mutants, and it was nearly completely abolished in the AMPK mutants (Fig 3A). These observations suggest AMPK plays a critical role in the regulation of proteasome degradation under low glucose conditions and implicate the ESCRT machinery as well.

Considering that proteasomes accumulated adjacent to the vacuolar membrane in ESCRT mutants under nitrogen starvation (Fig 2B and S2A Fig), we tested if they were found at similar cellular sites in these same mutants during glucose starvation. These could represent the aberrant stacked membranes called class E compartments in ESCRT mutants, such as those lacking VPS4 [42]. We examined the localization of Pre1-mC, Rpn5-mC, and Rpn2-mC in vps4Δ cells expressing a vacuolar membrane protein marker, Vph1-GFP, in low glucose conditions. In addition to the diffusely localized proteasomes and proteasome granules in the cytoplasm, a small
portion of proteasomes colocalized with Vph1-GFP at class E-like compartments based on confocal microscopy (Fig 3B). Similar juxta-vacuolar localization of proteasomes was also found in other ESCRT mutants (S2B Fig). These data suggest that the ESCRT machinery could promote proteasome sorting from the cytoplasm into the vacuole during glucose starvation and that AMPK regulates this process.

AMPK regulates IPOD association of CP-containing PSGs in glucose-starved cells

The perivacuolar IPOD compartment serves as a cellular protein quality control site by sequestering misfolded cytosolic proteins [43]. It also participates in proteasome quality control by distinguishing aberrant proteasomal subunits from normal ones during PSG formation [17]. To investigate whether the apparently irreversible PSGs that form in AMPK mutants are associated with the IPOD, we combined a gene encoding the IPOD marker Hsp42-GFP with \textit{snf4}\Delta or \textit{snf1}\Delta in strains also expressing Pre1-mC, Rpn2-mC, or Rpn5-mC. We measured the colocalization frequencies between mC-tagged proteasome subunits and Hsp42-GFP over seven days of glucose starvation.

We counted colocalization events as cells with at least one mC-tagged focus (PSG) that overlapped with Hsp42-GFP (IPOD) staining (Fig 4A, Pre1-mC). The mC-tagged proteasome subunits that marked PSGs colocalized with Hsp42-GFP in \textasciitilde10\% of WT cells during the 7-day time course under either low glucose or glucose-free conditions (Fig 4B and 4D, S3 Fig). This may represent a basal level of PSG maturation into or direct proteasome exchange with IPODs. Rpn5-mC or Rpn2-mC colocalized with Hsp42-GFP to a similar degree in the AMPK mutants (S3 Fig), suggesting that RP-containing PSGs associate with or mature into IPODs independently of AMPK signaling. Surprisingly, the fraction of cells with PSGs marked with Pre1-mC (CP) increasingly
colocalized with IPODs in the AMPK mutants, reaching nearly 60% after seven days in low glucose (Fig 4B). Under glucose-free starvation, however, Pre1-mC-containing PSGs maintained a basal (~WT) level of association with Hsp42-GFP (Fig 4D). These data suggest that AMPK specifically regulates CP-containing PSG association with the IPOD under low glucose conditions and that the kinase transduces small differences in glucose concentration into alternative proteasome trafficking and quality control pathways.

In light of the strongly increased colocalization between Pre1-mC and Hsp42-GFP in AMPK mutants after extended low-glucose incubation (Fig 4B), we examined their localization upon glucose refeeding. Pre1-mC foci did not dissipate, as noted above, and colocalization between Pre1-mC and Hsp42-GFP dropped only partially: by ~46% and ~34% in the snf4Δ and snf1Δ cells, respectively, after 30 min glucose recovery (Fig 4C). By comparison, colocalization dropped by ~79% in WT cells due to PSG dissipation and proteasome nuclear reimport. This suggests that a fraction of the CP-containing foci in AMPK mutants have either become terminal PSGs or have matured into IPODs (the expected difference between these two structures being the greater compositional complexity expected for the latter).

Proteasome fragmentation in glucose-depleted cells requires vacuolar proteases but not macroautophagy

Proteasomes are targeted to the vacuole through macroautophagy when cells are grown in the absence of nitrogen or carbon, leading to free GFP release [26, 28]. To assess whether macroautophagy is necessary for the more complex proteasome fragmentation observed under low-glucose conditions, we examined yeast strains lacking macroautophagy-related genes. Loss of ATG8 eliminates all types of macroautophagy [44], while ATG11 and ATG39 are needed for
specific classes of selective macroautophagy [18]. ATG17 is required only for non-selective macroautophagy [20], and ATG15 encodes an intravacuolar lipase [45]. Under low-glucose conditions, anti-GFP immunoblot analysis showed that cleavages of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP within the proteasome subunit moieties of the fusions (“fragments” in Fig 5A) were not significantly different from WT cells in atg8Δ, atg11Δ, atg17Δ, atg39Δ, or atg15Δ mutants, but release of free GFP in these fusions was completely blocked in atg8Δ and atg17Δ cells (Fig 5A). PSGs continued to be observed in all of the tested atg mutants (Fig 5B and S4A Fig); upon glucose refeeding, the PSGs dissipated normally, and nuclear reimport of proteasomes was efficient (S4B Fig). By contrast, proteasome macroautophagy was blocked in nitrogen-starved atg15Δ cells (S4C Fig), in agreement with a previous study [28], although proteasome trafficking to the vacuole continued in the mutant, inasmuch as GFP-tagged full-length proteasome subunits accumulated in the vacuole lumen (S4D Fig). Collectively, these data suggest that complex proteasome fragmentation (in contrast to free GFP release), PSG assembly, PSG dissipation, and nuclear reimport of proteasomes are independent of macroautophagic components in cells cultured in low glucose.

To determine whether the complex proteasome fragmentation depended on vacuolar proteases, we introduced Pre10-GFP, Rpn5-GFP, and Rpn2-GFP into yeast strains lacking PEP4, encoding vacuolar protease A; PRB1, encoding protease B; or both genes [46]. GFP immunoblot analysis showed that fragmentation of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP was partially inhibited in the prb1Δ and pep4Δ single mutants and completely blocked in the pep4Δ prb1Δ double mutant during glucose deprivation (Fig 5A). PSGs formed normally in these mutants, along with retention of GFP-tagged proteasomes in the vacuole lumen (Fig 5B); PSG dissipation and nuclear reimport of proteasomes upon glucose refeeding was also similar to WT cells (S4B Fig).
These results indicate that the complex proteasome fragmentation requires vacuolar proteases and that these macroautophagy-independent cleavages could serve as a signature for an alternative mode of vacuolar import.

**ESCRT-dependent microautophagy is required for proteasome fragmentation**

Another recently described mechanism for protein import and degradation in the yeast vacuole is ESCRT-dependent microautophagy [22]. Vph1 resides in the vacuolar membrane and is subjected to vacuolar degradation by microautophagy when cells are grown beyond the diauxic shift stage; this is independent of macroautophagy factors [22]. We first examined Vph1-GFP degradation under our three starvation conditions: nitrogen-free, glucose-free, and low glucose. Under nitrogen starvation, immunoblot analysis showed that free GFP release from Vph1-GFP was normal in *snf1Δ* and *snf4Δ* cells, while a minor reduction in degradation was seen in *atg15Δ* and *atg8Δ* cells; degradation was completely blocked in *vps4Δ* cells (Fig 6A). This suggests that under nitrogen starvation, vacuolar degradation of Vph1-GFP requires the ESCRT machinery but not AMPK, consistent with our data on AMPK and ESCRT factors in proteasome macroautophagy under nitrogen starvation (Fig 2A and S4C Fig).

This contrasts with immunoblot analyses showing that free GFP release from Vph1-GFP was strongly inhibited in *snf1Δ* and *snf4Δ* cells as well as *vps4Δ* cells under both low-glucose and glucose-free conditions. Interestingly, GFP release was also partially inhibited in *atg15Δ* and *atg8Δ* cells under glucose-free conditions, but was normal in these mutants in low glucose (Fig 6B). Therefore, vacuolar degradation of Vph1-GFP requires AMPK and the ESCRT pathway but is independent of macroautophagy under low-glucose conditions. These results closely parallel the data on proteasome fragmentation under glucose starvation (Figs 3A and 5A). The implication is
that like Vph1 cleavage, proteasome fragmentation in cells grown in low-glucose conditions results from ESCRT-dependent microautophagy.

To observe proteasome microautophagy cytologically, we fused the GFP sequence to different proteasome subunit genes in a triple mutant lacking \textit{PRB1}, \textit{PEP4}, and \textit{ATG8}; the latter mutations block all macroautophagy and most or all vacuolar hydrolysis reactions. Confocal microscopy of cells in low glucose suggested that PSGs marked by Pre10-GFP, Rpn5-GFP, or Rpn2-GFP were tightly associated with vacuolar membrane invaginations (Fig 6C and S1-S3 Videos). Strikingly, by anti-CP immunogold electron microscopy, large numbers of membrane vesicle-like structures accumulated in the vacuole in \textit{atg8Δ pep4Δ prb1Δ} cells, often with gold bead-marked proteasomes on their edges (Fig 6D). The vesicles or tubules fill a large fraction of the vacuole lumen and were often delimited by apparent double membranes, possibly due to folding of invaginated membranes around them (Fig 6E). By contrast, in glucose-limited \textit{vps4Δ pep4Δ prb1Δ} cells, which lack the full ESCRT pathway and therefore microautophagy, CP-labeled autophagic bodies (ABs) were observed in the vacuole lumen instead (Fig 6D and 6E). This is consistent with the known role of macroautophagy in proteasome degradation when cells are carbon-starved [31] and our GFP release assays (Figs 3A and 5A). Moreover, the membrane vesicle-like structures and ABs were induced simultaneously as both structures formed in \textit{pep4Δ prb1Δ} cells, while both disappeared in \textit{atg8Δ vps4Δ pep4Δ prb1Δ} cells (Fig 6D).

\textbf{Aberrant proteasomes are more prone to microautophagy during glucose starvation}

Proteasome microautophagy might represent a type of protein triage or quality control under nutrient stress conditions. We hypothesized that aberrant proteasomes would sort into microautophagy structures rather than reversible PSGs in cells subjected to glucose limitation. To
create cells bearing large numbers of misassembled or abnormal proteasomes, we used yeast
strains lacking either of two nonessential proteasome subunits, Sem1/Rpn15 or Pre9/α3, which
affect assembly of the RP and CP, respectively [47, 48].

Detection of free GFP and novel cleavage fragments from Pre10-GFP, Rpn5-GFP, and
Rpn2-GFP was enhanced in sem1Δ and pre9Δ cells under both low-glucose and glucose-free
conditions (Fig 7A). At the same time, formation of PSGs from GFP-tagged CPs and RPs was
abolished in the sem1Δ cells, as was PSG assembly of Pre10-GFP-tagged CPs in pre9Δ cells (Fig
7B). PSG assembly of Rpn2-GFP and Rpn5-GFP was significantly inhibited but not fully blocked
in the pre9Δ mutant, consistent with formation of RP-only PSGs (Fig 7B and 7C). Rpn2-GFP-
containing PSGs were observed in only ~36% of pre9Δ cells compared to ~75% of WT cells, and
Rpn5-GFP-marked PSGs were observed in ~23% of pre9Δ cells versus ~79% of WT cells (Fig
7C). The PSGs of Rpn2-GFP and Rpn5-GFP still dissipated, and proteasomes were reimported to
the nucleus in the pre9Δ mutant after glucose recovery (Fig 7D). The novel fragments generated
from RP subunit Rpn2-GFP and CP subunit Pre10-GFP specifically in the sem1Δ RP mutant and
pre9Δ CP mutant, respectively, and the greater sensitivity of Pre10-GFP localization to PSGs in
the pre9Δ CP mutant support the idea that CP and RP trafficking through PSGs is likely regulated
through at least partly distinct mechanisms.

To test further the notion that aberrant proteasomes are channeled into the microautophagy
pathway rather than assembled into PSGs during low-glucose starvation, we tested if inhibition of
CP catalytic activity by the drug MG132 affected proteasome fragmentation and PSG assembly
under these conditions. Immunoblot analysis showed that fragmentation of Pre6-GFP (α4), Rpn5-
GFP, and Rpn2-GFP was enhanced by MG132 compared to the DMSO control (S5A Fig).
Strikingly, assembly of these tagged proteasomes into PSGs was completely blocked in MG132-
treated cells (S5B Fig). These findings suggest that cells can recognize inactive or abnormal proteasomes and preferentially remove them by autophagy under low-glucose conditions.

Discussion

The results presented here demonstrate that subcellular proteasome trafficking and degradation are highly regulated during glucose limitation by the integrated action of AMPK (Snf1 kinase) and the ESCRT machinery (Fig 8). In particular, we discovered that AMPK promotes ESCRT-dependent microautophagy of proteasomes under low glucose conditions, and this degradation mechanism appears to be biased toward inactive or abnormal proteasomes. This selectivity allows functional proteasomes to accumulate in PSGs, which after glucose refeeding rapidly dissipate, with reimport of proteasomes into the nucleus. In the absence of AMPK, when ESCRT-dependent microautophagy is compromised, the IPOD serves as an alternative cellular site to sequester and remove proteasome granules that fail to disassemble normally, especially those containing only CP complexes.

Independent regulation of CP and RP during nutrient limitation

In proliferating yeast and most mammalian cells, proteasomes concentrate in the nucleus, but large-scale proteasome relocalization from the nucleus to other intracellular compartments is triggered by nutrient limitation. During nitrogen starvation, proteasomes are routed by macroautophagy to the vacuole for degradation, whereas they mostly relocate to the cytoplasm into large, membraneless granules (PSGs) during carbon starvation.

In nitrogen-starved cells, nuclear proteasomes have been reported to disassemble into CP and RP lid and base subcomplexes [28], and the CP and RP are regulated by different mechanisms.
during their trafficking from the nucleus to the vacuole. For example, CP macroautophagy depends
on the deubiquitylating enzyme Ubp3, but RP macroautophagy does not [27]. Here we have shown
that many components of the ESCRT pathway are required for proteasome macroautophagy,
probably by facilitating the sealing of proteasome-containing autophagosomes; ESCRT factors
have been implicated directly in this stage of macroautophagy [49]. Surprisingly, the Vps4 AAA-
ATPase, while required for RP macroautophagy, is not essential for CP macroautophagy. Vps4
disassembles and recycles the ESCRT-III polymers responsible for membrane fission/fusion
during autophagosome closure [50]. In light of the similar modes of action of Vps4 and the AAA-
ATPase ring of the proteasome RP, it is possible that Vps4 activity in CP macroautophagy can
somehow be replaced by that of the RP ATPase complex [50, 51]. The molecular mechanisms of
Vps4 and potentially the RP in CP macroautophagy during nitrogen starvation will require further
investigation.

Reversible cytoplasmic PSGs are assembled under glucose starvation in yeast and plants
[13, 31]. Previous studies reported that proteasomes also disassemble into CP and RP during
prolonged incubation in stationary phase (which is also a form of carbon starvation) or under
carbon starvation [31, 52], but CP and RP eventually accrete into the same 1-2 large PSGs per cell
[13, 17]. We have found that mutations in either the CP or RP inhibit PSG assembly (Fig 7B-7D).
Alteration of the lid by deletion of SEM1 completely blocked PSG formation from CP, base, and
lid subcomplexes (Fig 7B). This is consistent with previous studies showing that the integrity of
lid subunits (Rpn5 and Rpn11) is required for PSG assembly [12, 16, 17]. Interestingly, creation
of an alternative form of the CP by deletion of PRE9 completely blocked CP incorporation into
PSGs while incompletely inhibiting PSG assembly from base and lid components (Fig 7B-7D).
This supports the inference that proteasomes separate into CP and RP complexes during glucose
starvation and further suggests that PSGs assemble from RP complexes alone in cells expressing a defective CP.

**ESCR T-dependent microautophagy versus reversible PSG assembly**

Our data suggest that AMPK and ESCRT-dependent microautophagy play an important role in sorting and eliminating aberrant proteasomes in cells starved for glucose. Complex proteasome fragmentation is more strongly induced under low glucose (0.025% glucose) than glucose-free conditions (Figs 3A, 5A, and 7A) and requires AMPK; the ESCRT machinery also contributes to this fragmentation by vacuolar proteases (Figs 3A and 5A), but it is largely independent of macroautophagy factors (Fig 5A). ESCRT-dependent microautophagy during glucose starvation was only recently demonstrated [22]. We have now shown that AMPK also plays a critical role in microautophagy of the vacuolar membrane protein Vph1, the primary model substrate used in the earlier study (Fig 6B).

In light of the similar requirements for proteasome and Vph1 degradation in response to glucose starvation, we surmised that proteasome fragmentation could also be through a type of microautophagy under low glucose conditions. We found direct evidence of proteasome microautophagy by immunogold EM labeling of CPs cells (and time-lapse analysis of both CP and RP by confocal microscopy) in *atg8Δ pep4Δ prb1Δ*, which accumulate intravacuolar agglomerations of membrane tubules and vesicles that stain with anti-CP antibodies (Fig 6D). These cells, which cannot carry out macroautophagy and are incapable of degrading their vacuolar contents, collect enormous amounts of this intravacuolar material (Fig 6E). Thus, under conditions of extended glucose deprivation, which are expected to occur in the wild, microautophagy is likely to be a highly active and potent means of cellular remodeling and protein quality control.
The finding that aberrant proteasomes appear to augment their clearance by microautophagy in low-carbon conditions and at the same time compromise reversible PSG assembly (Fig 7 and S5 Fig) raises the question of how abnormal or inactive proteasomes are distinguished and sorted into the degradation pathway. The ESCRT machinery can mediate the selective autophagy of soluble ubiquitylated cargos in fission yeast via the MVB pathway [53]. This is independent of the core macroautophagy machinery [53]. Moreover, by examining the degradation of a vacuolar lysine transporter, Ypq1, a recent study reported that the ESCRT machinery functions directly on the vacuolar membrane for sorting ubiquitylated Ypq1 to the vacuole lumen [54]. We propose that aberrant or inactive proteasomes, which are known to get ubiquitylated [26], are sorted for microautophagy in a similar fashion.

Among the ESCRT proteins, Vps27, a component of the ESCRT-0 complex, recognizes ubiquitylated cargos and recruits ESCRT-I, II, and III for delivering cargos to the vacuole for degradation [55]. It can also work directly at the vacuolar membrane [54]. At the vacuolar membrane, Vps27 may recognize aberrant ubiquitylated proteasomes and initiate ESCRT assembly, leading to the selective elimination of these proteasomes by microautophagy. Proteasome ubiquitylation may occur at the vacuolar membrane; both our fluorescence microscopy data on PSG localization (Figs 4A, 5B, 7B and S2B Fig) and prior studies [17] have shown that the majority of PSGs localize adjacent to the vacuolar membrane during PSG assembly. Therefore, aberrant proteasomes might be accessible to E3 ubiquitin ligases on the vacuolar surface even if initially sorted into PSGs; ubiquitylated particles could then be sorted via the ESCRT machinery to the vacuole lumen. This model posits a direct proteasome quality-control mechanism at the PSG-vacuole interface, an idea we are currently testing.
AMPK regulation of proteasome trafficking and degradation

AMPK can promote autophagy by phosphorylation of the serine/threonine kinase Ulk1 [38, 39]. Ulk1 is a homolog of yeast Atg1 kinase, which is pivotal in macroautophagy initiation during nitrogen starvation [56]. But how does AMPK regulate proteasome microautophagy? Recently, microautophagy has been classified into three types according to the overt membrane dynamics (morphology and location) involved. They are microautophagy with lysosomal protrusion, microautophagy with lysosomal invagination, and microautophagy with endosomal invagination [57]. Proteasome microautophagy in our model (Fig 8), which is based on our cell biological data, is akin to vacuolar (lysosomal) invagination, although we cannot completely exclude endosomal invagination.

In microautophagy with vacuolar invagination, distinct vacuolar membrane domains, classified as liquid-ordered (Lo) and liquid-disordered (Ld), facilitate the invagination of vacuolar membranes in response to glucose depletion [58]. When cells enter stationary phase, for example, lipid droplets (LDs) undergo microautophagy and likely enter the vacuole lumen through sterol-enriched Lo domains [59]. AMPK is activated in response to low glucose levels and promotes the redistribution of Atg14 from ER exit sites onto Lo domains to initiate LD microautophagy [60]. It is possible that AMPK promotes lipid phase separation or stabilizes these vacuolar membrane domains and that PSG association with Ld domains safeguards reversible PSG assembly but allows aberrant proteasomes to partition into Lo domains where the ESCRT machinery initiates proteasome microautophagy. In another scenario, AMPK may directly phosphorylate ESCRT proteins or proteasome subunits and thus enhance interaction of ESCRT factors and proteasomes.

Regulation of proteasome trafficking by the IPOD during glucose starvation
Over a 7-day period of glucose limitation, we found a steady state of ~10% colocalized CP and RP foci with IPODs in WT cells (Fig 4 and S3 Fig). Consistent with our results, a previous study, while reporting increased colocalization between PSGs and IPODs at an early stage of PSG assembly during glucose starvation, observed that the majority of proteasomes eventually separated from the IPOD, resulting in ~10% of cells showing colocalization [17]. We also saw that in the absence of AMPK, the IPOD selectively sequesters excess CP granules that form during low glucose starvation but fail to dissipate upon glucose restoration in these mutants (Figs 4B, 4C, and 8). The IPOD may thus serve as an alternative means of regulating proteasome homeostasis by collecting aggregated proteasomes that escaped from AMPK and ESCRT-dependent proteasome quality controls during PSG formation.

In summary, our findings extend the developing view that proteasomes, which are key components of the cellular protein homeostasis network, are themselves subject to diverse surveillance mechanisms when cells experience nutritional and other stresses. In glucose-limited cultures, yeast cells relocate most proteasomes to 1-2 large cytoplasmic foci, and these PSGs are subject to various types of dynamic control. Our data reveal a novel AMPK- and ESCRT-regulated pathway of proteasome microautophagy under these conditions that preferentially removes abnormal or inactive proteasomes from the cellular pool. How proteasomes are exchanged between PSGs and free proteasome pools both in the cytoplasm and nucleus, and how microautophagy, macroautophagy, and IPOD sequestration control these proteasome populations under different conditions will be important questions to pursue.

Materials and Methods

High-throughput yeast non-essential gene deletion library screening
A synthetic genetic array (SGA) compatible yeast strain [61], harboring a gene encoding the proteasome lid subunit Rpn5-GFP, was introduced into the yeast gene deletion library [35] using automated mating approaches [36]. The resulting strains were glucose starved to induce PSGs, and then imaged using a high-content screening platform [37] to uncover mutants that interfere with PSG dissipation and nuclear reimport of proteasomes upon addition of glucose to the cells. In short, cells were transferred from agar plates into 384-well polystyrene plates (Greiner) for growth in liquid media using the RoToR arrayer robot (Singer Instruments). The cultures in the plates were then transferred by the liquid handler into glass-bottom 384-well microscope plates (Matrical Bioscience) coated with Concanavalin A (Sigma-Aldrich). The plates were then transferred to the ScanR automated inverted fluorescent microscope system (Olympus) using a robotic swap arm (Hamilton). Images of cells in the 384-well plates were recorded at 24°C using a 60× air lens (NA 0.9) and with an ORCA-ER charge-coupled device camera (Hamamatsu). Images were acquired in the GFP channel (excitation filter 490/20 nm, emission filter 535/50 nm). All images were taken at a single focal plane.

Yeast strains and cell growth

Yeast manipulations were performed according to standard protocols [62]. Yeast strains used in this study are listed in S1 Table. Yeast cells were grown overnight in synthetic complete (SC) medium [41] at 30°C with vigorous agitation. Cells were then diluted in fresh SC medium and grown to mid-log phase. Mid-log cells were pelleted, washed once with sterile H2O, followed by different treatments. For nitrogen starvation, cells were resuspended in synthetic minimal medium lacking nitrogen (0.17% [w/v] yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose) and cultured for ~1 day at 30°C. For glucose starvation, cells were
resuspended in SC medium containing 0.025% glucose or lacking glucose, and cultured for ~4 days at 30°C. For proteasome inhibitor MG132 treatment, cells harboring a PDR5 deletion (which allows efficient intracellular accumulation of the drug) 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 # A1118) dissolved in DMSO and cultured for ~1 day at 30°C. For glucose recovery, starved cells were washed once with sterile H2O and resuspended in SC medium (2% glucose) and cultured at room temperature (RT) for microscopy.

**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 equipped with a CCD camera (AxioCam MRm; Carl Zeiss) and a HBO100W/2 light source. Images were taken using AxioVision software. Chemical fixation of cells in Fig 4C was performed as described previously with minor modifications [63]. Briefly, glucose-starved cells were recovered in SC medium for 30 min at RT and fixed with 2% (v/v) formaldehyde and incubated for 5 min at RT. The fixed cells were pelleted and washed once with 0.1 M KPO₄ pH 6.5, and then resuspended in 0.1 M KPO₄ pH 7.5 for imaging with a fluorescence microscope as above.

For confocal microscopy and time-lapse videos, 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 DPSS laser at 561 nm, and emission was collected in the range of 493-556 nm or 579-624 nm for GFP and mCherry.
imaging, respectively. Images were acquired using ZEN software and processed using Adobe Photoshop CS6 software.

Electron microscopy

For immunogold labeling EM: yeast cells grown in SC medium containing 0.025% glucose for ~4 days were collected and fixed with 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde in PBS for 30 min followed by further fixation in 4% PFA for 1 hr. The fixed cells were rinsed with PBS, and resuspended in 10% gelatin. The 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 on a Leica Cryo-EM UC6 UltraCut, and 60 nm thick sections were collected using the Tokuyasu method [64] and 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 antibody rabbit anti-20S (Enzo Life Sciences, catalog # BML-PW9355) at a dilution of 1:200, and 10 nm Protein A gold (Utrecht Medical Center) was used as a secondary antibody. All grids were rinsed in PBS, fixed with 1% glutaraldehyde for 5 min, rinsed again and transferred to a uranyl acetate/methylcellulose drop before being collected and dried.

For regular EM: yeast cells grown in SC medium containing 0.025% glucose for ~4 days were collected and fixed with 2.5% glutaraldehyde and 2% PFA in phosphate buffer pH 6.9 for 1 hr, then rinsed in PBS followed by rinsing in 0.1 M sodium acetate buffer pH 6.1. The cells were post fixed in 2% osmium in 0.1 M sodium acetate for 30 min at RT, rinsed with distilled water and resuspended in aqueous 2% uranyl acetate for 60 min at RT in the dark. Cells were then rinsed in
distilled water and dehydrated in 95% and 100% ethanol. The pelleted cells were resuspended in propylene oxide, then infiltrated using LX112 (Ladd) epon resin and baked overnight at 60°C. Hardened blocks were cut using a Leica UltraCut UC7 and 60 nm sections were collected and stained using 2% uranyl acetate and lead citrate. Grids were all 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 CS6 software.

**Protein extraction and Western blotting**

Total proteins were extracted by the alkaline lysis method [65], and Western blotting was performed as described previously with minor modifications [63]. Cells equivalent to one optical density unit at 600 nm (OD_{600}) were collected by centrifugation and washed once with sterile H_2O. Cells were resuspended in 400 µl 0.1 M NaOH and incubated for 5 min at RT. Cells were pelleted, 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 were pelleted 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 # R8EA4245). The membranes were incubated with the primary antibodies Living Colors A.v. monoclonal antibody (JL-8; anti-GFP) (TaKaRa, catalog # 632381, lot # A5033481) at 1:2,000 dilution or anti-Pgk1 monoclonal antibody (Invitrogen, catalog # 459250, lot # TG2598062B) at 1:10,000 dilution, followed by ECL anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (GE Healthcare, catalog # NA931V, lot # 16889300)
at 1:10,000 dilution. The membranes were incubated in ECL detection reagent [66], and the protein
signals were detecting using autoradiography film (Thomas Scientific, catalog # E3018).

578  **Statistical analysis**

579  ANOVA single factor analysis was performed using Microsoft Excel software. The
580  number of cells counted for each assay are shown in the figure legends. Each experiment was
581  repeated at least three times and the percentages shown in the figures represent the average of all
582  the experiments. Error bars represent standard deviations.

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590  **Competing interests**

591  The authors declare that no competing interests exist.

593  **Author contributions**

594  M.H. coordinated the study. J.L. and M.H. conceived the experiments and analyzed the data. J.L.
595  performed the experiments as described in the manuscript. M.S. conceived and M.B. performed
596  the yeast genetic screening. M.G. processed yeast samples for EM. J.L. drafted the manuscript.
597  J.L. and M.H. edited the manuscript. All authors read the manuscript.
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Fig 1

A

Pre1-mC Rpn2-mC Rpn5-mC

WT

vps27Δ

vps4Δ

Glucose starvation

B

Nup49-GFP Pre1-mC Merge

Intensity

WT

24 min

vps27Δ

25 min

vps4Δ

Glucose recovery

Nucleus (white line)

C

Pre1-mC Rpn2-mC Rpn5-mC

snf4Δ

snf1Δ

Glucose starvation

D

Nup49-GFP Pre1-mC Merge

Intensity

snf4Δ

22 min

snf1Δ

16 min

Glucose recovery

Nucleus (white line)

E

WT snf4Δ snf1Δ

Cyto

Nuc PSG

500 nm

Glucose starvation

500 nm
Fig 1. AMPK and ESCRT proteins are required for reversible PSG assembly under glucose starvation.

(A) Epifluorescence microscopy images of Pre1-mC (mCherry), Rpn2-mC, and Rpn5-mC in WT and ESCRT-defective mutant cells (vps27Δ and vps4Δ) during low glucose starvation. PSG assembly is largely disrupted in the mutants. (B) Confocal microscopy images of Pre1-mC in the ESCRT mutant cells after glucose recovery at the indicated time points. Defective nuclear reimport of proteasomes was also observed in the ESCRT mutants. (C) Epifluorescence images of Pre1-mC, Rpn2-mC, and Rpn5-mC in AMPK-defective cells (snf4Δ and snf1Δ) under low glucose. PSGs observed in AMPK mutants were comparable to those in WT cells. (D) Confocal images of Pre1-mC in the AMPK mutant cells after glucose recovery at the indicated time points. The PSGs in AMPK mutant cells failed to dissipate upon glucose refeeding. (E) Cryo-immunogold electron micrographs of PSGs in the WT and AMPK mutant cells during glucose starvation. Cells were immunolabeled with anti-CP primary antibody and 10-nm Protein A-gold-bead-conjugated secondary antibody. Nuc: nucleus; Cyto: cytoplasm. Cells were harvested from cultures grown in SC medium containing 0.025% glucose for ~4 days at 30°C in panels (A), (C), and (E). White arrowheads point to PSGs in panels (A) and (C). Scale bars, 5 µm in panels (A) and (C). In panels (B) and (D), the times indicate when the images were taken after glucose add-back; Nup49-GFP served as a nuclear envelope marker; white dotted circles indicate the approximate outlines of cells. The line charts quantify signal intensity of Pre1-mC in the indicated white line across the nucleus after glucose add-back.
Fig 2

(A) Immunoblot analyses of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in WT, ESCRT mutant (vps27Δ, vps28Δ, vps37Δ, vps25Δ, snf7Δ, vps4Δ), and AMPK mutant (snf4Δ, snf1Δ) cells cultured in nitrogen starvation medium (-N) for ~1 day at 30°C. JL-8 monoclonal anti-GFP antibody was used to detect full-length proteasome subunit fusions and free GFP. Pgk1 served as a loading control. (B) Epifluorescence images of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in WT and ESCRT mutant (vps27Δ and vps4Δ) cells from panel (A). GFP-tagged proteasome signals were observed in the vacuole lumen in WT cells but adjacent to the vacuolar membrane (marked with dotted white line) in vps27Δ and vps4Δ cells under nitrogen starvation, with the exception that Pre10-GFP was observed in the vacuole lumen in vps4Δ cells. BF: bright field. V: vacuole. 4×: 4× enlargement of the squared regions in cells. Scale bar, 5 µm. Representative blots and images are shown.

Fig 2. Proteasome macroautophagy requires the ESCRT machinery but not AMPK under nitrogen starvation.
Fig 3

A

$0.025\% \text{ C}$  

| ESCRT | AMPK |
|-------|------|
| WT    | WT    |
| $\text{vps24}^{\Delta}$ | $\text{snf}^{\Delta}$ |
| $\text{vps24}^{\Delta}$ | $\text{snf}^{\Delta}$ |
| $\text{Pre10-GFP}$ | $\text{free GFP}$ |
| $\text{fragments}$ | $\text{Rpn5-GFP}$ |
| $\text{free GFP}$ | $\text{Rpn2-GFP}$ |

B

$0.025\% \text{ C}$  

| ESCRT | Vph1-GFP | mCherry | Merge |
|-------|----------|---------|-------|
| WT    | $\text{Pre1-mC}$ | $\text{vps4}^{\Delta}$ | $\text{Rpn5-mC}$ |
| $\text{Rpn2-mC}$ | $\text{Rpn5-mC}$ | $\text{Rpn2-mC}$ |
Fig 3. AMPK and ESCRT machinery are required for proteasome fragmentation under glucose starvation.

(A) Anti-GFP immunoblot analyses of free GFP release and proteasome fragmentation of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in WT and ESCRT mutants (vps27Δ, vps28Δ, vps37Δ, vps25Δ, snf7Δ, vps4Δ) and AMPK mutants (snf4Δ, snf1Δ) under glucose starvation. Cells were harvested from cultures in SC medium containing 0.025% glucose (0.025% C) or lacking glucose (-C) for ~4 days at 30°C. Low glucose conditions induced more proteasome fragmentation than did glucose-free starvation. (B) Confocal images of Pre1-mC, Rpn5-mC, and Rpn2-mC in an ESCRT-defective mutant (vps4Δ) expressing the vacuolar membrane marker Vph1-GFP. Cells were harvested from cultures in SC medium containing 0.025% glucose for ~1 day at 30°C. White arrowheads point to proteasomes that were blocked at apparent class E compartments. Scale bar, 2 µm.
Fig 4. CP-containing irreversible PSGs are enriched for IPOD marker Hsp42 in AMPK mutants in low glucose.

(A) Epifluorescence images of Pre1-mC and Hsp42-GFP in WT, snf4Δ, and snf1Δ cells in low glucose for ~4 days. Representative images of Pre1-mC where it separates from or colocalizes with Hsp42-GFP are shown. White arrows point to colocalized Pre1-mC and Hsp42-GFP. BF: bright field. Scale bar, 5 µm. (B) Quantification of colocalized Pre1-mC and Hsp42-GFP in WT (438 cells counted [1d], 493 [2d], 338 [3d], 390 [4d], 546 [7d]), snf4Δ (n=334 [1d], 845 [2d], 947 [3d], 652 [4d], 705 [7d]), and snf1Δ (n=273 [1d], 551 [2d], 514 [3d], 531 [4d], 453 [7d]) live cells in 0.025% glucose. (C) Quantification of colocalized Pre1-mC and Hsp42-GFP after 30 min recovery in 2% glucose in cells fixed after 7 d in 0.025% glucose from panel (B). Cells counted: WT (789), snf4Δ (692), snf1Δ (396). (D) Percentage of living cells with colocalized Pre1-mC and Hsp42-GFP. WT (433 cells counted [1d], 456 [2d], 693 [3d], 495 [4d], 355 [7d]), snf4Δ (519 [1d], 688 [2d], 473 [3d], 361 [4d], 220 [7d]), and snf1Δ (559 [1d], 484 [2d], 500 [3d], 412 [4d], 147 [7d]) cultures were grown in glucose-free medium. Results plotted as mean±sd. *, P<0.05; **, P<0.01; ***, P<0.001 (ANOVA single factor analysis comparing snf4Δ or snf1Δ to WT).
Fig 5

A 0.025% C

| WT | atg8Δ | atg11Δ | atg17Δ | pep4Δ | pep4Δ prb1Δ | WT | atg15Δ |
|----|-------|--------|--------|-------|-------------|----|--------|
| kDa | 250   | 150    | 75     | 50    | 37          | 25 | 20     |
| Pre10-GFP fragments | free GFP | Pgk1 |
| Rpn5-GFP fragments | free GFP | Pgk1 |

B

0.025% C

Vacuole lumen

| BF | Pre10-GFP 4X | BF Rpn5-GFP 4X | BF Rpn2-GFP 4X |
|----|--------------|----------------|----------------|
| atg8Δ |            |                |                |
| prb1Δ |            |                |                |
| pep4Δ |            |                |                |
| pep4Δ prb1Δ | |                |                |
Fig 5. Proteasome fragmentation in glucose-starved cells requires vacuolar proteases but not macroautophagy factors.

(A) Anti-GFP immunoblot analyses of WT, macroautophagy mutant (atg8Δ, atg11Δ, atg17Δ, atg39Δ, atg15Δ), and vacuolar protease-deficient mutant (prb1Δ, pep4Δ, pep4Δ prb1Δ) cells under glucose limitation. Cells were harvested from cultures in SC medium containing low glucose (0.025% C) or no glucose (-C) for ~4 days at 30°C. (B) Epifluorescence images of a core macroautophagy mutant (atg8Δ) and vacuolar protease-defective mutants (prb1Δ, pep4Δ, pep4Δ prb1Δ) from panel (A). Black arrows mark PSGs. BF: bright field. 4×: 4× enlargement of the square regions in the vacuole lumen. Scale bar, 5 µm.
Fig 6. Evidence for proteasome microautophagy in low glucose conditions.

(A) Anti-GFP immunoblot analysis of free GFP release from the vacuolar membrane protein Vph1-GFP in WT, macroautophagy mutant (atg8Δ and atg15Δ), ESCRT mutant (vps4Δ), and AMPK mutant (snf4Δ and snf1Δ) cells under nitrogen starvation for ~1 day at 30°C. (B) Immunoblot analyses of free GFP release from Vph1-GFP in the same strains as in (A) but during glucose starvation. Cells were harvested from cultures after ~4 days at 30°C. (C) Confocal microscopy images of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in atg8Δ pep4Δ prb1Δ cells in low glucose. Related videos are provided in the supporting information. BF: bright field. Scale bar, 2 μm. (D) Cryo-immunogold electron micrographs of proteasomes in the indicated cells grown in low glucose. Cells were immunolabeled with anti-CP primary antibody and Protein A gold-conjugated secondary antibody (black arrows). (E) Electron micrographs of microautophagic vesicles and autophagic bodies formed in atg8Δ pep4Δ prb1Δ and vps4Δ pep4Δ prb1Δ cells, respectively, during low glucose starvation. “*” indicates highlighted phenotypes in left panels that enlarged as shown in right panels. Nuc: nucleus; Cyto: cytoplasm; Vac: Vacuole; AB: autophagic body. Cells were harvested from cultures in SC medium containing 0.025% glucose for ~1 day in panel (C) and ~4 days in panels (D, E) at 30°C.
Fig 7

A. 0.025% C

B. 0.025% C

C. Percentage of PSGs

D. 2% C

42
Fig 7. Defective proteasomes are channeled to the vacuole rather than PSGs in glucose-starved cells.

(A) Anti-GFP immunoblot analyses of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in WT and proteasome mutant (sem1Δ and pre9Δ) cells during glucose starvation. Cells were cultured for ~4 days at 30°C in SC media with low or no glucose. “*” indicates novel fragments not seen in WT.

(B) Epifluorescence images of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP from the same cultures grown in low (0.025%) glucose from panel (A). CP-containing PSGs were no longer observed in sem1Δ or pre9Δ cells, and RP-containing PSGs were not formed in sem1Δ cells and were significantly reduced in pre9Δ cells. White arrows point to PSGs.

(C) Percentage of cells with PSGs visualized with Rpn5-GFP and Rpn2-GFP in WT (793 cells counted [Rpn2-GFP], 890 [Rpn5-GFP]) and pre9Δ (938 cells counted [Rpn2-GFP], 819 [Rpn5-GFP]) cells. Results shown as mean±sd. *, P<0.05 (ANOVA single factor analysis).

(D) Epifluorescence images of Rpn5-GFP and Rpn2-GFP in WT and pre9Δ cells after glucose add-back for the indicated times. Cultures were the same as in panel (B). White arrows point to nuclei with reimported proteasomes. BF: bright field; scale bar, 5 µm in panels (B, D).
Fig 8. Model for cellular control of proteasome trafficking and degradation during low glucose starvation and glucose recovery.

In this working model, AMPK links glucose status with the trafficking and degradation of proteasomes. Normally, AMPK will either directly or indirectly promote formation of reversible PSGs in low glucose conditions. A fraction of proteasomes will be inactive or defective after prolonged starvation, and ESCRT-dependent microautophagy preferentially targets this fraction for degradation in the vacuole. In AMPK mutants, proteasome microautophagy is blocked (as indicated by the absence of proteasome fragmentation), and PSGs accumulate both aberrant and normal proteasomes. In this case, PSGs mature into or exchange proteasomes with the IPOD compartment to sequester potentially defective or inactive proteasomes, particularly irreversible CP-containing granules. These structures might be degraded by macroautophagy or, upon glucose refeeding and re-entry into the cell cycle, might utilize retention in mother cells as a means to clear aberrant proteasomes.
S1 Fig. ESCRT proteins are required for reversible PSG assembly under glucose starvation.

(A) Epifluorescence images of Pre1-mC, Rpn2-mC, and Rpn5-mC in ESCRT-defective mutant cells (ESCRT-II: vps25Δ, and ESCRT-III: did2Δ, vps2Δ, vps24Δ, snf7Δ) during low glucose starvation. PSG assembly is largely disrupted in the mutants. Scale bar, 5 μm. (B) Confocal images of Pre1-mC in the ESCRT mutant cells after glucose recovery at the indicated time points. Defective nuclear reimport of proteasomes was also observed in the ESCRT mutants. The times indicate when the images were taken after glucose add-back; Nup49-GFP served as a nuclear envelope marker; white dotted circles indicate the approximate outlines of cells. The line charts quantify signal intensity of Pre1-mC in the indicated white line across the nucleus after glucose recovery.
### S2 Fig

#### A

|        | -N | BF | Pre10-GFP | 4X | BF | Rpn5-GFP | 4X | BF | Rpn2-GFP | 4X |
|--------|----|----|-----------|----|----|----------|----|----|----------|----|
| ESCRT-I | vps28Δ | | | | | | | | | |
| ESCRT-II | vps25Δ | | | | | | | | | |
| ESCRT-III | snf7Δ | | | | | | | | | |
| snf4Δ | | | | | | | | | | |
| AMPK | | | | | | | | | | |
| snf1Δ | | | | | | | | | | |

#### B

|        | 0.025% C | BF | Pre10-GFP | 4X | BF | Rpn5-GFP | 4X | BF | Rpn2-GFP | 4X |
|--------|----------|----|-----------|----|----|----------|----|----|----------|----|
| WT | | | | | | | | | | |
| ESCRT-0 | vps27Δ | | | | | | | | | |
| ESCRT-I | vps28Δ | | | | | | | | | |
| ESCRT-II | vps25Δ | | | | | | | | | |
| ESCRT-III | snf7Δ | | | | | | | | | |
| vps4Δ | | | | | | | | | | |
S2 Fig. ESCRT machinery is required for proteasome trafficking to the vacuole during nitrogen and glucose starvation conditions.

(A) Epifluorescence images of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in nitrogen-starved ESCRT mutant (vps28Δ, vps25Δ, and snf7Δ) and AMPK mutant (snf4Δ, snf1Δ) cells from figure panel (2A). (B) Epifluorescence images of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in low glucose-starved WT and ESCRT mutant cells from figure panel (3A). The vacuolar membrane is marked with dotted white line. BF: bright field. V: vacuole. 4×: 4× enlargement of the squared regions in cells. Scale bars, 5 μm.
S3 Fig

A

Percentage of cells with colocalized Rpn5-mC and Hsp42-GFP

WT
snf4Δ
snf1Δ

0%
10%
20%
30%
40%
50%
60%
70%

Days under 0.025% glucose starvation

1d 2d 3d 4d 7d

B

Percentage of cells with colocalized Rpn5-mC and Hsp42-GFP

WT
snf4Δ
snf1Δ

0%
10%
20%
30%
40%
50%
60%
70%

Days under glucose free starvation

1d 2d 3d 4d 7d

C

Percentage of cells with colocalized Rpn2-mC and Hsp42-GFP

WT
snf4Δ
snf1Δ

0%
10%
20%
30%
40%
50%
60%
70%

Days under 0.025% glucose starvation

1d 2d 3d 4d 7d

D

Percentage of cells with colocalized Rpn2-mC and Hsp42-GFP

WT
snf4Δ
snf1Δ

0%
10%
20%
30%
40%
50%
60%
70%

Days under glucose free starvation

1d 2d 3d 4d 7d
S3 Fig. RP-containing irreversible PSGs maintain a basal colocalization level with an IPOD marker Hsp42 in AMPK mutants under glucose starvation.

(A) Quantification of colocalized Rpn5-mC and Hsp42-GFP in WT (577 cells counted [1d], 542 [2d], 748 [3d], 792 [4d], 446 [7d]), snf4Δ (546 [1d], 790 [2d], 838 [3d], 368 [4d], 524 [7d]), and snf1Δ (316 [1d], 656 [2d], 530 [3d], 374 [4d], 266 [7d]) live cells in 0.025% glucose. (B) Percentage of living cells with colocalized Rpn5-mC and Hsp42-GFP. WT (292 cells counted [1d], 580 [2d], 457 [3d], 419 [4d], 301 [7d]), snf4Δ (622 [1d], 547 [2d], 403 [3d], 440 [4d], 164 [7d]), and snf1Δ (555 [1d], 465 [2d], 422 [3d], 434 [4d], 212 [7d]) cultures were grown in glucose-free medium. (C) Quantification of colocalized Rpn2-mC and Hsp42-GFP in WT (469 cells counted [1d], 402 [2d], 498 [3d], 460 [4d], 435 [7d]), snf4Δ (355 [1d], 573 [2d], 704 [3d], 494 [4d], 499 [7d]), and snf1Δ (459 [1d], 445 [2d], 555 [3d], 535 [4d], 348 [7d]) live cells in 0.025% glucose. (D) Percentage of living cells with colocalized Rpn2-mC and Hsp42-GFP. WT (347 cells counted [1d], 481 [2d], 601 [3d], 396 [4d], 367 [7d]), snf4Δ (471 [1d], 541 [2d], 352 [3d], 385 [4d], 183 [7d]), and snf1Δ (563 [1d], 415 [2d], 325 [3d], 347 [4d], 138 [7d]) cultures were grown in glucose-free medium. Results plotted as mean±sd.
S4 Fig

**A** 0.025% C  Pre10  BF  Merge  Rpn5  BF  Merge  Rpn2  BF  Merge

- *atg11Δ*
- *atg17Δ*
- *atg39Δ*
- *atg15Δ*

**B** 2% C  Pre10  BF  Merge  Rpn5  BF  Merge  Rpn2  BF  Merge

- *atg8Δ*
- *atg11Δ*
- *atg17Δ*
- *atg39Δ*
- *atg15Δ*
- *prb1Δ*
- *pep4Δ*
- *pep4Δ prb1Δ*

**C**

| kDa | 250 | 150 | 100 | 75 | 50 | 37 | 25 | 20 |
|-----|-----|-----|-----|----|----|----|----|----|

- Rpn2-GFP
- Rpn5-GFP
- Pre10-GFP
- free GFP

**D**

| -N | Pre10 | BF | Merge |
|-----|-------|----|-------|

- WT
- *atg15Δ*

| -N | Pre10 | BF | Merge |
|-----|-------|----|-------|

- WT
- *atg15Δ*
S4 Fig. Reversible PSGs are assembled in macroautophagy mutant and vacuolar protease-deficient mutant cells.

(A) Epifluorescence images of Pre10-GFP, Rpn5-GFP, and Rpn2-GFP in low glucose-starved core macroautophagy mutants (atg11Δ, atg17Δ, atg39Δ, atg15Δ) from figure panel (5A). (B) Epifluorescence images of core macroautophagy mutants (atg8Δ, atg11Δ, atg17Δ, atg39Δ, atg15Δ) and vacuolar protease-deficient mutants (prb1Δ, pep4Δ, pep4Δ prb1Δ) cells at the indicated time recovery in 2% glucose. Cells were from figure panel (5A). White arrows mark PSGs in panel (A) and the nucleus in panel (B). (C) Anti-GFP immunoblot analyses of WT and a vacuolar lipase-deficient mutant atg15Δ cells under nitrogen starvation for ~1 day at 30°C. (D) Epifluorescence images of nitrogen-starved WT and atg15Δ cells from panel (C). White arrowheads mark GFP-tagged full length proteosomes in the vacuole lumen in atg15Δ cells. BF: bright field. Scale bars, 5 µm.
S5 Fig

Inactive proteasomes enhance proteasome fragmentation but compromise PSG assembly.

(A) Anti-GFP immunoblot analyses of Pre6-GFP, Rpn5-GFP, and Rpn2-GFP in pdr5Δ mutant cells. Cells were harvested from cultures in SC medium containing low glucose (0.025% C) with DMSO or 50 µM MG132 treatment for ~1 day at 30°C. (B) Epifluorescence images of DMSO and MG132 treated pdr5Δ mutant cells from panel (A). White arrows mark PSGs. BF: bright field. Scale bar, 5 µm.
S1, S2, and S3 Videos. PSGs are associated with the vacuolar membrane invagination in \textit{atg8}\textsuperscript{\Delta} \textit{pep4}\textsuperscript{\Delta} \textit{prb1}\textsuperscript{\Delta} mutant in low glucose conditions.

Confocal time lapse images of Pre10-GFP (S1 Video), Rpn5-GFP (S2 Video), and Rpn2-GFP (S3 Video) showing that PSGs were associated with the vacuolar membrane invagination in \textit{atg8}\textsuperscript{\Delta} \textit{pep4}\textsuperscript{\Delta} \textit{prb1}\textsuperscript{\Delta} mutant cells in low glucose for ~1 day at 30°C. The time lapse videos were composed from 40 frames of images with 1.27s scanning time for each frame and played at 4 frames per second, the real time length was 49.47s for each video.
### Table 1. Yeast strains used in this study

| Strain    | Genotype                                                                 | Reference |
|-----------|---------------------------------------------------------------------------|-----------|
| MHY500    | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 vps4Δ::TRP1             | [1]       |
| MHY2443   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1                         | [2]       |
| MHY6377   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3       | This study|
| MHY6614   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 prb1Δ::kanMX pep4Δ::natMX | This study|
| MHY7791   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::kanMX pdr5Δ::kanMX | This study|
| MHY7797   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 prb1Δ::kanMX pep4Δ::natMX | This study|
| MHY8595   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX | This study|
| MHY8599   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX | This study|
| MHY8602   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX | This study|
| MHY8629   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX did2Δ::HIS3 | This study|
| MHY8630   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX did2Δ::HIS3 | This study|
| MHY8632   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX did2Δ::HIS3 | This study|
| MHY8633   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX vps4Δ::HIS3 | This study|
| MHY8634   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX vps4Δ::HIS3 | This study|
| MHY8636   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX vps24Δ::HIS3 | This study|
| MHY8637   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX vps24Δ::HIS3 | This study|
| MHY8639   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX vps24Δ::HIS3 | This study|
| MHY8641   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX vps27Δ::LEU2 | This study|
| MHY8642   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX vps27Δ::LEU2 | This study|
| MHY8643   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX vps27Δ::LEU2 | This study|
| MHY8645   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX vps27Δ::LEU2 | This study|
| MHY8647   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX vps4Δ::TRP1 | This study|
MHY8649  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX vps4Δ::TRP1 This study
MHY8652  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX vps4Δ::TRP1 This study
MHY9736  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 snf1Δ::kanMX This study
MHY9738  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 snf4Δ::kanMX This study
MHY9744  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 prb1Δ::kanMX This study
MHY9746  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 pep4Δ::natMX This study
MHY9749  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 prb1Δ::kanMX pep4Δ::natMX This study
MHY9812  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf4Δ::kanMX This study
MHY9813  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 snf1Δ::kanMX This study
MHY9848  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX snf1Δ::kanMX This study
MHY9849  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX snf1Δ::kanMX This study
MHY9851  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX snf1Δ::kanMX This study
MHY9856  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX snf7Δ::kanMX This study
MHY9859  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX snf7Δ::kanMX This study
MHY9860  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX snf7Δ::kanMX This study
MHY9866  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX snf4Δ::kanMX This study
MHY9867  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX snf4Δ::kanMX This study
MHY9868  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ PRE1-mC::natMX vps25Δ::kanMX This study
MHY9870  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN2-mC::natMX vps25Δ::kanMX This study
MHY9872  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX vps25Δ::kanMX This study
MHY9885  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 NUP49-GFP::his5+ RPN5-mC::natMX snf4Δ::kanMX This study
MHY9943  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 PRE1-mC::natMX This study
MHY9945  MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 RPN2-mC::natMX This study
| Strain | Description |
|--------|-------------|
| MHY9946 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 RPN5-mC::natMX |
| MHY9948 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 PRE1-mC::natMX snf4Delta::kanMX |
| MHY9950 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 RPN2-mC::natMX snf1Delta::kanMX |
| MHY9953 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 PRE1-mC::natMX snf1Delta::kanMX |
| MHY9956 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 RPN5-mC::natMX snf4Delta::kanMX |
| MHY9958 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 RPN5-mC::natMX snf1Delta::kanMX |
| MHY9967 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HSP42-GFP::HIS3 RPN2-mC::natMX snf4Delta::kanMX |
| MHY9989 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 vps27A::LEU2 |
| MHY9992 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 vps25A::kanMX |
| MHY9993 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 vps4Delta::TRP1 |
| MHY9998 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 snf7Delta::kanMX |
| MHY10011 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 snf4Delta::kanMX |
| MHY10015 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 vps28A::kanMX |
| MHY10018 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 vps37A::kanMX |
| MHY10021 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 |
| MHY10042 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 atg8Delta::hphMX |
| MHY10044 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 atg8Delta::hphMX |
| MHY10047 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 snf4Delta::kanMX |
| MHY10048 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 snf1Delta::kanMX |
| MHY10051 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 snf7Delta::kanMX |
| MHY10052 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 vps37Delta::kanMX |
| MHY10055 | MATa his3-A200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 vps28Delta::kanMX |

This study
| Experiment | Description |
|------------|-------------|
| MHY10057   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10058   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10061   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10072   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10073   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10075   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10081   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10083   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10110   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10114   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10115   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10119   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10126   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10128   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10130   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10131   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10147   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10151   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10162   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10164   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10166   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10169   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| MHY10170   | MATa his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 |
| ID     | Strain Details                                                                 | Comment     |
|--------|-------------------------------------------------------------------------------|-------------|
| MHY10173 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 VPH1-GFP::HIS3 vps4Δ::TRP1 | This study  |
| MHY10175 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 VPH1-GFP::HIS3 snf4Δ::kanMX | This study  |
| MHY10179 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 vps4Δ::TRP1 | This study  |
| MHY10181 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 vps25Δ::kanMX | This study  |
| MHY10182 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 vps27Δ::LEU2 | This study  |
| MHY10183 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 vps37Δ::kanMX | This study  |
| MHY10185 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 snf7Δ::kanMX | This study  |
| MHY10187 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 vps28Δ::kanMX | This study  |
| MHY10190 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 atg8Δ::hphMX | This study  |
| MHY10192 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 atg11Δ::hphMX | This study  |
| MHY10193 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 atg17Δ::hphMX | This study  |
| MHY10195 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 atg39Δ::hphMX | This study  |
| MHY10326 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 VPH1-GFP::HIS3 atg15Δ::hphMX | This study  |
| MHY10328 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 sem1Δ::kanMX | This study  |
| MHY10330 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 sem1Δ::kanMX | This study  |
| MHY10332 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 sem1Δ::kanMX | This study  |
| MHY10335 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE10-GFP::HIS3 pre9Δ::hphMX | This study  |
| MHY10336 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-GFP::HIS3 pre9Δ::hphMX | This study  |
| MHY10339 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-yEGFP::HIS3 pre9Δ::hphMX | This study  |
| MHY10354 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 PRE1-mC::natMX VPH1-GFP::HIS3 vps4Δ::TRP1 | This study  |
| MHY10361 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN5-mC::natMX VPH1-GFP::HIS3 vps4Δ::TRP1 | This study  |
| MHY10371 | MATa his3-D200 leu2-3, 112 ura3-52 lys2-801 trp1-1 RPN2-mC::natMX VPH1-GFP::HIS3 vps4Δ::TRP1 | This study  |
Supporting References

1. Chen P, Johnson P, Sommer T, Jentsch S, Hochstrasser M. Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MATα2 repressor. Cell. 1993;74(2):357-69. doi: 10.1016/0092-8674(93)90426-Q.

2. Amerik A, Sindhi N, Hochstrasser M. A conserved late endosome–targeting signal required for Doa4 deubiquitylating enzyme function. J Cell Biol. 2006;175(5):825-35. doi: 10.1083/jcb.200605134.