Proteasome autophagy is specifically regulated and requires factors dispensible for general autophagy

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ABSTRACT

Changing physiological conditions can increase the need for protein degradative capacity in eukaryotic cells. Both the ubiquitin-proteasome system and autophagy contribute to protein degradation. However, proteasomes are also an autophagy substrate. Thus, these processes must be differentially regulated depending on the physiological conditions presented. The signals and molecular mechanisms that govern proteasome autophagy are only partly elucidated. Our data indicate that chemical inhibition of TORC1 with rapamycin induces a bi-phasic response where proteasome levels are upregulated followed by an autophagy-dependent reduction. Surprisingly, several conditions that result in inhibited TORC1 exclusively induce proteasome autophagy (i.e. without any proteasome upregulation), suggesting a convergence of signals upstream of proteasome autophagy under different physiological conditions. Indeed, several conditions that activate general autophagy did not induce proteasome autophagy further distinguishing between proteasome autophagy and general autophagy. Consistent with this, we found that Atg11, the receptor for selective autophagy, and the map kinases Mpk1, Mkk1, and Mkk2, all play a role in autophagy of proteasomes, while they are dispensible for general autophagy. In all, our data provide new insights into the molecular regulation of proteasome autophagy by demonstrating that these complexes are specifically regulated under different autophagy inducing conditions.

There are two major pathways for eukaryotic cells to recycle proteins, lysosomal targeting and the ubiquitin proteasome system (UPS). Within the UPS, an E3 ubiquitin ligase recognizes substrates destined for degradation. In concert with a ubiquitin
conjugating enzyme, lysines of the substrate are labeled with ubiquitin or a chain of ubiquitins. The ubiquitinated substrates are recognized by the proteasome and subsequently unfolded and hydrolyzed into short peptides. The peptides are further processed by peptidases into amino acids that become intermediates for various metabolic processes (1, 2). Lysosomes receive substrates either from endocytosis (extracellular and plasma membrane components) or through autophagy (intracellular components). Autophagy, in this paper referring to macroautophagy, utilizes a process where substrates are engulfed into a double membrane compartment known as the autophagosome (3–5). The targeting of substrates to autophagosomes, like the UPS, is often initiated by ubiquitination of target proteins. Ubiquitinated proteins are recognized by autophagy adapters, such as p62/SQSTM1 in humans and Cue5 in yeast, that bind LC3 (Atg8 in yeast) found on expanding pre-autophagosomes (6–9). LC3 binding is facilitated by LC3 interacting regions (LIRs and Atg8 interacting motifs, AIMs, in yeast) in autophagy adapters. Closure of the pre-autophagosome captures the substrates. The formed autophagosome then fuses with lysosomes (or vacuoles in yeast and plants) and the content is degraded. Specific transporters export generated amino acids to the cytosol (10). The ability of both the UPS and lysosomal degradation to contribute to the amino acid pool in cells becomes particularly important during physiological conditions that reduce amino acid levels. For example, under conditions of nitrogen starvation, autophagy becomes essential and is the major route for amino acid recycling (11, 12).

Autophagy and the UPS both replenish the amino acid pool as well as utilize ubiquitination as a signal for degradation. Therefore, it makes sense that these processes are coordinated and have some level of redundancy. Indeed, the upregulation of one pathway can, to some extent, compensate for the impairment of the other (13–15). For example, proteasome inhibitors have been shown to induce an autophagic response, and mTOR inhibition by rapamycin, which induces autophagy, was shown to relieve proteotoxic stress caused by proteasome inhibition (16, 17). Further, an increase in proteasome activity was reported in yeast and mammalian cells deleted for the essential autophagy gene ATG5 as well as upon chemical inhibition of autophagy (18).

Similarly, atg32Δ cells upregulate proteasome activity in an effort to compensate for an inability to induce non-selective autophagy upon antimycin A treatment (18). Some functions are however clearly unique to each pathway. Proteasomes are, in large, responsible for the degradation of short-lived proteins like IκB (during NFκB signaling) and cyclins (during cell cycle progression) (19, 20).

Autophagy, on the other hand, can dispose of aggregated proteins or defective (parts of) organelles directly through the extension of autophagic membranes.

Autophagy is induced by a number of cellular stresses such as nutrient starvation, mitochondrial dysfunction, and various infections (3, 10, 21). This process can capture cytosolic material non-specifically, as well as be selective for specific cargo. Examples of the latter are mitophagy, pexophagy, and ER-phagy, where unique receptors (Atg32, Pex14, and Atg39/40 respectively) ensure specific and efficient targeting to autophagosomes (4, 22–24). Besides organelles, selective degradation has also been observed for ribosomes in yeast and mammalian cells, a process named ribophagy (24–27). Proteasomes are another multisubunit complex that can be targeted for autophagic degradation, a process referred to as proteaphagy. In yeast, nitrogen starvation and proteasome inhibitor treatment induce proteaphagy (28, 29). The process is conserved as it has also been observed in Arabidopsis and human cells (30–32) and appears to be selective. First, receptors have been identified that target proteasomes to autophagosomes: For example, p62 upon amino acid starvation in mammalian cells, (30) and Rpn10 and Cue5 upon proteasome inhibition in plants and yeast respectively (29, 31). Furthermore, several factors that are dispensable for bulk autophagy are important for proteasome autophagy upon nitrogen starvation in yeast (28, 31, 33). How this
selective proteasome autophagy is regulated, however, remains poorly understood.

The target of rapamycin complex 1 (TORC1) is a master regulator controlling cell growth and metabolic activity based on the cell's physiological state. Under nutrient rich conditions, TORC1 is active and general autophagy is inhibited through the phosphorylation and inactivation of Atg13 and Atg1/Ulk1 (34–36). Interestingly, treatment of cells with the TORC1 inhibitor rapamycin has been shown to increase proteasome levels in yeast and mammalian cells (34, 37, 38). Further, an increase in K48-linked ubiquitinated substrates has been observed, along with an increase in proteasomal proteolysis in mammalian cells treated with mTOR inhibitors. Apparently, the capacity of protein degradation by proteasomes becomes important under conditions where TORC1 is not active. Surprisingly, proteasomes undergo autophagic degradation in yeast, plants, and mammalian cells upon starvation conditions well-known to cause TORC1 inhibition (28, 30, 31). To better understand the response of proteasomes to autophagic stimuli, we sought to determine how proteasome autophagy was regulated in yeast. We used various chemical treatments and physiological conditions known to induce general autophagy. Our data show a biphasic response upon inhibition of TORC1 with rapamycin. During the first four hours of treatment, proteasome levels and activity increased. After this, proteasomes were targeted for vacuolar degradation. However, nitrogen starvation, which induces TORC1 inhibition naturally, did not induce a similar response. Indeed, various stimuli that cause TORC1 inhibition and induce general autophagy, showed little to no proteasome autophagy. Thus, proteasome autophagy is regulated distinctly from general autophagy. Consistent with this, we identified several genes required for proteasome autophagy, namely, those encoding the regulatory kinases Mpk1, Mkk1 and Mkk2 as well as the selective autophagy receptor Atg11.

RESULTS

Rapamycin induces a bi-phasic proteasome response

Upon nitrogen starvation, proteasomes are targeted for vacuolar degradation via an autophagy dependent pathway. The extent of this degradation appears to vary (28, 31, 33). To elucidate signaling pathways involved, we sought to determine the role of TORC1 inhibition in this process as TORC1 inhibition occurs upon nitrogen starvation and induces autophagy. However, the TORC1 inhibitor rapamycin has been reported to increase proteasome levels and activity (38, 39). To study this contradiction, we monitored the response of proteasomes to rapamycin treatment over an extended time by native gel analyses of yeast lysates. We introduced the sequence of eGFP before the stop codon at the endogenous locus of the core particle (CP) subunit α1 to ensure that α1 transcription is regulated as in wild type and no untagged subunits are produced (40). Upon rapamycin treatment, these cells showed an upregulation of 26S proteasome levels as determined by α1-GFP signal detected on native gel (Fig. 1A, top panel). Coinciding with the increased levels of GFP, we observed more hydrolysis of the suc-LLVY-AMC fluorogenic substrate (Fig. 1A, lower panel). This increase was transient as levels peaked at 30 to 60 minutes. The reduction after the peak was accompanied by an increase in a faster migrating GFP species on the fluorescent scan of the native gel. The accumulation of this species showed kinetics similar to the accumulation of a 25 kDa GFP positive band observed on immunoblots (Fig. 1B) and migrated where we anticipate free GFP to migrate on native gel (41). This “free GFP” is generally indicative of vacuolar targeting (28, 42). We observed the same dynamics when the regulatory particle (RP) subunit Rpn1 was tagged with GFP (Supplementary Fig. 1). Growth for 24 hours in YPD did not yield such an increase in this GFP species and resulted in little to no free GFP on immunoblots (Supplementary Fig. 2). In sum, rapamycin treatment of Saccharomyces cerevisiae resulted in an initial upregulation of proteasomes followed by vacuolar degradation.
Thus, treatment with tunicamycin caused proteasome upregulation (38, 46). Cells treated with tunicamycin showed a bi- phasic response similar to rapamycin, however proteasome levels peaked at 4 to 6 hours instead of 1 hour post treatment (Fig. 2A). The increase in free GFP reflects the induction of proteasome autophagy. However, less proteasome autophagy occurred compared to rapamycin treatment as native gels and immunoblots showed a higher ratio of Rpn1- GFP to free GFP and more proteasome peptidase activity was detected 24 hours post tunicamycin addition (Fig. 2B). The accumulation of free GFP was dependent on Atg7, confirming that tunicamycin induced autophagy of proteasomes (Fig. 2B). Since tunicamycin induces both ER autophagy and general autophagy (47), we wondered if the autophagy of proteasomes we observed resulted from ER-associated proteasomes that traffic to the vacuole via ER-phagy. To test this, we deleted ATG39 and ATG40, two genes required for ER/nucleophagy in yeast (23). Neither gene product was required for proteasome autophagy under conditions of nitrogen starvation (29, 33) and we observed no reduction in the amount of free GFP generated upon tunicamycin treatment when ATG39, ATG40 or both genes were deleted (Fig. 2C). Thus, proteasome autophagy observed upon tunicamycin treatment is distinct from ER-phagy.

We next tested another inhibitor of TORC1, caffeine (48, 49). We did not detect an upregulation of proteasome levels or activity when caffeine was added to growing cells, (Supplementary Fig. 3). This difference between caffeine and rapamycin could result from differential inhibition of TORC1 downstream pathways with these drugs, or

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Nitrogen starvation and rapamycin treatment both lead to the inactivation of TORC1 with various downstream effects, such as nuclear translocation of the nitrogen responsive transcription factor Gln3 (34, 45).

Thus, rapamycin and nitrogen starvation elicit, at least in part, a similar response through TORC1 inhibition. However, we observed proteasome upregulation only with rapamycin and not upon nitrogen starvation (Fig. 1) (41). This suggests that proteasomes respond differently to these two conditions that induce general autophagy. Therefore, we followed the proteasome response to other conditions and drugs that induce general autophagy.

Tunicamycin is a drug that induces ER stress, the unfolded protein response (UPR), general autophagy, and has been shown to cause proteasome upregulation (38, 46). Cells treated with tunicamycin showed a bi-phasic response similar to rapamycin, however proteasome levels peaked at 4 to 6 hours instead of 1 hour post treatment (Fig. 2A). The increase in free GFP reflects the induction of proteasome autophagy. However, less proteasome autophagy occurred compared to rapamycin treatment as native gels and immunoblots showed a higher ratio of Rpn1-GFP to free GFP and more proteasome peptidase activity was detected 24 hours post tunicamycin addition (Fig. 2B). The accumulation of free GFP was dependent on Atg7, confirming that tunicamycin induced autophagy of proteasomes (Fig. 2B). Since tunicamycin induces both ER autophagy and general autophagy (47), we wondered if the autophagy of proteasomes we observed resulted from ER-associated proteasomes that traffic to the vacuole via ER-phagy. To test this, we deleted ATG39 and ATG40, two genes required for ER/nucleophagy in yeast (23). Neither gene product was required for proteasome autophagy under conditions of nitrogen starvation (29, 33) and we observed no reduction in the amount of free GFP generated upon tunicamycin treatment when ATG39, ATG40 or both genes were deleted (Fig. 2C). Thus, proteasome autophagy observed upon tunicamycin treatment is distinct from ER-phagy.

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TORC1-independent effects of rapamycin. However, rapamycin is potent in yeast and both drugs show a similar response in transcriptional profile (50, 51). Consistent with this, proteasome autophagy was also detected following caffeine treatment. However, free GFP accumulated slower and to a lesser extent compared with rapamycin treatment or nitrogen starvation, perhaps because caffeine is less potent as an inhibitor of TORC1 (48, 49).

Treating S. cerevisiae with the different general autophagy inducing drugs, as shown above, led to proteasome autophagy, although each drug elicited different responses with regard to proteasome levels and activity over time. Starving cells for different nutrients such as nitrogen, phosphate, amino acids, or carbon, also induces general autophagy (52–55). To compare general autophagy with proteasome autophagy under these conditions, we utilized GFP-Atg8, which has been used to monitor bulk autophagy (56, 57). While we detected GFP cleaved from Atg8 under starvation conditions, we also observed cleavage when cells were grown in rich media for the same amount of time, a condition where little to no bulk autophagy has been reported (Fig. 3A). Considering Atg8 is not only involved in bulk autophagy, but also a number of selective autophagy pathways such as the cytoplasm-to-vacuole targeting pathway (56), general autophagy, but proteasomes were not always robustly targeted for degradation. This utilized as a second reporter overexpressed GFP (GFP^OE) not linked to any other protein. The conditions utilized above induced general autophagy, but proteasomes were not this read-out might not be ideal. Therefore, we utilized as a second reporter overexpressed GFP (GFP^OE) not linked to any other protein. The conditions utilized above induced general autophagy, but proteasomes were not always robustly targeted for degradation. This suggests proteasomes are not subject to selective process. On the other hand, considering the majority of proteasomes are nuclear, our results could also reflect a lag time grown in rich media, this reporter did not produce any significant amount of free GFP due to the need for nuclear export (Fig. 3A). When we starved cells for amino acids, we observed increased free GFP from general autophagy from selective autophagy is similar increase as seen with nitrogen starvation (Fig. 3A and B). This indicates substrates into autophagosomes. For comparable autophagic flux for Atg8 under example, selective autophagy of peroxisomes these conditions, consistent with previous work utilizes the receptor Atg30, ER-phagy utilizes (53). Free GFP formation in our GFP^OE strain Atg39 and/or Atg40, and mitophagy Atg32 or was also observed upon amino acid or nitrogen starvation. Proteasome autophagy, on the other hand, was not induced to a similar extent following amino acid starvation compared to nitrogen starvation, as indicated by the reduction in generated free GFP (Fig. 3A). Phosphate starvation induces general autophagy, albeit to a lesser extent than nitrogen starvation (54). Consistent with this, we found that there was only a modest level of autophagy for GFP^OE (Fig. 3A) even though GFP-Atg8 was targeted to vacuoles robustly. This suggests there is little general autophagy induced by phosphate starvation in our strain and the GFP-Atg8 processing we observed could be derived mainly from selective autophagy. This is supported by the reported requirement for ATG11 in phosphate starvation induced autophagy (54) as Atg11 is a protein normally involved in selective autophagy. Proteasomes also appeared to undergo phosphate starvation induced (indicative of vacuolar targeting) under phosphate starvation, as we did not detect a reduction in the amount of free GFP for the RP upon ATG11 deletion (Fig. 3C).
Atg11 (58, 59). General autophagy, on the other hand, does not depend on Atg11 (60). We have previously reported that nitrogen starvation induced autophagy of an Rpn1-GFP fusion strain was not entirely abolished by a deletion of ATG17 (28). Interestingly, the residual autophagy of proteasomes in the atg17Δ strain was abolished following deletion of ATG11 (Fig. 4A). For rapamycin induced proteasome autophagy, we were unable to detect free GFP in the atg17Δ strain and no difference in free GFP was detected in the atg11Δ strain. This suggests that Atg11 is not essential for any form of proteasome autophagy induced by rapamycin. However, less proteasome autophagy is induced with this drug in general, and the levels might be below our detection limit. Other factors required for proteasome autophagy, such as p62 in humans, and Cue5 and Atg24 in yeast, (30, 31, 33), further support the notion that some forms of proteaphagy proceed through a selective pathway.

To identify other factors involved in proteasome autophagy, we focused on a key MAP kinase signaling pathway in yeast which utilizes the kinase Mpk1. Mpk1 is not involved in general autophagy (61) but regulates proteasome abundance upon rapamycin addition (38). Deletion of MPK1 in our Rpn1 GFP-tagged proteasome strain resulted in ~35% reduction in the amount of cleaved GFP (going from 62% in wild type to 40% in mppk1Δ, p=0.067). When α1 was tagged with GFP, we observed a reduction of ~33% (going from 40% in the wild type to 27% in the knock out; p=0.0005) (Fig. 4B). This indicates a role for Mpk1 in efficient proteaphagy upon nitrogen starvation. To trace the kinase pathway involved in this process, we next examined the two kinases directly upstream of Mpk1 in the cell wall integrity pathway, Mkk1 and Mkk2. Mkk1 and Mkk2 are redundant in function and strains deleted of either MKK1 or MKK2 displayed normal proteasome autophagy (Fig. 4C). However, a significant reduction in proteasome autophagy was observed in the M KK1 and M KK2 double deletion mutant upon nitrogen starvation (Fig. 4C, D). For Rpn1, we observed ~39% reduction in cleaved GFP upon deletion of M KK1 and M KK2 (going from 44% in the wild type to 27% in the double knock out; p=0.030). For α1 GFP-tagged strains, the observed reduction in free GFP formed was ~ 40% (going from 25% in the wild type to 15% in mkk1Δ mkk2Δ strain; p=0.119). These data show that Mkk1 and Mkk2 play redundant roles and, like Mpk1, are important for efficient proteaphagy. Surprisingly, deletion of Bck1, which acts upstream of Mkk1 and Mkk2, did not result in a detectable decrease in proteasome autophagy following nitrogen starvation (Supplementary Fig. 4). This might indicate the signaling does not go through the standard cell wall integrity pathway. Indeed Bck1 has been shown to be dispensible for Mpk1 activation in a number of conditions (62, 63) Nevertheless, the identification of roles for signal transduction (MPK1, M KK1/2) and selective autophagy (Atg11) in proteasome autophagy reinforces the model that proteasomes are specifically targeted for vacuolar degradation.

**DISCUSSION**

As an important regulator of many cellular processes, including the cell cycle, the UPS fulfills an essential function. Similarly, the ability to degrade proteins via autophagy is crucial during mammalian development (64). However, individual cells, like MEFs, grow and multiply without the need for autophagy (65). Similarly, yeast strains lacking the ability to perform autophagy grow at wild type rates under optimal conditions. Autophagy becomes essential, however, upon exposure to certain stress conditions like nitrogen starvation (66). While this indicates important and distinct functions for the UPS and autophagy, it has become clear in recent years that proteasomal and autophagic protein degradation can be synergistic. Here, one system can, to a certain extent, compensate for the impairment of the other (15, 18). Interestingly, in the context of this synergy, recent observations show proteasomes themselves are substrates for autophagic degradation (28–31, 33). While
such degradation makes sense under conditions where proteasomes are damaged or not functional (e.g. as a result of inhibition). Proteasome upregulation by rapamycin likely reflects a similar cellular response that benefits cell survival under specific conditions. In line with this, the lack of proteasome autophagy upon nitrogen deprivation has recently been shown as well as several other genes, is required for cell survival after inducing ER stress with tunicamycin (70). Proteasome degradation to replenish amino acid pools might be important early in nitrogen starvation. Indeed, we observed delayed proteaphagy compared to general autophagy in yeast (starting ~6 hrs versus ~2 hours) (41, 72).

Approximately 70% of proteasomes in yeast are nuclear and direct autophagy of nuclear material is not responsible for vacuolar targeting of proteasomes. Instead, nuclear export is required for their efficient degradation and turnover factors by the proteasome, like Pop2, a deadenylase subunit, and Dcp2, a capping enzyme, has been reported upon both nitrogen starvation and rapamycin treatment (73). In addition, degradation of translation and RNA export is required for their efficient degradation. Here, we show that MAPK signalling is important for the reactivation of TORC1 signaling upon early in autophagy induction may facilitate extended arginine starvation of HEK-293 cells to cellular reprogramming and stress responses. In support of this idea, TORC1 inhibition upon nitrogen starvation and rapamycin treatment (73). These data support a role for proteasome degradation after nutrient deprivation has recently been shown in yeast (31, 33).

A master regulator of metabolic signal transduction is TORC1. This kinase is inhibited under nitrogen starvation, a condition that can be mimicked with the drug rapamycin. Therefore, the observation that proteasomes are upregulated upon TORC1 inhibition was surprising (38).

Our extended analyses showed that their synergy, and facilitate the appropriate proteasomes are indeed initially upregulated. However, following upregulation proteasomes are degraded through autophagy. A similar biphasic response was also observed with proteasome autophagy comes from the identification of specific factors that are required for proteasome autophagy but are dispensable for bulk autophagy. Examples are as proteasomes are important for clearance of misfolded proteins (68, 69). Consistent with this, the transcriptional regulator Rpn4, responsible for upregulation of proteasome...
proteasome autophagy upon nitrogen starvation. We have previously reported that a subset of the regulatory particle was still degraded in an Atg17 mutant (28). The autophagic degradation of the remaining proteasomes depended on Atg11. Atg11 is known to be required for the specific recognition of autophagic cargo in processes like mitophagy, pexophagy, and the CVT pathway (58, 59, 74, 75). Whether or not these proteasomes utilize a selective autophagy receptor that binds Atg11 remains to be determined.

In addition to Atg11, we also observed an important role for the MAP kinase Mpk1 in proteasome autophagy. Importantly, Mpk1 is not involved in general autophagy (61). Currently, it is unclear if Mpk1 facilitates proteasome autophagy by directly phosphorylating proteasomes, regulatory factors, or is part of a longer signaling cascade that ultimately leads to proteasome autophagy. Intriguingly, this kinase is also required for proteasome upregulation following rapamycin addition (38). The role of Mpk1 in rapamycin induced proteasome autophagy could not be determined as mpk1Δ cells died approximately 4 hours following rapamycin addition, which is before robust proteasome autophagy is detectable. Mpk1 upregulates proteasomes by inducing expression of regulatory particle assembly chaperones including Adc17. Since, we did not detect a role for Adc17 in proteasome autophagy (supplementary Fig. 5), different factors are likely involved in Mpk1’s roles in the process, a subject we are currently exploring.

Experimental procedures

Yeast strains – All strains and oligos used in this study are reported in supplementary table 1. Yeast strains are the W303 derived SUB61 (Matα, lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1) and SUB62 (MatA, lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1) that arose from a dissection of DF5 (78). Standard PCR based procedures were used to delete specific genes from the genome or introduce mammalian cells, the nuclear ribophagy receptor NUFIP1 still translocated to the cytosol in ATG7 deleted cells following starvation (27). Thus, proteasomes remaining nuclear suggests that the signals that govern proteasome nuclear export are not present when autophagy is blocked. Proteasome nuclear export potentially does not depend on an import function. Conversely, autophagy flux. Consistent with this, autophagy is not observed until approximately six hours post induction (Fig. 1B). Alternatively, it is possible that an equilibrium exists for proteasomes between the cytoplasm and the nucleus. Since only cytoplasmic proteasomes appear to be autophagy substrates, proteasome autophagy results in a drop in the cytosolic proteasome levels. This would induce nuclear export to maintain the nuclear-cytosolic equilibrium of proteasomes. As such, autophagy of nuclear proteasomes would be propagated passively. However, given the dependence of proteasome autophagy on map kinase signaling, this alone does not explain why proteasomes remain nuclear since general autophagy continues. One possibility is that the nuclear cytoplasmic equilibrium of proteasomes is maintained through cellular signaling. This suggest that cells might regulate proteasome autophagy, at least for the nuclear population, by controlling nuclear export. This mechanism is different from protection against autophagy via the formation of proteasome storage granules (77). In all, our data support a model where proteasomes are selective cargo for autophagy regulated through MAP kinase signaling independently from general autophagy.
of GFP or mCherry (79–81). GFP-atg8 expressing strains were generated by mixing equal amounts of lysates for each transformant with BS-Ura3-GFP-Atg8, a gift from Zhiping Xie (Addgene plasmid #69194), and harvested by centrifugation. Raspamycin at 10 \(\mu\)M and tunicamycin at 6 \(\mu\)M were added, and 600 \(\mu\)L ddH2O. This suspension was frozen dropwise in liquid nitrogen and stored at -80 °C. Lysis was completed using previously prepared lysate and electrophoresis, samples were transferred to PVDF membranes and immuno-blotted with antibodies against indicated proteins or tags followed by the appropriate horseradish-peroxidase conjugated secondary antibodies. This produced a linear DNA fragment for targeted integration in the URA3-TIM9 region of the yeast genome. To identify successful integration of the expression modules in cells, transformants were grown on plates lacking histidine. Integration was confirmed by PCR.

### Yeast Growth Conditions – Overnight cultures

Yeast Growth Conditions – Overnight cultures of yeast were diluted to an OD\textsubscript{600} of 0.5 and grown in YPD medium to an OD\textsubscript{600} of 1.5 (approximately 4 hours). Cultures were then treated with drugs for indicated time periods and harvested by centrifugation. Rapamycin was used at 200 nM final concentration.

To induce starvation, cultures growing logaritihmically in YPD (2% dextrose) were centrifuged, washed with the respective starvation medium, re-inoculated at an OD\textsubscript{600} of 1.5, and incubated at 30 °C with constant shaking.

### Protein lysates and electrophoresis – For native gel analyses, 50 OD’s of cells were pelleted, washed in ddH20, and resuspended in 50 \(\mu\)L ddH2O. This suspension was frozen dropwise in liquid nitrogen and stored at -80 °C until further processing. Protein lysates were obtained by cryogrinding cell pellets using CryoCooler, mortar, and pestle from OPS (84)(https://www.youtube.com/watch?v=ZrZVBFg9NE8). All imaging by fluorescence microscopy was done within 10 minutes after cryogrinding, lysates were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM ATP, 5 mM MgCl\textsubscript{2}, 1 mM EDTA) for native gel analyses, equal volumes were assigned based on phase contrast images. In addition, we regularly stained vacuolar membranes with the dye FM4-64 and CMAC-Arg (Invitrogen; microscope settings: excitation filter 350/50 nm, emission filter of 457/50 nm). To confirm nuclear localization, proteasome complexes. Samples for SDS-PAGE and western analyses were prepared by mixing equal amounts of lysates for each sample with 1/5 volume of 6X SDS sample buffer (10% SDS, 40% glycerol, 60M DTT, from Invitrogen; cat. nr. #11814460001) and anti-Pgk1 (1:5000; Invitrogen, cat. nr. #459250). Horseradish-peroxidase activity was visualized using the Immobilon Forte Western HRP substrate (Millipore) and images were acquired using the G-box imaging system (Syngene) with Genesnap software. Data shown are representative of consistently observed trends from at least three independent biological replicates.

### Fluorescence Microscopy – All microscopy was done with live yeast strains where proteasome subunits where fluorescently tagged (Rpn1-GFP, \(\alpha\)-1-GFP) at their endogenous locus with expression driven by the endogenous promoter. After indicated treatments, approximately 2 ODs of cells were pelleted, washed with PBS, then resuspended in 30 \(\mu\)L of PBS, and 3 \(\mu\)L mounted on 1% soft agar slides as described by E. Muller (82). After cryogrinding, lysates were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM ATP, 5 mM MgCl\textsubscript{2}, 1 mM EDTA). For native gel analyses, equal volumes were assigned based on phase contrast images. In addition, we regularly stained vacuolar membranes with the dye FM4-64 and CMAC-Arg (Invitrogen; microscope settings: excitation filter 350/50 nm, emission filter of 457/50 nm). To confirm nuclear localization, proteasome complexes. Samples for SDS-PAGE and western analyses were prepared by mixing equal amounts of lysates for each sample with 1/5 volume of 6X SDS sample buffer (10% SDS, 40% glycerol, 60M DTT, from Invitrogen; cat. nr. #11814460001) and anti-Pgk1 (1:5000; Invitrogen, cat. nr. #459250). Horseradish-peroxidase activity was visualized using the Immobilon Forte Western HRP substrate (Millipore) and images were acquired using the G-box imaging system (Syngene) with Genesnap software. Data shown are representative of consistently observed trends from at least three independent biological replicates.

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nm, emission filter of 457/50 nm). Images were acquired at room temperature using a Nikon Eclipse TE2000-S microscope at 600X magnification with a Plan Apo 60x/1.40 objective. Equipped with a Retiga R3™ camera.

**Data Availability Statements**

All data are contained within the manuscript or the Supplementary Information.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. Rapamycin induces a bi-phasic proteasome response. (A) Yeast expressing α1-GFP were grown in YPD medium and treated with 200 nM rapamycin. Equal amounts of lysates were separated on native gels and imaged for GFP (top panel) or peptidase activity in the presence of 0.02 % SDS using the proteasome substrate suc-LLVY-AMC (lower panel). Graphs show the quantifications of the levels of GFP and LLVY peptidase activity corresponding to the RP2-CP species on native gel, both normalized using PGK1 signal (see (B)). Error bars represent SEM. (B) Samples from Fig. 1A were denatured and separated on SDS-PAGE and immunoblotted for GFP. Upper band shows α1-GFP and lower band “free” GFP resulting from α1-GFP cleavage by vacuolar hydrolysis. Pgk1 was used as a loading
control. (C) Wild type and atg7Δ yeast expressing α1-GFP or Rpn1-GFP, were inoculated in YPD medium at OD600 of 0.5 and grown to log phase (~ 4 hrs). Cells were treated with rapamycin as in (A) and lysed at indicated time points. α1-GFP, Rpn1-GFP, and cleaved free GFP (indicative of vacuolar targeting) were monitored by immunoblotting for GFP. Pgd1 was used as a loading control. Graph shows quantification of immunoblots. (D) Rpn1-GFP expressing yeast were stained for the vacuole membrane using FM464 and the nucleus using Hoechst 33342. Microscopy was performed at log phase and following 24hrs growth in rich media (YPD) (top). Rpn1-GFP expressing yeast growing logarithmically were incubated with the vacuole lumen marker CMAC-arg (bottom). Scale bars represent 0.5µm (E) Microscopic analysis of yeast collected at log phase and 24 hours after rapamycin treatment. In top image, arrow heads point to nuclei and arrows to vacuoles. In rapamycin treated cells, filled arrow heads indicate cells with vacuolar fluorescent signal, while open arrow heads show subset of non-responding cells. Scale bars represent 0.5µm. Values indicate the percentage of cells in WT and atg7Δ with vacuolar GFP signal following rapamycin treatment. Vacuolar GFP signal was rarely (<1 %) observed in non-treated cells. N>100 from 3 biological replicates.
Figure 2. Tunicamycin induces a bi-phasic proteasome response (A) Yeast expressing α1-GFP were treated with tunicamycin (6 µM) for indicated time periods. Cells were collected, lysed under native conditions and equal amounts of lysates were loaded on native gel. Following separation, gels were imaged for GFP fluorescence (top) or suc-LLVY-AMC peptidase activity in the presence of 0.02% SDS (bottom). Quantifications show the amount of GFP signal and LLVY peptidase activity associated with the RP$_2$-CP proteasome complexes. GFP and LLVY activity were normalized to Pgk1 intensity using SDS-PAGE immunoblots of the same samples. (B) Wild type and atg7Δ yeast were treated with tunicamycin and lysates analyzed as in A (left) and denatured for western blotting (right). (C) WT, atg39Δ, atg40Δ, and atg39Δ atg40Δ yeast expressing Rpn1-GFP were treated with tunicamycin as in A. 2 ODs of cells were lysed using the alkaline lysis method. Samples were separated on SDS-PAGE and immunoblotting for GFP and Pgk1 was carried out as described above.
Figure 3. Proteasome autophagy is distinct from general autophagy. (A) Yeast strains expressing GFP-atg8, GFP, or α1-GFP, were grown in YPD and subsequently starved 24 hours for nitrogen (-N), amino acids (-AA), or phosphate (-PO₄). The equivalent of 50 ODs of cells was lysed by cryogrinding. Equal volumes of lysate were blotted for GFP and Pgk1. Value below immunoblots indicate the free GFP signal as percentage of free + fused GFP normalized to PGK1. (B) Localization of fluorescent proteins in starved cells from A was monitored by microscopy. Scale bar represents 5µm. (C) WT and atg11Δ yeast were grown to log phase in rich medium and switched to SD medium lacking phosphate. 2 ODs of cells were harvested at indicated timepoints and analyzed as above.
Figure 4. Proteasome autophagy requires factors dispensible for general autophagy. (A) WT, atg11Δ, atg17Δ, atg11Δ atg17Δ yeast expressing Rpn1-GFP were starved for nitrogen or treated with rapamycin for 24hrs. (B) WT and mpk1Δ cells expressing Rpn1-GFP or α1-GFP were starved for nitrogen and 2 ODs were harvested at indicated time points. Immunoblotting for GFP and Pgk1 were performed as described above. We observed an ~35% reduction in cleaved GFP from Rpn1 and ~33% from α1 (p value 0.067 and 0.0005 respectively) in the mutants compared to WT 24 hours following starvation. (C) WT, mkk1Δ, mkk2Δ and mkk1Δ mkk2Δ cells expressing Rpn1-GFP were starved for nitrogen and 2 ODs harvested at 24 hours. Immunoblotting for GFP and Pgk1 were performed as described above. (D) WT and mkk1Δ mkk2Δ cells expressing Rpn1-GFP or α1-GFP were starved for nitrogen and 2 ODs harvested at indicated time points. We observed an ~39% reduction in cleaved GFP from Rpn1 and an ~40% reduction from α1 (p=0.030 and 0.119 respectively)