

Molecular regulation of proteasome abundance and localization

by

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B.S., Kansas State University, 2012

AN ABSTRACT OF A DISSERTATION

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Abstract

Proteasomes are molecular complexes that degrade more than 80% of proteins in eukaryotes. This makes these proteases essential for processes associated with cell survival and proliferation. Proteasomes are localized to both the cytosol and nucleus, with the majority in the nucleus of dividing cells. In recent years, much progress has been made in understanding how the 33 unique subunits of proteasomes assemble into active protease complexes. The identification of ten dedicated chaperones that facilitate assembly suggest that this is an intricate process. Thus, quality control dependent degradation for complexes that fail to assemble correctly likely exist. However, there is little known about when and how proteasomes are degraded in cells. Considering the large size and complex assembly of proteasomes, we hypothesized that their degradation would be carried out via autophagy. Autophagy involves the de-novo synthesis of double membrane vesicles capable of engulfing large cytosolic cargo. In this work, we show that proteasomes are indeed degraded through autophagy. This degradation requires the canonical autophagy genes required for general autophagy. Importantly, factors required for selective autophagy play a role in the degradation of a sub-set of proteasomes, and proteasome autophagy is dependent on several factors not required for general autophagy. Further, we show that several general autophagy-inducing stimuli do not result in proteasome autophagy, but instead result in proteasomes remaining nuclear or being targeted to cytosolic puncta termed proteasome storage granules (PSGs). Thus, our data support the model that proteasomes, depending on the physiological conditions, are selectively regulated. To understand the signaling events involved, we sought to determine how cells make the choice between PSG formation and proteasome autophagy. We hypothesized that carbon starvation, a PSG-inducing condition, unlike autophagy-inducing stimuli, affected proteasomes through a strong reduction in cellular energy. Proteasomes need to hydrolyze ATP for normal biological function, and in vitro proteasome complexes are unstable in the absence of ATP. When we tested conditions that inhibit mitochondrial function, and thus cellular energy levels, we observed PSG formation. Consistent with our model, we observed a reduction in ATP levels in several PSG-inducing conditions,

though not for all. If destabilization due to low ATP levels is a key step in the re-localization of proteasomes, we predicted factors that stabilize proteasome complexes, such as proteasome inhibitors or association with *Ecm29*, would prevent PSGs. Indeed, we found that both proteasome inhibitors and *Ecm29* reduced the amount of PSGs formed, and restricted proteasomes to the nucleus. This suggests that proteasome disassembly into subcomplexes plays a role in PSG formation. Further, we identified several proteins that play a role in proteasome re-localization. In all, our data provide new insight into the proteasome's cellular dynamics under a variety of physiological conditions. Our current model is that the re-localization of proteasomes facilitates cell survival under stress. Here, proteasome autophagy-inducing conditions benefit from the conversion of excess proteasomes to amino acids required for survival. On the other hand, proteasome granules restrict ATP use and proteasome activity under PSG-inducing conditions.

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Dedication

In memory of the past I dedicate this work to my mother, Deloris Adina Clarke, and my brother, Javayne Romario Rhoden. I think of you every day and am driven by your memory.

With hope for the future I dedicate this work to my daughter, Penelope Adina Waite. I named you after my mother not knowing you would inherit her spirit. I am incredibly proud of you and hope that you will work hard for your dreams.

Chapter 1 - Introduction

Proteasomes

Throughout evolution, the ability to efficiently degrade proteins has been conserved and is essential for life in eukaryotes. The major protease involved in this process has been observed in different but related forms in organisms varying from bacteria to archaea and eukaryotes (1–4). In eukaryotes, these proteases are called proteasomes and they are responsible for the degradation of more than 80% of intracellular proteins (5, 6). The substrates of proteasomes include signaling molecules, unfolded proteins, and proteins that might otherwise aggregate. Thirty-three different subunits are assembled into two distinct subcomplexes, the regulatory particle (RP) and the core particle (CP). RP binds one or both faces of CP to form active 26S proteasomes (7–9). The RP serves as a CP activator that recognizes, sequesters, and unfolds target proteins. Protein unfolding at the RP is energy dependent and requires ATP. Recent cryo-EM structures have shown how this molecular machine works to unfold and translocate substrates into the narrow CP channel for degradation (1, 10). Seven conformational states of the proteasome were identified as being required for substrate processing through three modes of action. Sequential ATP hydrolysis in the proteasome RP was shown to regulate these conformational changes and facilitate the recognition/de-ubiquitination of substrates, followed by translocation initiation and ubiquitin release, and finally CP gate opening and subsequent protein degradation. These data show the absolute requirement for ATP in RP function. The CP is composed of four hetero-heptameric rings that form a cylindrical structure with a central channel (11–13). Alpha subunits 1-7 form the outer rings of the CP while beta subunits 1-7 form the inner rings (α 1-7 β 1-7 β 1-7 α 1-7). Following translocation, protein targets are exposed to the caspase- (β 1), trypsin- (β 2), and chymotrypsin- (β 5) like proteolytic activities of the CP (14). Access to the proteolytic active sites is restricted by the alpha subunits forming a functional gate with their N-terminal tails. The 19S RP is the primary activator of the CP; however, others exist and bind to the CP under different conditions. For instance, PA200 (Blm10 in yeast), and the 11S family (PA28, PA26 and REG) can bind and activate the CP in an ATP-independent manner (15). The biological function of

these activators is less understood than the function of the ATP-dependent RP. However, different proteasome arrangements are apparent in different contexts, thus providing clues as to their biological role. For example, upon oxidative stress or when cells experience an immunological insult, proteasomes dissociate, and free CPs can interact with PA28. It was shown that this activator was required for the efficient removal of oxidized proteins in this condition. Since this degradation is independent of ubiquitination and ATP, it is proposed to allow proteasomal degradation of a variety of oxidized/un-folded proteins. The CP itself is also modified under these conditions. Oxidation and interferon- γ induce the translation of three alternative CP active site subunits. The difference in specificity allows for the generation of immune peptides for antigen presentation (16–19). Other proteasome interacting proteins (PIP) that facilitate ubiquitin and ATP independent protein degradation, such as Blm10 in yeast, likely facilitate degradation of similarly unfolded material. Thus, proteasome function is tightly regulated and includes assembly and disassembly of complexes to achieve correct biological outcomes.

Proteasome assembly and the ubiquitin proteasome system

The assembly of the 26S active complex requires the concerted action of at least ten molecular chaperones. As mentioned previously, thirty-three different polypeptides are involved in the formation of CPs and RPs. The chaperones that govern proteasome assembly are conserved from yeast to mammals and appear to function similarly. The yeast assembly pathway has been described in more detail and is discussed here. CP assembly requires five chaperones. Proteasome biogenesis associated (Pba)1, 2, 3 and 4 facilitate the assembly of the CP alpha rings. Pba1 and 2 bind the outer surface of the rings while 3 and 4 bind the inner surface. The role of Pba3 and 4 appears to be prevention of improper alpha ring complexes, whereas Pba1 and 2 prevent premature interaction of the RP with the CP (8, 12, 20). Following alpha ring assembly, a fifth CP chaperone Ump1 and beta subunits associate with this complex forming half CPs. Ump1 is thought to prevent interaction of half CPs before proper beta ring assembly. Upon completion of assembly, Ump1 is degraded as this chaperone remains inside the CP (21). Assembly of the proteasome RP is achieved by building base and lid sub-complexes. Base is assembled with the aid of four chaperones that facilitate the

incorporation of regulatory particle ATPases 1-6 (RPTs) into a ring structure. These chaperones, Nas2, Nas6, Hsm3, and Rpn14, ensure the proper assembly of this ring, as well as interaction with the remaining proteasome-base subunits regulatory particle non-ATPase (RPN) 1, 2, and 13 (8, 22, 23). Assembly of the proteasome lid appears to be independent of molecular chaperones. However, lid assembly was shown to be hierarchical where subunits incorporate in an ordered fashion. Here, the last subunit that incorporates into the lid is Rpn12, which is required for interaction of lid and base subcomplexes (24). Mutations in proteasome chaperones or subunits that affect assembly or function result in the sensitization of cells to protein stress (25, 26). When there are large amounts of damaged or misfolded proteins, proteasome activity is necessary for their removal (5, 13). The requirement for these chaperones show that proteasome assembly is tightly regulated, and this is required for cell survival. This is supported by the fact that the expression of proteasome subunits, and associated proteins such as their chaperones, is regulated through a feedback loop requiring proteasomal degradation. When the transcription factor Rpn4 is stabilized, it signals the production of more proteasomes. Rpn4 is also a proteasome substrate, thus higher proteasome levels result in its degradation and stops proteasome production (27).

Not only is proteasome assembly regulated, proteasome quality also appears to be monitored following assembly. This is achieved through the conserved chaperone Ecm29 (KIAA0368 in mammals) that exclusively binds assembled proteasomes (28–30). Ecm29 is a large (210 kDa) protein and depending on the proteasome configuration (singly capped CP vs doubly capped), either one or two Ecm29 molecules can bind proteasomes (31, 32). When Ecm29 is bound to proteasomes, it inhibits their proteolytic activity by restricting the ability of the proteasomal ATPases to hydrolyze ATP, and thus to translocate substrates (29). Mutations affecting proteasome assembly or function result in increased Ecm29 on proteasome complexes. Indeed, Ecm29 has been shown to correct 26S assembly when added to lysates containing improperly assembled CPs. This suggested a scaffolding function for Ecm29 where this protein binds misassembled proteasomes to allow their repair (31). Here it appears that Ecm29 facilitates proteasome dissociation in the process of repair. Other studies have reported a similar requirement for Ecm29 in proteasome dissociation, for example upon oxidative stress

(17, 33), This however is somewhat controversial as Ecm29 has been shown by us and others to stabilize RP-CP interactions (29, 32). Further, over-expression of Ecm29 did not result in RP-CP dissociation, which would be expected if that was its function (31). The differences observed may result from changes in how Ecm29 engages with different types of proteasomes. For instance, a complex in an oxidized condition may have characteristics that distinguish it from a mutant complex, thus allowing Ecm29 to engage them differently. Though a structure of Ecm29-bound proteasomes remains to be determined, experimental evidence suggest that it binds near the RP subunit Rpt5 and the CP subunit $\alpha 7$ (29, 34). Here, it appears that phosphorylation of the C-terminal tail of $\alpha 7$ was required for Ecm29 binding, suggesting that alterations in phosphorylation play a role in regulating Ecm29-proteasome associations. This could also serve to regulate Ecm29 association with different types of proteasomes (ex. oxidized vs. mutated). The function of Ecm29 in different biological context remains to be fully elucidated. Nevertheless, cells regulate proteasome complexes through Ecm29 following assembly.

Regulation of proteasome function also occurs at the level of substrate interaction. Selectivity in proteasomal degradation is controlled through a cascade of enzymes that ultimately conjugate ubiquitin to a target protein. First, an E1 activating enzyme activates ubiquitin using ATP energy. Activated ubiquitin is covalently attached to this enzyme and transferred to an E2 conjugating enzyme. This E2 interacts with an E3 ligating enzyme and either transfers the ubiquitin to this E3 or the E3 facilitates interaction of the E2 with a substrate (35). Ultimately, the ubiquitin is attached to substrate lysine residues. Ubiquitin itself contains internal lysine residues that act as ubiquitin conjugation sites. Thus, besides the addition of a single ubiquitin to the lysine of a substrate, ubiquitin-ubiquitin conjugations allow for the formation of ubiquitin chains. These chains can vary in length as well as linkages. Among the large number of possible arrangements are chains with different linkages (ex. building K11 linkages on existing K63 chains) or branches (one ubiquitin having ubiquitins attached to two lysine residues) (36, 37). These different ubiquitin linkages are still poorly understood but have been shown to signal different events or serve as signaling scaffolds. For instance, mono-ubiquitination of histones has been shown to regulate gene expression, and

ubiquitination governs the localization of p53 and G-protein coupled receptors (38–41). Mono-ubiquitination, as well as Lys-63 linkages, is also known to play a role in autophagy regulation and protein degradation (42, 43). Proteasomal degradation of substrates is signaled when lysine 48 is used to build chains. However, other linkages have also been reported to lead to proteasomal degradation (36, 44). Chain-specific de-ubiquitinating enzymes (DUBs) serve to regulate ubiquitin signaling events by removing these tags from substrates (45). Ubiquitination and de-ubiquitination ensure the ability of the ubiquitin proteasome system (UPS) to degrade proteins in a temporally and spatially regulated manner to facilitate biological outcomes (46–48). UPS-dependent processes include cell division, immunological responses, and various stages of development among others (49–51). Consequently, disruptions in the UPS are associated with numerous human pathologies (52, 53). As proteasomes play a central role in this system, it is not surprising that mutations that affect this complex or delivery of its substrates have negative health outcomes.

Proteasomes in health

As the major cellular protease, the proteasome has a role in several pathologies such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease and Parkinson's disease. A hallmark of these neurodegenerative diseases is failure to efficiently degrade particular substrates resulting in their accumulation and toxicity (47, 54–62). The specific substrate that aggregates, and the cell type they aggregate in, results in different diseases. However, a commonality among most of these conditions is a loss of proper UPS function (60, 63, 64). Many of the aggregates that form are decorated with ubiquitin and/or ubiquitin like proteins. They also contain components of the UPS including different ligases and proteasome subunits (40, 63, 65, 66). Thus, it appears that cells, at least in part, attempt to clear these aggregates through the UPS. Indeed, it is known that when certain cell types encounter aggregated material, they upregulate immuno-proteasomes (67). Loss of proteasome activity, which occurs with aging, compromises the UPS response and aggregation becomes more likely (68, 69). Ongoing work is exploring the possibility of modifying the UPS for therapeutic purposes. For instance, activation of 26S proteasome activity through phosphorylation of the complex or inhibition of proteasome associated DUBs , and increasing targeting of

substrates to proteasomes, are being explored as therapeutic techniques (70–72). These strategies would lead to increased degradation of substrates before toxic levels can be reached. Indeed, increased degradation of prion proteins as well as proteins known to aggregate in Alzheimer's disease and ALS has been shown upon inhibition of proteasome associated DUBs (73, 74). Some diseases, on the other hand, are treated by reducing proteasome activity. The proteasome inhibitor bortezomib (ps341) was approved by the FDA in 2003 for the treatment of multiple myeloma (75). It was subsequently found to be an effective treatment for mantle cell lymphoma, leading researchers to develop new inhibitors or otherwise disrupt the UPS to treat different types of cancers (72, 76–78). The mechanism of action that makes these drugs cytotoxic to cancer cells is not fully elucidated. One model suggest that stabilization of the inhibitor of (I)- κ B, and subsequent cytoplasmic retention of nuclear factor (nf)- κ B, results in increased apoptosis of cancer cells (79, 80). However, others have shown that proteasome inhibitors can also increase nf- κ B activation in certain cell types (81). Another model proposes that these cancers have high rates of protein translation, making proteasome quality control essential for survival (72). It appears a full understanding of how these inhibitors work remains to be determined. Nevertheless, modulation of the UPS is a viable method of treating at least some types of cancer.

Autophagy

Along with the UPS, eukaryotes degrade intracellular proteins via three main pathways, collectively classified as autophagy. These distinct pathways facilitate the degradation of a variety of protein substrates by lysosomes (vacuoles in yeast). One pathway is micro-autophagy, which involves the direct engulfment of cytosolic or nuclear material by lysosomes (82, 83). In this form of autophagy, the vacuolar membrane directly interacts with a substrate, and invaginates to deliver cargo into the vacuolar lumen for degradation. Piecemeal autophagy of the nucleus (PMT) is one such pathway in yeast for the degradation of nuclear membranes and some constituents (84). This is in contrast to chaperone-mediated autophagy (CMA), where a specific amino acid sequence on substrates is exposed and recognized by molecular chaperones (85, 86). These substrates are then delivered to vacuoles where a specific channel facilitates their translocation into the vacuolar lumen (87, 88). The major type of autophagy that

has been described is macro-autophagy, which is responsible for most autophagic degradation. More than 30 autophagy related genes serve to regulate this process (89). Autophagy constitutively active at a basal level functions to maintain cellular health under normal conditions by selectively degrading proteins, organelles and aggregates (90, 91). These include mitochondria, nuclear membranes, peroxisomes and endoplasmic reticulum among others. In yeast, a biosynthetic autophagic pathway delivers the hydrolases needed in the vacuole to carry out protein degradation. This specialized pathway requires specific receptors and is termed the cytoplasm to vacuole targeting (CVT) pathway (92, 93). Thus, selective types of autophagy operate at basal levels to help maintain cellular homeostasis. Induction of autophagy above basal levels occurs upon TORC1 inactivation. The target of rapamycin complex 1 (TORC1) kinase acts as a sensor of metabolic conditions and regulates autophagic flux accordingly. When nutrients are abundant, TORC1 represses autophagy to a basal level by phosphorylating *Atg13* and *Atg1*, factors required for autophagy induction (94). Inactivation of TORC1 upon stress results in rapid dephosphorylation of *Atg13* and subsequent formation of the *Atg1* autophagy initiating kinase complex (94, 95). This upregulates macro-autophagy (hereafter referred to as autophagy) and occurs under numerous cellular conditions including starvation for various nutrients, as well as damage to organelles or viral and bacterial insults (96–101).

Proteins involved in autophagy serve to sequester membranes from other organelles, deliver these membranes to specific locations, and decorate them with the autophagy receptor *Atg8* (LC3 in mammals) (89, 102, 103). This occurs through a ubiquitin-like system that conjugates *Atg8* to the lipid phosphatidyl ethanolamine. Ultimately, double membrane autophagosomes are produced that engulf cytoplasmic substrates. These include selective autophagy substrates such as mitochondria and ribosomes, but other cellular constituents as well (104–107). In the case of starvation, general autophagy is upregulated and serves to recycle cellular material in bulk. Here, metabolic intermediates, such as amino acids and lipids, are generated from degraded substrates and used for cell survival (97). Like the UPS, autophagic degradation involves the ubiquitination of target substrates. Covalently attached ubiquitins bind autophagy receptors such as p62(SQSTM1) or *Atg11* at their ubiquitin interacting motifs

(108–110). Specific proteins in particular organelles interact with the cargo recognition domain of autophagy receptors during selective autophagy (105, 111). For example, *Atg32* and *Atg33* are mitochondrial membrane proteins that interact with *Atg11* to facilitate selective autophagy of mitochondria (112–114). Other than the cargo recognition domain, receptors like *Atg11* contain intrinsic *Atg8* (LC3) interacting motifs (AIM), and thus bridge the interaction between target substrates and *Atg8*-conjugated expanding autophagosomes. Because of its important role in cellular homeostasis, disruptions in autophagy result in a variety of pathologies caused by divergent molecular mechanisms. Among these are a failure to correctly degrade material in lysosomes, failure to deliver material to lysosomes, or failure in recognizing particular cargo (115–117). As a result, the observed pathologies include cardiomyopathies, lysosomal storage disorders, failures in development, cancer and neurodegeneration. These observations again point to the important role for protein degradation in maintaining cellular health.

Synergy between proteasomes and autophagy

It is not surprising that a loss of proteolytic capacity when one of the major protein degradation pathways is disrupted results in adverse health outcomes. Proper balance between protein synthesis and degradation is required for cells to maintain normal function. Evidence in recent years point to a synergistic relationship between proteasomal and autophagic degradation systems. Ubiquitination of substrates is required for both pathways, and thus some receptors that bind ubiquitin are able to deliver cargo to proteasomes or autophagic membranes, for example p62 (118–120). Indeed, certain substrates can be degraded through either pathway, and simultaneous activation of both pathways occurs upon TORC1 inhibition (121–125). Proteasomes have further been shown to help regulate autophagic flux by degrading the autophagy initiator Ulk1 (*Atg1*) to prevent uncontrolled induction and facilitate termination (126, 127). Deletion of the autophagy gene *ATG5* results in proteasome activation. Further, inhibiting proteasome activity or disrupting recruitment of polyubiquitinated proteins induces autophagy (128–132). The idea of synergy is exemplified at the endoplasmic reticulum (ER). Here, cells undergoing ER stress upregulate proteasomal degradation via the ER-associated degradation (ERAD) pathway to clear misfolded proteins.

However, if proteasomes are unable to clear these proteins and relieve stress on the ER, autophagy is activated (119, 133, 134). Autophagy is also activated when proteasomes are impaired in a *Drosophila* model of neurodegeneration (135). On the other hand, inhibiting autophagy has been shown to result in upregulation of proteasome activity (132, 136). Thus, when one system is compromised, it appears cells can compensate by upregulating the other. The fact that these systems are interconnected is further shown through their common function of adapting to stress. Proteasome abundance and activity are increased upon rapamycin stress, and both systems function to recycle amino acids back to cells (122, 123). Both pathways also play a role in the cellular response to oxidative stress (19, 86). Proteasomes themselves have recently been shown by us and others to be autophagy substrates under specific conditions (137, 138). However, as would be expected for these intricately connected systems, proteasome degradation is specifically regulated under different autophagy inducing conditions. For example, carbon starvation induces general autophagy, but proteasomes are instead targeted to cytosolic granules. Though originally termed proteasome storage granules (PSGs), they have recently been proposed to protect proteasomes from autophagic degradation (139, 140). However, this appears to be inconsistent with previously published data (140). Thus, the function of these proteasome granules during autophagy remains to be fully understood.

Rationale, results and significance

The focus of this work is to identify the mechanisms cells use to control proteasome abundance and localization in response to different conditions. We became interested in this question when it was demonstrated that proteasomes undergo quality control (29, 31). We reasoned that dysfunctional proteasomes would be degraded. Until recently, very little was known about how cells degrade proteasomes. As these large complexes are made of multiple unique proteins, we hypothesized that they were degraded through autophagy. Our data show that that proteasomes are specifically and differentially regulated under numerous conditions that induce general autophagy. In chapter 2, we present work showing that proteasomes are degraded through autophagy upon nitrogen but not carbon starvation (137). We found here that starvation for both nutrients did not result in proteasome autophagy, suggesting a dominant signal

when carbon was limiting. Degradation of proteasomes under nitrogen starvation was dependent on vacuolar hydrolases and proteins required for general autophagy. We found in this work that other autophagy pathways, including CVT and nucleophagy, were not required for proteasome autophagy. Our data suggested that proteasome sub-complexes underwent autophagy via different pathways. Here, we observed CP localization in cytoplasmic granules when autophagy was blocked, while RP remained nuclear. Subsequent data from other groups supports this model of proteasome disassembly prior to autophagy (141, 142). However, in chapter 3, we show that CP phenotypes observed were apparent only when the CP was tagged on the $\beta 5$ subunit. These $\beta 5$ -GFP induced granules were dependent on specific factors that have been shown to regulate proteasome autophagy in other contexts. Nevertheless, we report that proteasomes in yeast are degraded through autophagy, an event that has since been observed in mammalian cells, showing this is evolutionarily conserved (143). Carbon starvation does not induce proteasome autophagy despite the induction of general autophagy (99, 137). Instead proteasomes are re-localized to cytosolic granules. Thus, we sought to determine under what autophagy inducing conditions proteasomes were degraded. Surprisingly, rapamycin, an inhibitor of TORC1 and starvation mimic, was shown to induce proteasome upregulation and protein degradation (122, 123). We show in chapter 3 that this upregulation is part of a bi-phasic response that ultimately leads to proteasome autophagy. This response was not observed with other TORC1 inhibitors, suggesting it may be specific to rapamycin. However, we identified several conditions that result in proteasome autophagy. More interestingly, we identified conditions that lead to general autophagy but not proteasome autophagy, as well as factors that show specificity in its regulation. This suggests proteasomes are not bulk autophagy substrates, but rather, autophagy of proteasomes is a specific and regulated process. We further show that clinically relevant proteasome inhibitors restrict proteasome granule formation in carbon starvation and stationary phase. Intriguingly, CP and RP appeared to be affected differently by these inhibitors. In chapter 4 we work to further characterize the molecular events that result in proteasome granule formation. We hypothesized that this was linked to cellular energy and found that several mitochondria inhibitors induce proteasome granules. We also

identified other conditions resulting in perturbations in cellular energy that induce these granules. These data show that there are signaling events that occur upon mitochondria dysfunction that result in proteasome granule formation in yeast. Consistently, we show that when mitochondria are the main source of ATP production, factors otherwise not required for granule formation become essential. Interestingly, factors predicted to stabilize proteasome complexes prevented granule formation. Thus, our data support a model whereby proteasomes become unstable and are more readily exported from the nucleus under granule-inducing stimuli. Within this model, proteasome granules promote cell survival in conditions of stress.

In all, we report that proteasomes are dynamically regulated under stress conditions. Upon nitrogen starvation, degradation of proteasomes contributes to the replenishment of amino acids. The work presented here is one of the first descriptions of proteasome autophagy. How and why cells degrade these essential proteases was previously unknown. Our data helped to fill knowledge gaps in both proteasome biology and autophagy. We hypothesize that the sequestration of proteasomes into cytoplasmic granules, other than providing proteasome storage, facilitates survival. We propose that proteasome granules help to restrict proteasome activity and ATP use in ATP limited conditions. The effect of proteasome inhibition on proteasome localization is of great interest. Future studies intend to investigate localization in mammalian cells. Finally, the link between mitochondrial function and proteasome localization has been further expanded. This is of interest as it may represent a general response of proteasomes to mitochondrial stress, something that requires further exploration as it may have relevance to proteasome localization and activity in mammals. Modulation of proteasome activity has emerged as treatments or potential treatments for disease. Understanding the basic biology behind how cells normally control proteasomes provides us with necessary knowledge to inform such exploration. Our work has elucidated many of the factors that govern proteasome abundance and localization.

References-Chapter 1

1. Bar-Nun, S., and Glickman, M. H. (2012) Proteasomal AAA-ATPases: Structure and function. *Biochim. Biophys. Acta - Mol. Cell Res.* **1823**, 67–82
2. Forouzan, D., Ammelburg, M., Hobel, C. F., Ströh, L. J., Sessler, N., Martin, J., and Lupas, A. N. (2012) The archaeal proteasome is regulated by a network of AAA ATPases. *J. Biol. Chem.* **287**, 39254–62
3. Maupin-Furlow, J. A. (2013) Archaeal Proteasomes and Sanylation. in *Sub-cellular biochemistry*, pp. 297–327, **66**, 297–327
4. Maupin-Furlow, J. A. (2014) Prokaryotic Ubiquitin-Like Protein Modification. *Annu. Rev. Microbiol.* **68**, 155–175
5. Finley, D., Ulrich, H. D., Sommer, T., and Kaiser, P. (2012) The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. *Genetics*. **192**, 319–360
6. Xie, Y. (2010) Structure, assembly and homeostatic regulation of the 26S proteasome. *J. Mol. Cell Biol.* **2**, 308–317
7. Finley, D., Chen, X., and Walters, K. J. (2016) Gates, Channels, and Switches: Elements of the Proteasome Machine. *Trends Biochem. Sci.* **41**, 77–93
8. Tomko, R. J., and Hochstrasser, M. (2013) Molecular architecture and assembly of the eukaryotic proteasome. *Annu. Rev. Biochem.* **82**, 415–45
9. Huang, X., Luan, B., Wu, J., and Shi, Y. (2016) An atomic structure of the human 26S proteasome. *Nat. Struct. Mol. Biol.* **23**, 778–785
10. Dong, Y., Zhang, S., Wu, Z., Li, X., Wang, W. L., Zhu, Y., Stoilova-McPhie, S., Lu, Y., Finley, D., and Mao, Y. (2019) Cryo-EM structures and dynamics of substrate-engaged human 26S proteasome. *Nature*. **565**, 49–55
11. Gerards, W. L., de Jong, W. W., Boelens, W., and Bloemendal, H. (1998) Structure and assembly of the 20S proteasome. *Cell. Mol. Life Sci.* **54**, 253–62
12. Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4Å resolution. *Nature*. **386**, 463–471
13. Heinemeyer, W., Ramos, P. C., and Dohmen, R. J. (2004) Ubiquitin-proteasome system. *Cell. Mol. Life Sci.* **61**, 1562–78
14. Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U., and Wolf, D. H. (1997) The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. *J. Biol. Chem.* **272**, 25200–9
15. Stadtmueller, B. M., and Hill, C. P. (2011) Proteasome activators. *Mol. Cell.* **41**, 8–19
16. Aiken, C. T., Kaake, R. M., Wang, X., and Huang, L. (2011) Oxidative stress-mediated regulation of proteasome complexes. *Mol. Cell. Proteomics*. **10**, R110.006924

17. Wang, X., Yen, J., Kaiser, P., and Huang, L. (2010) Regulation of the 26S proteasome complex during oxidative stress. *Sci. Signal.* **3**, ra88
18. Schmidt, M., Hanna, J., Elsasser, S., and Finley, D. (2005) Proteasome-associated proteins: regulation of a proteolytic machine. *Biol. Chem.* **386**, 725–37
19. Pickering, A. M., Koop, A. L., Teoh, C. Y., Ermak, G., Grune, T., and Davies, K. J. A. (2010) The immunoproteasome, the 20S proteasome and the PA28 $\alpha\beta$ proteasome regulator are oxidative-stress-adaptive proteolytic complexes. *Biochem. J.* **432**, 585–94
20. Wani, P. S., Rowland, M. A., Ondracek, A., Deeds, E. J., and Roelofs, J. (2015) Maturation of the proteasome core particle induces an affinity switch that controls regulatory particle association. *Nat. Commun.* **6**, 6384
21. Ramos, P. C., Höckendorff, J., Johnson, E. S., Varshavsky, A., and Dohmen, R. J. (1998) Ump1p Is Required for Proper Maturation of the 20S Proteasome and Becomes Its Substrate upon Completion of the Assembly. *Cell.* **92**, 489–499
22. Li, F., Tian, G., Langager, D., Sokolova, V., Finley, D., and Park, S. (2017) Nucleotide-dependent switch in proteasome assembly mediated by the Nas6 chaperone. *Proc. Natl. Acad. Sci.* **114**, 1548–1553
23. Park, S., Roelofs, J., Kim, W., Robert, J., Schmidt, M., Gygi, S. P., and Finley, D. (2009) Hexameric assembly of the proteasomal ATPases is templated through their C termini. *Nature.* **459**, 866–70
24. Tomko, R. J., Hochstrasser, M., and Hochstrasser, M. (2011) Incorporation of the Rpn12 subunit couples completion of proteasome regulatory particle lid assembly to lid-base joining. *Mol. Cell.* **44**, 907–17
25. Roelofs, J., Park, S., Haas, W., Tian, G., McAllister, F. E., Huo, Y., Lee, B.-H., Zhang, F., Shi, Y., Gygi, S. P., and Finley, D. (2009) Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature.* **459**, 861–5
26. Nahar, A., Fu, X., Polovin, G., Orth, J. D., and Park, S. (2019) Two Alternative Mechanisms Regulate the Onset of Chaperone-mediated Assembly of the Proteasomal ATPases. *J. Biol. Chem.* 10.1074/jbc.RA118.006298
27. Xie, Y., and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3056–61
28. Lee, S. Y.-C., De la Mota-Peynado, A., and Roelofs, J. (2011) Loss of Rpt5 protein interactions with the core particle and Nas2 protein causes the formation of faulty proteasomes that are inhibited by Ecm29 protein. *J. Biol. Chem.* **286**, 36641–51
29. De La Mota-Peynado, A., Lee, S. Y.-C., Pierce, B. M., Wani, P., Singh, C. R., and Roelofs, J. (2013) The proteasome-associated protein Ecm29 inhibits proteasomal ATPase activity and in vivo protein degradation by the proteasome. *J. Biol. Chem.* **288**, 29467–81

30. Gorbea, C., Goellner, G. M., Teter, K., Holmes, R. K., and Rechsteiner, M. (2004) Characterization of mammalian Ecm29, a 26 S proteasome-associated protein that localizes to the nucleus and membrane vesicles. *J. Biol. Chem.* **279**, 54849–61
31. Lehmann, A., Niewianda, A., Jechow, K., Janek, K., and Enenkel, C. (2010) Ecm29 Fulfills Quality Control Functions in Proteasome Assembly. *Mol. Cell.* **38**, 879–888
32. Kleijnen, M. F., Roelofs, J., Park, S., Hathaway, N. A., Glickman, M., King, R. W., and Finley, D. (2007) Stability of the proteasome can be regulated allosterically through engagement of its proteolytic active sites. *Nat. Struct. Mol. Biol.* **14**, 1180–1188
33. Haratake, K., Sato, A., Tsuruta, F., and Chiba, T. (2016) KIAA0368 -deficiency affects disassembly of 26S proteasome under oxidative stress condition. *J. Biochem.* **159**, 609–618
34. Wani, P. S., Suppahia, A., Capalla, X., Ondracek, A., and Roelofs, J. (2016) Phosphorylation of the C-terminal tail of proteasome subunit $\alpha 7$ is required for binding of the proteasome quality control factor Ecm29. *Sci. Rep.* **6**, 27873
35. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–79
36. Komander, D., and Rape, M. (2012) The Ubiquitin Code. *Annu. Rev. Biochem.* **81**, 203–229
37. Rittinger, K., and Ikeda, F. (2017) Linear ubiquitin chains: enzymes, mechanisms and biology. *Open Biol.* **7**, 170026
38. Carter, S., Bischof, O., Dejean, A., and Vousden, K. H. (2007) C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. *Nat. Cell Biol.* **9**, 428–435
39. Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R. S., and Zhang, Y. (2004) Role of histone H2A ubiquitination in Polycomb silencing. *Nature.* **431**, 873–878
40. Nanduri, P., Hao, R., Fitzpatrick, T., and Yao, T.-P. (2015) Chaperone-mediated 26S proteasome remodeling facilitates free K63 ubiquitin chain production and aggresome clearance. *J. Biol. Chem.* **290**, 9455–64
41. Skieterska, K., Rondou, P., and Van Craenenbroeck, K. (2017) Regulation of G Protein-Coupled Receptors by Ubiquitination. *Int. J. Mol. Sci.* 10.3390/ijms18050923
42. Tan, J. M. M., Wong, E. S. P., Kirkpatrick, D. S., Pletnikova, O., Ko, H. S., Tay, S.-P., Ho, M. W. L., Troncoso, J., Gygi, S. P., Lee, M. K., Dawson, V. L., Dawson, T. M., and Lim, K.-L. (2008) Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. *Hum. Mol. Genet.* **17**, 431–439
43. Shaid, S., Brandts, C. H., Serve, H., and Dikic, I. (2013) Ubiquitination and selective autophagy. *Cell Death Differ.* **20**, 21–30

44. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science*. **243**, 1576–83
45. Finley, D. (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu. Rev. Biochem.* **78**, 477–513
46. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature*. **349**, 132–138
47. Ciechanover, A., and Brundin, P. (2003) The Ubiquitin Proteasome System in Neurodegenerative Diseases. *Neuron*. **40**, 427–446
48. Tai, H.-C., Besche, H., Goldberg, A. L., and Schuman, E. M. (2010) Characterization of the brain 26S proteasome and its interacting proteins. *Front. Mol. Neurosci.* **3**, 12
49. Bax, M., McKenna, J., Do-Ha, D., Stevens, C. H., Higginbottom, S., Balez, R., Cabral-da-Silva, M. e C., Farrowell, N. E., Engel, M., Poronnik, P., Yerbury, J. J., Saunders, D. N., and Ooi, L. (2019) The Ubiquitin Proteasome System Is a Key Regulator of Pluripotent Stem Cell Survival and Motor Neuron Differentiation. *Cells*. **8**, 581
50. No Title (2017)
51. Sijts, E. J. A. M., and Kloetzel, P. M. (2011) The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell. Mol. Life Sci.* **68**, 1491–502
52. Schmidt, M., and Finley, D. (2014) Regulation of proteasome activity in health and disease. *Biochim. Biophys. Acta*. **1843**, 13–25
53. Schwartz, PhD, MD, A. L., and Ciechanover, MD, PhD, A. (1999) THE UBIQUITIN-PROTEASOME PATHWAY AND PATHOGENESIS OF HUMAN DISEASES. *Annu. Rev. Med.* **50**, 57–74
54. Cliffe, R., Sang, J. C., Kundel, F., Finley, D., Klenerman, D., and Ye, Y. (2019) Filamentous Aggregates Are Fragmented by the Proteasome Holoenzyme. *Cell Rep.* **26**, 2140-2149.e3
55. Tseng, B. P., Green, K. N., Chan, J. L., Blurton-Jones, M., and LaFerla, F. M. (2008) Abeta inhibits the proteasome and enhances amyloid and tau accumulation. *Neurobiol. Aging*. **29**, 1607–18
56. Chiti, F., and Dobson, C. M. (2006) Protein Misfolding, Functional Amyloid, and Human Disease. *Annu. Rev. Biochem.* **75**, 333–66
57. Zhao, X., and Yang, J. (2010) Amyloid- β peptide is a substrate of the human 20S proteasome. *ACS Chem. Neurosci.* **1**, 655–660
58. Bennett, E. J., Shaler, T. A., Woodman, B., Ryu, K.-Y., Zaitseva, T. S., Becker, C. H., Bates, G. P., Schulman, H., and Kopito, R. R. (2007) Global changes to the ubiquitin system in Huntington's disease. *Nature*. **448**, 704–708

59. Keller, J. N., Hanni, K. B., and Markesbery, W. R. (2001) Impaired Proteasome Function in Alzheimer's Disease. *J. Neurochem.* **75**, 436–439
60. Bedford, L., Hay, D., Devoy, A., Paine, S., Powe, D. G., Seth, R., Gray, T., Topham, I., Fone, K., Rezvani, N., Mee, M., Soane, T., Layfield, R., Sheppard, P. W., Ebendal, T., Usoskin, D., Lowe, J., and Mayer, R. J. (2008) Depletion of 26S Proteasomes in Mouse Brain Neurons Causes Neurodegeneration and Lewy-Like Inclusions Resembling Human Pale Bodies. *J. Neurosci.* **28**, 8189–8198
61. Bendotti, C., Marino, M., Cheroni, C., Fontana, E., Crippa, V., Poletti, A., and De Biasi, S. (2012) Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: Implication for protein aggregation and immune response. *Prog. Neurobiol.* **97**, 101–126
62. Junn, E., Lee, S. S., Suhr, U. T., and Mouradian, M. M. (2002) Parkin accumulation in aggresomes due to proteasome impairment. *J. Biol. Chem.* **277**, 47870–7
63. van Tijn, P., de Vrij, F. M. S., Schuurman, K. G., Dantuma, N. P., Fischer, D. F., van Leeuwen, F. W., and Hol, E. M. (2007) Dose-dependent inhibition of proteasome activity by a mutant ubiquitin associated with neurodegenerative disease. *J. Cell Sci.* **120**, 1615–23
64. Wang, J., Wang, C.-E., Orr, A., Tydlacka, S., Li, S.-H., and Li, X.-J. (2008) Impaired ubiquitin–proteasome system activity in the synapses of Huntington's disease mice. *J. Cell Biol.* **180**, 1177–1189
65. Schipper-Krom, S., Juenemann, K., Jansen, A. H., Wiemhoefer, A., van den Nieuwendijk, R., Smith, D. L., Hink, M. A., Bates, G. P., Overkleeft, H., Ovaas, H., and Reits, E. (2014) Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies. *FEBS Lett.* **588**, 151–159
66. Suhr, S. T., Senut, M.-C., Whitelegge, J. P., Faull, K. F., Cuizon, D. B., and Gage, F. H. (2001) Identities of Sequestered Proteins in Aggregates from Cells with Induced Polyglutamine Expression. *J. Cell Biol.* **153**, 283–294
67. Jansen, A. H. P., Reits, E. a. J., and Hol, E. M. (2014) The ubiquitin proteasome system in glia and its role in neurodegenerative diseases. *Front. Mol. Neurosci.* **7**, 1–14
68. Douglas, P. M., and Dillin, A. (2010) Protein homeostasis and aging in neurodegeneration. *J. Cell Biol.* **190**, 719–729
69. Martín-Aparicio, E., Yamamoto, A., Hernández, F., Hen, R., Avila, J., and Lucas, J. J. (2001) Proteasomal-dependent aggregate reversal and absence of cell death in a conditional mouse model of Huntington's disease. *J. Neurosci.* **21**, 8772–81
70. Myeku, N., and Duff, K. E. (2018) Targeting the 26S Proteasome To Protect Against Proteotoxic Diseases. *Trends Mol. Med.* **24**, 18–29
71. Huang, Q., and Figueiredo-Pereira, M. E. (2010) Ubiquitin/proteasome pathway impairment in neurodegeneration: therapeutic implications. *Apoptosis.* **15**, 1292

72. Hanna, J., Guerra-Moreno, A., Ang, J., and Micoogullari, Y. (2019) Protein Degradation and the Pathologic Basis of Disease. *Am. J. Pathol.* **189**, 94–103
73. Lee, B.-H., Lee, M. J., Park, S., Oh, D.-C., Elsasser, S., Chen, P.-C., Gartner, C., Dimova, N., Hanna, J., Gygi, S. P., Wilson, S. M., King, R. W., and Finley, D. (2010) Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature.* **467**, 179–184
74. Boselli, M., Lee, B.-H., Robert, J., Prado, M. A., Min, S.-W., Cheng, C., Silva, M. C., Seong, C., Elsasser, S., Hatle, K. M., Gahman, T. C., Gygi, S. P., Haggarty, S. J., Gan, L., King, R. W., and Finley, D. (2017) An inhibitor of the proteasomal deubiquitinating enzyme USP14 induces tau elimination in cultured neurons. *J. Biol. Chem.* **292**, 19209–19225
75. Crawford, L. J., Walker, B., and Irvine, A. E. (2011) Proteasome inhibitors in cancer therapy. *J. Cell Commun. Signal.* **5**, 101–110
76. Hoeller, D., and Dikic, I. (2009) Targeting the ubiquitin system in cancer therapy. *Nature.* **458**, 438–444
77. Sato, K., Rajendra, E., and Ohta, T. (2008) The UPS: a promising target for breast cancer treatment. *BMC Biochem.* **9 Suppl 1**, S2
78. Yu, D., Carroll, M., and Thomas-Tikhonenko, A. (2007) p53 status dictates responses of B lymphomas to monotherapy with proteasome inhibitors. *Blood.* **109**, 4936–4943
79. Traenckner, E. B., Wilk, S., and Baeuerle, P. A. (1994) A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. *EMBO J.* **13**, 5433
80. McDade, T. P., Perugini, R. A., Vittimberga, F. J., and Callery, M. P. (1999) Ubiquitin-proteasome inhibition enhances apoptosis of human pancreatic cancer cells. *Surgery.* **126**, 371–7
81. Nemeth, Z. H., Wong, H. R., Odoms, K., Deitch, E. A., Szabó, C., Vizi, E. S., and Haskó, G. (2004) Proteasome Inhibitors Induce Inhibitory B (I B) Kinase Activation, I B Degradation, and Nuclear Factor B Activation in HT-29 Cells. *Mol. Pharmacol.* **65**, 342–349
82. Li, W., Li, J., and Bao, J. (2012) Microautophagy: lesser-known self-eating. *Cell. Mol. Life Sci.* **69**, 1125–1136
83. Oku, M., and Sakai, Y. (2018) Three Distinct Types of Microautophagy Based on Membrane Dynamics and Molecular Machineries. *BioEssays.* **40**, 1800008
84. Roberts, P., Moshitch-Moshkovitz, S., Kvam, E., O'Toole, E., Winey, M., and Goldfarb, D. S. (2003) Piecemeal Microautophagy of Nucleus in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* **14**, 129–141
85. Kaushik, S., and Cuervo, A. M. (2018) The coming of age of chaperone-mediated

- autophagy. *Nat. Rev. Mol. Cell Biol.* **19**, 365–381
86. Kiffin, R., Christian, C., Knecht, E., and Cuervo, A. M. (2004) Activation of Chaperone-mediated Autophagy during Oxidative Stress. *Mol. Biol. Cell.* **15**, 4829–4840
 87. Cuervo, A. M., and Dice, J. F. (1996) A Receptor for the Selective Uptake and Degradation of Proteins by Lysosomes. *Science (80-.).* **273**, 501–503
 88. Bandyopadhyay, U., Kaushik, S., Varticovski, L., and Cuervo, A. M. (2008) The Chaperone-Mediated Autophagy Receptor Organizes in Dynamic Protein Complexes at the Lysosomal Membrane. *Mol. Cell. Biol.* **28**, 5747–5763
 89. Feng, Y., He, D., Yao, Z., and Klionsky, D. J. (2014) The machinery of macroautophagy. *Cell Res.* **24**, 24–41
 90. Chinchwadkar, S., Padmanabhan, S., Mishra, P., Singh, S., Suresh, S. N., Vats, S., Barve, G., Ammanathan, V., and Manjithaya, R. (2017) Multifaceted Housekeeping Functions of Autophagy. *J. Indian Inst. Sci.* **97**, 79–94
 91. Anding, A. L., and Baehrecke, E. H. (2017) Cleaning House: Selective Autophagy of Organelles. *Dev. Cell.* **41**, 10–22
 92. Kim, J., Scott, S. V., Oda, M. N., and Klionsky, D. J. (1997) Transport of a Large Oligomeric Protein by the Cytoplasm to Vacuole Protein Targeting Pathway. *J. Cell Biol.* **137**, 609–618
 93. Lynch-Day, M. A., and Klionsky, D. J. (2010) The Cvt pathway as a model for selective autophagy. *FEBS Lett.* **584**, 1359–66
 94. Kamada, Y., Yoshino, K. -i., Kondo, C., Kawamata, T., Oshiro, N., Yonezawa, K., and Ohsumi, Y. (2010) Tor Directly Controls the Atg1 Kinase Complex To Regulate Autophagy. *Mol. Cell. Biol.* **30**, 1049–1058
 95. Kabeya, Y., Kamada, Y., Baba, M., Takikawa, H., Sasaki, M., and Ohsumi, Y. (2005) Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. *Mol. Biol. Cell.* **16**, 2544–53
 96. Beck, T., and Hall, M. N. (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature.* **402**, 689–692
 97. Onodera, J., and Ohsumi, Y. (2005) Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *J. Biol. Chem.* **280**, 31582–6
 98. Yokota, H., Gomi, K., and Shintani, T. (2017) Induction of autophagy by phosphate starvation in an Atg11-dependent manner in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **483**, 522–527
 99. Adachi, A., Koizumi, M., and Ohsumi, Y. (2017) Autophagy induction under carbon starvation conditions is negatively regulated by carbon catabolite repression. *J. Biol. Chem.* **292**, 19905–19918

100. Huang, W.-P., and Klionsky, D. J. (2002) Autophagy in yeast: a review of the molecular machinery. *Cell Struct. Funct.* **27**, 409–20
101. Sharma, V., Verma, S., Seranova, E., Sarkar, S., and Kumar, D. (2018) Selective Autophagy and Xenophagy in Infection and Disease. *Front. Cell Dev. Biol.* **6**, 147
102. Jin, M., and Klionsky, D. J. (2014) Regulation of autophagy: modulation of the size and number of autophagosomes. *FEBS Lett.* **588**, 2457–63
103. Yamamoto, H., Fujioka, Y., Suzuki, S. W., Noshiro, D., Suzuki, H., Kondo-Kakuta, C., Kimura, Y., Hirano, H., Ando, T., Noda, N. N., and Ohsumi, Y. (2016) The Intrinsically Disordered Protein Atg13 Mediates Supramolecular Assembly of Autophagy Initiation Complexes. *Dev. Cell.* **38**, 86–99
104. Wyant, G. A., Abu-Remaileh, M., Frenkel, E. M., Laqtom, N. N., Dharamdasani, V., Lewis, C. A., Chan, S. H., Heinze, I., Ori, A., and Sabatini, D. M. (2018) NUFIP1 is a ribosome receptor for starvation-induced ribophagy. *Science.* **360**, 751–758
105. Schuck, S., Gallagher, C. M., and Walter, P. (2014) ER-phagy mediates selective degradation of endoplasmic reticulum independently of the core autophagy machinery. *J. Cell Sci.* **127**, 4078–4088
106. Kraft, C., Deplazes, A., Sohrmann, M., and Peter, M. (2008) Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat. Cell Biol.* **10**, 602–610
107. Kraft, C., Reggiori, F., and Peter, M. (2009) Selective types of autophagy in yeast. *Biochim. Biophys. Acta - Mol. Cell Res.* **1793**, 1404–1412
108. Suzuki, H., and Noda, N. N. (2018) Biophysical characterization of Atg11, a scaffold protein essential for selective autophagy in yeast. *FEBS Open Bio.* **8**, 110–116
109. Delorme-Axford, E., and Klionsky, D. J. (2015) A missing piece of the puzzle: Atg11 functions as a scaffold to activate Atg1 for selective autophagy. *Autophagy.* **11**, 2139–2141
110. Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* **282**, 24131–45
111. Farré, J.-C., Burkenroad, A., Burnett, S. F., and Subramani, S. (2013) Phosphorylation of mitophagy and pexophagy receptors coordinates their interaction with Atg8 and Atg11. *EMBO Rep.* **14**, 441–9
112. Mochida, K., Oikawa, Y., Kimura, Y., Kirisako, H., Hirano, H., Ohsumi, Y., and Nakatogawa, H. (2015) Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus. *Nature.* **522**, 359–362
113. Mao, K., Wang, K., Liu, X., and Klionsky, D. J. (2013) The Scaffold Protein Atg11

- Recruits Fission Machinery to Drive Selective Mitochondria Degradation by Autophagy. *Dev. Cell.* **26**, 9–18
114. Kanki, T., Wang, K., Cao, Y., Baba, M., and Klionsky, D. J. (2009) Atg32 Is a Mitochondrial Protein that Confers Selectivity during Mitophagy. *Dev. Cell.* **17**, 98–109
 115. Levine, B., and Kroemer, G. (2008) Autophagy in the pathogenesis of disease. *Cell.* **132**, 27–42
 116. Jiang, P., and Mizushima, N. (2014) Autophagy and human diseases. *Cell Res.* **24**, 69–79
 117. Levine, B., and Klionsky, D. J. (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev. Cell.* **6**, 463–77
 118. Danieli, A., and Martens, S. (2018) p62-mediated phase separation at the intersection of the ubiquitin-proteasome system and autophagy. *J. Cell Sci.* **131**, jcs214304
 119. Dikic, I. (2017) Proteasomal and Autophagic Degradation Systems. *Annu. Rev. Biochem.* **86**, 193–224
 120. Myeku, N., and Figueiredo-Pereira, M. E. (2011) Dynamics of the degradation of ubiquitinated proteins by proteasomes and autophagy: association with sequestosome 1/p62. *J. Biol. Chem.* **286**, 22426–40
 121. Webb, J. L., Ravikumar, B., Atkins, J., Skepper, J. N., and Rubinsztein, D. C. (2003) α -Synuclein Is Degraded by Both Autophagy and the Proteasome. *J. Biol. Chem.* **278**, 25009–25013
 122. Rousseau, A., and Bertolotti, A. (2016) An evolutionarily conserved pathway controls proteasome homeostasis. *Nature.* **536**, 184–189
 123. Zhao, J., Zhai, B., Gygi, S. P., and Goldberg, A. L. (2015) mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 15790–7
 124. Zhang, N., Quan, Z., Rash, B., and Oliver, S. G. (2013) Synergistic effects of TOR and proteasome pathways on the yeast transcriptome and cell growth. *Open Biol.* **3**, 120137
 125. Kelly, S. P., and Bedwell, D. M. (2015) Both the autophagy and proteasomal pathways facilitate the Ubp3p-dependent depletion of a subset of translation and RNA turnover factors during nitrogen starvation in *Saccharomyces cerevisiae*. *RNA.* **21**, 898–910
 126. Nazio, F., Carinci, M., Valacca, C., Bielli, P., Strappazzon, F., Antonioli, M., Ciccocanti, F., Rodolfo, C., Campello, S., Fimia, G. M., Sette, C., Bonaldo, P., and Cecconi, F. (2016) Fine-tuning of ULK1 mRNA and protein levels is required for autophagy oscillation. *J. Cell Biol.* **215**, 841–856
 127. Liu, C.-C., Lin, Y.-C., Chen, Y.-H., Chen, C.-M., Pang, L.-Y., Chen, H.-A., Wu, P.-R., Lin, M.-Y., Jiang, S.-T., Tsai, T.-F., and Chen, R.-H. (2016) Cul3-KLHL20 Ubiquitin Ligase

- Governs the Turnover of ULK1 and VPS34 Complexes to Control Autophagy Termination. *Mol. Cell.* **61**, 84–97
128. BAO, W., GU, Y., TA, L., WANG, K., and XU, Z. (2016) Induction of autophagy by the MG-132 proteasome inhibitor is associated with endoplasmic reticulum stress in MCF-7 cells. *Mol. Med. Rep.* **13**, 796–804
 129. Tang, B., Cai, J., Sun, L., Li, Y., Qu, J., Snider, B. J., and Wu, S. (2014) Proteasome Inhibitors Activate Autophagy Involving Inhibition of PI3K-Akt-mTOR Pathway as an Anti-Oxidation Defense in Human RPE Cells. *PLoS One.* **9**, e103364
 130. Harada, M., Hanada, S., Toivola, D. M., Ghorri, N., and Omary, M. B. (2008) Autophagy activation by rapamycin eliminates mouse Mallory-Denk bodies and blocks their proteasome inhibitor-mediated formation. *Hepatology.* **47**, 2026–2035
 131. Demishtein, A., Fraiberg, M., Berko, D., Tirosh, B., Elazar, Z., and Navon, A. (2017) SQSTM1/p62-mediated autophagy compensates for loss of proteasome polyubiquitin recruiting capacity. *Autophagy.* **13**, 1697–1708
 132. Athané, A., Buisson, A., Challier, M., Beaumatin, F., Manon, S., Bhatia-Kiššová, I., and Camougrand, N. (2015) Insights into the relationship between the proteasome and autophagy in human and yeast cells. *Int. J. Biochem. Cell Biol.* **64**, 167–173
 133. Bernales, S., McDonald, K. L., and Walter, P. (2006) Autophagy Counterbalances Endoplasmic Reticulum Expansion during the Unfolded Protein Response. *PLoS Biol.* **4**, e423
 134. Yorimitsu, T., Nair, U., Yang, Z., and Klionsky, D. J. (2006) Endoplasmic Reticulum Stress Triggers Autophagy. *J. Biol. Chem.* **281**, 30299–30304
 135. Pandey, U. B., Nie, Z., Batlevi, Y., McCray, B. A., Ritson, G. P., Nedelsky, N. B., Schwartz, S. L., DiProspero, N. A., Knight, M. A., Schuldiner, O., Padmanabhan, R., Hild, M., Berry, D. L., Garza, D., Hubbert, C. C., Yao, T.-P., Baehrecke, E. H., and Taylor, J. P. (2007) HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. *Nature.* **447**, 859–63
 136. Havé, M., Balliau, T., Cottyn-Boitte, B., Déron, E., Cuff, G., Soulay, F., Lornac, A., Reichman, P., Dissmeyer, N., Avic, J.-C., Gallois, P., Rajjou, L., Zivy, M., and Masclaux-Daubresse, C. (2018) Increases in activity of proteasome and papain-like cysteine protease in Arabidopsis autophagy mutants: back-up compensatory effect or cell-death promoting effect? *J. Exp. Bot.* **69**, 1369–1385
 137. Waite, K. A., De La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2015) Starvation Induces Proteasome Autophagy with Different Pathways for Core and Regulatory Particle. *J. Biol. Chem.* 10.1074/jbc.M115.699124
 138. Marshall, R. S., Li, F., Gemperline, D. C., Book, A. J., Vierstra Correspondence, R. D., and Vierstra, R. D. (2015) Autophagic Degradation of the 26S Proteasome Is Mediated by the Dual ATG8/Ubiquitin Receptor RPN10 in Arabidopsis. *Mol. Cell.* **58**, 1053–1066

139. Marshall, R. S., and Vierstra, R. D. (2018) Proteasome storage granules protect proteasomes from autophagic degradation upon carbon starvation. *Elife*. 10.7554/eLife.34532
140. van Deventer, S., Menendez-Benito, V., van Leeuwen, F., and Neefjes, J. (2014) N-terminal acetylation and replicative age affect proteasome localization and cell fitness during aging. *J. Cell Sci.* **128**, 109–117
141. Nemec, A. A., Howell, L. A., Peterson, A. K., Murray, M. A., and Tomko, R. J. (2017) Autophagic clearance of proteasomes in yeast requires the conserved sorting nexin Snx4. *J. Biol. Chem.* **292**, 21466–21480
142. Marshall, R. S., McLoughlin, F., and Vierstra, R. D. (2016) Autophagic Turnover of Inactive 26S Proteasomes in Yeast Is Directed by the Ubiquitin Receptor Cue5 and the Hsp42 Chaperone. *Cell Rep.* **16**, 1717–1732
143. Cohen-Kaplan, V., Livneh, I., Avni, N., Fabre, B., Ziv, T., Kwon, Y. T., and Ciechanover, A. (2016) p62- and ubiquitin-dependent stress-induced autophagy of the mammalian 26S proteasome. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E7490–E7499

Chapter 2 - Starvation Induces Proteasome Autophagy with Different Pathways for Core and Regulatory Particles

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Abstract

The proteasome is responsible for the degradation of many cellular proteins. If and how this abundant and normally stable complex is degraded by cells is largely unknown. Here we show that in yeast, upon nitrogen starvation, proteasomes are targeted for vacuolar degradation through autophagy. Using GFP-tagged proteasome subunits, we observed that autophagy of a core particle (CP) subunit depends on the deubiquitinating enzyme Ubp3, although a regulatory particle (RP) subunit does not. Furthermore, upon blocking of autophagy, RP remained largely nuclear, although CP largely localized to the cytosol as well as granular structures within the cytosol. In all, our data reveal a regulated process for the removal of proteasomes upon nitrogen starvation. This process involves CP and RP dissociation, nuclear export, and independent vacuolar targeting of CP and RP. Thus, in addition to the well characterized transcriptional up-regulation of genes encoding proteasome subunits, cells are also capable of downregulating cellular levels of proteasomes through proteaphagy.

Introduction

The degradation of proteins in eukaryotic cells is largely mediated by two machineries, the lysosome (or vacuole in yeast and plants) and the proteasome. The lysosome is a membrane-surrounded organelle that houses numerous acidic hydrolases. These hydrolases can degrade a large variety of substrates, including soluble proteins, aggregates, structured complexes, and even whole bacteria. Entry into

the lysosome or vacuole normally requires substrates to be packaged as vesicular material. This includes, among others, endosomes, multivesicular body-derived material, and material packaged within a double-membrane structure through the process of macro-autophagy (referred to as autophagy hereafter) (1, 2). The proteasome, as predetermined by its structure, is a protease with different requirements for its substrates (3, 4). The proteolytic active sites of the proteasome are housed inside a cylindrically shaped core particle (CP or 20S) that is formed by four heptameric rings (α 1–7, β 1–7, β 1–7, and α 1–7). Both faces of the CP cylinder are identical and have a regulated entry site known as the gate. Folded polypeptides are too large to enter this gate. Hence, functional proteasomes require one or two regulatory particles (RPs or 19S). The RP provides several important functions in the degradation of substrates as follows: docking of RP on CP opens the gate, RP contains receptors that recognize the substrates (normally ubiquitin modifications found on proteins destined for degradation), and RP contains a ring of six AAA-ATPases that help to unfold and translocate substrates through the gate (3). The latter explains why proteasomal degradation is an energy-requiring process. Thus, proteasomes can readily degrade free soluble proteins; however, the degradation of aggregates or protein complexes requires processing by chaperones prior to degradation (4).

The cell has many large molecular complexes. These complexes are often stable and the proteasome might not be readily able to degrade them. Ribosomes are an example of such a large and stable molecular complex. Upon nitrogen starvation, the need for ribosomes is reduced as cells lack resources to produce large amounts of proteins. Furthermore, the degradation of ribosomes can provide crucial nitrogen-containing compounds required for cell survival (5, 6). Consistent with this, ribosomes have been shown to undergo vacuolar degradation upon nitrogen starvation through an autophagy-dependent pathway (6– 8). This is a process of selective autophagy, named ribophagy, based on the dynamics of ribosome packaging and the requirement of specific genes that are dispensable for non-selective bulk autophagy (6– 8).

Like ribosomes, the proteasome is a stable, abundant, and large protein complex in the cell (3, 9). Several studies have looked at the relationship between autophagy and the proteasome, and increased proteasome levels and activity have been observed

upon inhibition of autophagy (10–14). Nevertheless, the fate of proteasomes under starvation conditions is unclear, with only one report identifying proteasomes in the lysosome of starved rat livers (15). As a large and abundant protein complex, it can provide a substantial source of nutrients. Furthermore, a subset of proteasomes seem to work together with ribosomes. Thus, like ribosomes, proteasomes may be targeted for degradation upon starvation. However, proteasomes can degrade proteins and could, like vacuoles, be important in degrading proteins upon starvation (16).

In this study, we characterized how nitrogen starvation, but not glucose starvation, induces autophagy of proteasomes. Our data indicate that the vacuolar targeting of proteasomes requires nuclear export of proteasomes that are targeted for autophagy. Furthermore, CP and RP are targeted to the vacuole through different mechanisms, as only CP autophagy depends on the deubiquitinating enzyme Ubp3. Thus, our data show the autophagic removal of proteasomes from cells upon nitrogen starvation is not simple non-selective bulk autophagy but is a highly regulated process.

Materials and Methods

Yeast Strains—All strains used in this study are reported in Table 1 and were generated in a SUB61 background (lys2-801 leu2-3, 2-112 ura3-52 his3-200 trp1-1) (17). Strains deleted for specific genes or containing C-terminal GFP fusions of Rpn1 and Pre2 ($\beta 5$) were generated using standard PCR-based procedures (see Table 2) (18, 19).

Yeast Growth Conditions—Yeast cultures were grown in YPD medium or synthetic defined (SD) medium containing 0.17% yeast nitrogen base, 0.5% (NH₄)₂SO₄, 2% dextrose and supplemented with appropriate nutrients (specific amino acids, uracil, and adenine). For starvation, overnight cultures of yeast strains were diluted to an A₆₀₀ of 0.5 and grown in YPD medium to an A₆₀₀ between 1 and 2. Next, cells were washed with sterile water and resuspended in SD medium at an A₆₀₀ of 1.5. For nitrogen starvation, nutrients and ammonium sulfate were omitted from the SD medium; for glucose starvation dextrose was omitted, and for the combined starvation of nitrogen and glucose nutrients, ammonium sulfate, and dextrose were omitted. Cells were grown at 30 °C with constant shaking and harvested at the indicated time points by centrifugation. Cell pellets were stored at 80 °C until processing.

Table 2-1 Strain list

Strain	Genotype ^a (lys2-801 leu2-3, 2-112 ura3-52 his3- Δ 200 trp1-1)	Figure	Source
sJR786	MAT α rpn1::RPN1-GFP (HIS) atg7::CloNAT	3a, b	(1)
sJR858	MAT α pre2::PRE2-GFP (HIS)	1 to 6 and S	(1)
sJR861	MAT α rpn1::RPN1-GFP (HIS)	1 to 6 and S	(1)
sJR869	MAT α rpn11::RPN11-GFP (HIS)	S	(1)
sJR875	MAT α pre2::PRE2-GFP (HIS) pep4::CloNAT	2a, b, c	(1)
sJR876	MAT α rpn1::RPN1-GFP (HIS) pep4::CloNAT	2a, b, c	(1)
sJR877	MAT α rpn1::RPN1-GFP (HIS) nvj1::HYG	3c	(1)
sJR878	MAT α pre2::PRE2-GFP (HIS) nvj1::HYG	3b	(1)
sJR879	MAT α rpn1::RPN1-GFP (HIS) ubp3::HYG	5a, c	(1)
sJR880	MAT α pre2::PRE2-GFP (HIS) ubp3::HYG	5b, d	(1)
sJR881	MAT α pre2::PRE2-GFP (HIS) atg17::HYG	3d, 4a, b	(1)
sJR882	MAT α rpn1::RPN1-GFP (HIS) atg17::HYG	3c, 4a	(1)
sJR883	MAT α rpn1::RPN1-GFP (HIS) atg19::HYG	3c	(1)
sJR889	MAT α rpn1::RPN1-GFP (HIS) ufd3::HYG	6a, c	(1)
sJR890	MAT α pre2::PRE2-GFP (HIS) ufd3::HYG	6b	(1)
sJR896	MAT α pre2::PRE2-GFP (HIS) rpn10::HYG	S	(1)
sJR897	MAT α rpn1::RPN1-GFP (HIS) rpn10::HYG	S	(1)
sJR900	MAT α rpn1::RPN1-GFP (HIS) atg11::HYG	3c	(1)
sJR904	MAT α pre2::PRE2-GFP (HIS) atg11::HYG	3d	(1)
sRJ925	MAT α pre2::PRE2-GFP (HIS) atg7::CloNAT	3a,b	(1)
All strains have the DF5 background genotype (lys2-801 leu2-3, 2-112 ura3-52 his3- Δ 200 trp1-1)			
1. This study			

Table 2-2 Primer list

Primer	genotype	Template	Sequence (5' to 3')
Frwd/Beta5	pre2::PRE2-GFP (HIS)	pYM28 ¹	TATTTTGGAAAGGTCAAGGAAGAGGAAGGATCTTTCAACA ACGTTATTGGCCGTACGCTGCAGGTCGAC
Rvrs/Beta5	pre2::PRE2-GFP (HIS)	pYM28 ¹	TGTATCATTAATATAGATGTGCATATACATATGTTTGAT GCTTCTATTTAATCGATGAATTCGAGCTCG
Frwd/Rpn1	rpn1::RPN1-GFP (HIS)	pYM28 ¹	TTGAGGGCGTAGTAATTTTAAAGAAGAACCCCTGACTATC GTGAAGAGGAGCGTACGCTGCAGGTCGAC
Rvrs/Rpn1	rpn1::RPN1-GFP (HIS)	pYM28 ¹	TTTGAATTTTCTATTCTGGTTGATATTGCCCAAAGC TATTCAGTTTAATCGATGAATTCGAGCTCG
Frwd/Rpn11	rpn11::RPN11-GFP (HIS)	pYM28 ¹	ACAATATTGTTTCTGTGCTGACGGCGGGTGTTAATTCAG TGGCAATTAAACGTACGCTGCAGGTCGAC
Rvrs/Rpn11	Rpn11::RPN11-GFP (HIS)	pYM28 ¹	AGTGGGCTTTCTAGTTATTTAATGCATAATGACTTTATA AAATTTGTTTAATCGATGAATTCGAGCTCG
pRL53	pre6::pre6-CFP (HYG)	pBS4 [*]	GAGCAGCAAGAGCAGGACAAAAAGAAAAATCTAACC ATGGTCGACGGATCCCCGGG
pRL54	pre6::pre6-CFP (HYG)	pBS4 [*]	CTGTTATTTTATATAGGTTTATGCCCCAATATATATCGC CGATCGATGAATTCGAGCTCG
pRL61	pre5::pre5-CFP (HYG)	pBS4 [*]	CTTTTACAATTTACGATGGAGAAGCTGTTGCTAAATACA TCGGTCGACGGATCCCCGGG
pRL62	pre5::pre5-CFP (HYG)	pBS4 [*]	CTTTGACAAATTGCTTAGTGGAACGATACTTTGTGTCCA GATCGATGAATTCGAGCTCG
pRL236	atg7::CloNAT	pAG25 ²	TTCATTATATTTCAACAAATATAAGATAATCAAGAATAAA CGTACGCTGCAGGTCGACG
pRL237	atg7::CloNAT	pAG25 ²	CGGAAAGTGGCACCACAATATGTACCAATGCTATTATAT GCA ATCGATGAATTCGAGCTCG
pRL268	pep4::CloNAT	sMK-215 gDNA ³	TGGAGAGGCTGTAACCCGTC
pRL269	pep4::CloNAT	sMK-215 gDNA ³	TGACATTATGGGCAGCAGC
pRL281	ubp3::HYG	pFA6a-hphNT1 ¹	CCATCATCCAGGTACCGCTTTCCTTCCATCATCATTA CGTACGCTGCAGGTCGAC
pRL282	ubp3::HYG	pFA6a-hphNT1 ¹	TTATTTTTATGTATTTTGTCTATAATACCACCCCCGTC ATCGATGAATTCGAGCTCG
pRL285	nvj1::HYG	pFA6a-hphNT1 ¹	ATCAAAAAAGCTACAAATATAATTGTAAATATAAAGC CGTACGCTGCAGGTCGAC
pRL286	nvj1::HYG	pFA6a-hphNT1 ¹	GTAAGTGACGATGATAACCGAGATGACGGAAATATAGTA CA ATCGATGAATTCGAGCTCG
pRL289	atg19::HYG	pFA6a-hphNT1 ¹	CGGCACCTTGCTTCAGTAACGCCCAAAGGAGAGTTCTGGT A CGTACGCTGCAGGTCGAC
pRL290	atg19::HYG	pFA6a-hphNT1 ¹	GTGAAAAGGTACTCATTGCTGTATAAAAAATAGAGTTTGAC ATCGATGAATTCGAGCTCG
pRL293	atg17::HYG	pFA6a-hphNT1 ¹	ATTCGATACTGCGAGGATATTATCAACGTATTTAACACCT CGTACGCTGCAGGTCGAC
pRL294	atg17::HYG	pFA6a-hphNT1 ¹	GATACAATTATTGAATCTTTGTACCGTATCCTTTTTTCT ATCGATGAATTCGAGCTCG
pRL307	ufd3::HYG	pFA6a-hphNT1 ¹	TCATGTGTGATAGTAAGGTGTAGAGCAGCAGATTGGAG T CGTACGCTGCAGGTCGAC
pRL308	ufd3::HYG	pFA6a-hphNT1 ¹	TATCTAGACATTATGTGTTTTATGATTGCTGTAAAAGT ATCGATGAATTCGAGCTCG
pRL321	rpn10::HYG	pFA6a-hphNT1 ¹	GAAGGGAAAAGTCAATAAATACTCAGCAGTAACCGCAC A CGTACGCTGCAGGTCGAC
pRL322	rpn10::HYG	pFA6a-hphNT1 ¹	TTGGTTTATATATGACCCATATAGATATTTTGTACACTA ATCGATGAATTCGAGCTCG
pRL340	atg11::HYG	pFA6a-hphNT1 ¹	GTTGTTTCGAAAGTACTTCTTTTATTTCTTTTATACATC CGTACGCTGCAGGTCGAC
pRL341	atg11::HYG	pFA6a-hphNT1 ¹	ACATAATTAAATCTTGTCATTTGTGACAAACGTTTAGCA C ATCGATGAATTCGAGCTCG

1. Janke, C. *et al.* A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**, 947–62 (2004).
2. Goldstein, A. L. & McCusker, J. H. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**, 1541–53 (1999).
3. Kleijnen, M. F., Kirkpatrick, D. S. & Gygi, S. P. The ubiquitin-proteasome system regulates membrane fusion of yeast vacuoles. *EMBO J.* **26**, 275–87 (2007).

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Yeast Phenotype—Strains were grown to an A600 of 1 in YPD. 1 ml of cells was then harvested and washed with sterile water. Serial dilutions of the cells were made in 96-well plates, and cells were transferred to YPD plates or plates containing 0.2 µg/ml rapamycin. Plates were incubated for 24– 48 h at 30 °C.

Immunoblotting Analysis—Protein lysates were obtained by NaOH/TCA precipitation (6). In short, cell pellets were resuspended in 200 mM NaOH and incubated on ice for 10 min. Trichloroacetic acid was added to a final dilution of 5%, and the mixture was allowed to incubate on ice for 10 min. Cells were pelleted by centrifugation at 18,000 g for 2 min. Pellets were washed with ice-cold acetone and centrifuged at 18,000 g for 2 min. Next, pellets were resuspended in SDS-PAGE sample buffer and boiled at 96 °C for 5 min. Samples were centrifuged at 18,000 g for 5 min, and supernatant was collected. Samples were separated by SDS-PAGE and analyzed by immunoblotting using antibodies against GFP (Roche Applied Science, catalog no.11814460001) and Pgk1 (Invitrogen, catalog no.459250). All images were acquired using a Gbox imaging system (Syngene) with GeneSnap software. Quantifications were carried out by determining the peak volumes for the different bands using the GeneTools analysis software from Syngene. Values were corrected for loading using the Pgk1 signal. Data are displayed as value of free GFP (see under “Results” for definition) divided by the sum of the value of free and subunit associated GFP and are plotted over time. Data shown represent at least three independent biological replicates.

Fluorescence Microscopy—Live yeast cells expressing either Rpn1-GFP or β5-GFP with different mutations in these backgrounds were washed with sterile water either before or after starvation. To stain vacuoles, 0.4 µM FM 4-64 was added to logarithmically growing cells 2hrs prior to starvation. Cells were transferred to an L-lysine-coated slide, and the coverslip was sealed using VALAP (mixture of equal parts of Vaseline, lanolin, and paraffin wax). Images were acquired at room temperature on a Nikon Eclipse TE2000-S microscope at 600X magnification using a Plan Apo 60X/1.40 objective and the 490/528 nm (GFP images) and 555/617 nm (FM 4-64 images) Sedat Quad filter set (Chroma 86000v2, Bellows Falls, VT) using a CoolSNAP cf camera

(Photometrics). The images were collected using Metamorph (Molecular Devices). Images were prepared using Adobe.

Succinyl-LLVY-AMC Hydrolysis Assays—Lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM ATP, 5 mM MgCl₂, 1 mM EDTA) was added to nitrogen-starved and non-starved cell pellets equal to pellet volume. Cells were lysed by a combination of freeze-thaw and pestle grinding (at least three rounds) in a 1.5-ml microcentrifuge tube in the presence of liquid nitrogen. Lysates were spun at 18,000 g at 4 °C for 5 min, and the supernatant was collected. Protein concentrations were obtained by NanoDrop, and equal amounts were loaded in a well of a black 96-well plate (Greiner Bio-One, 655086) supplemented with buffer 2 (50 mM Tris-HCl (pH 7.5), 1 mM ATP, 5 mM MgCl₂, 1 mM EDTA) to a total volume of 50 µl. 50 µl of substrate buffer (buffer 2 supplemented with 200 µg/ml LLVY-AMC (Bachem, I-1395)) was added to the wells to start the reactions. Reactions were followed by measuring fluorescence (excitation 360 nm, 460 nm detection) using a Wallac Victor2 microplate reader (PerkinElmer Life Sciences) with plates at 30 °C. Data were normalized for input and displayed as relative fluorescent units.

Native Gels—To look at the incorporation of tagged subunits into proteasomes, native gel analyses, including in-gel LLVYAMC hydrolysis activity, were carried out as described previously (20). Cells were lysed by the liquid nitrogen method described above, and equal amounts of protein, 170 µg, were loaded onto gels. Gels were run for 3.5 h at 4 °C at 100 V and were scanned on Typhoon 9410 imager (excitation 488, 526SP filter)

Results

Vacuolar Targeting of Proteasomes upon Nitrogen Starvation—To test whether proteasomes are targeted for vacuolar degradation upon starvation, we generated strains with a gene fusion that encodes Rpn1-GFP to monitor RP as well as strains with a gene fusion that encodes Pre2-GFP (β5-GFP) to monitor CP. The GFP-encoding sequence was introduced into a genome at the endogenous locus to encode C-terminally GFP-tagged versions of the proteasome subunits Rpn1 and β5, thereby

ensuring that 100% of the subunits in the cell are GFP-tagged. Furthermore, the use of the endogenous promoter in its normal genomic environment minimizes artifacts resulting from abnormal expression levels. Both $\beta 5$ and Rpn1 are essential, and the successful integration of the GFP indicates that these GFP-tagged versions are functional and incorporated into the proteasome. We also confirmed this by using native gel analyses (Fig. 2-1A). Furthermore, these subunits have been successfully tagged at the C terminus and shown to be incorporated into the proteasome in previous studies as well (21–24). Strains with GFP-tagged proteasome subunits allow us to monitor vacuolar targeting of proteasomes by observing the accumulation of a band of 25 kDa that is recognized by the GFP antibody on immunoblots. In general, the appearance of this band (referred to as free GFP hereafter) results from rapid cleavage of the linker between GFP and the protein or the protein complex of interest (25, 26). The fold of the GFP protein resists fast vacuolar degradation resulting in the accumulation of free GFP (25, 26). We observed that both Rpn1-GFP and $\beta 5$ -GFP strains accumulate free GFP upon nitrogen starvation (Fig. 2-1B). Using this same approach, we also observed the appearance of free GFP for Rpn11-GFP, indicating these results reflect a common property of proteasomes and not a unique feature of specific proteasome subunits (Fig. 2-1, A and C).

Microscopic analyses of the Rpn1-GFP- or $\beta 5$ -GFP-expressing strains showed that, prior to starvation, the GFP signal is mainly nuclear (Fig. 2-1D, top row, and Hoechst 33342 colocalization, data not shown). This is consistent with reports that proteasomes in yeast are abundant in the nucleus (27, 28). Staining vacuolar membranes with FM 4-64 further revealed that the GFP signal was absent from vacuoles, indicating little or no proteasomes are in the vacuole. Upon nitrogen starvation, the majority of cells lost the nuclear enrichment of the GFP signal with a concurrent appearance of the GFP signal in vacuoles (Fig. 2-1, D and E). Please note that FM 4-64 staining was conducted prior to starvation and that in cells with active autophagy the FM 4-64 stain loses brightness over time. Nevertheless, staining still allowed us to unambiguously identify vacuoles. The observed vacuolar targeting is specific for nitrogen starvation, because glucose starvation did not result in accumulation of GFP on immunoblots (Fig. 2-1B). Microscopic analyses showed that

glucose starvation does result in re-localization of proteasomes (Fig. 2-1, D and E). However, vacuoles remained devoid of GFP signal under these conditions, and the signal was mostly concentrated in distinct structures that appear perinuclear. This localization is consistent with previous reports that show the translocation of proteasomes from the nucleus and cytosol into proteasome storage granules (PSGs) upon glucose starvation or upon entering into stationary phase (29, 30). Thus, proteasomes re-localize upon starvation; nitrogen starvation leads to vacuolar localization and glucose starvation to PSGs.

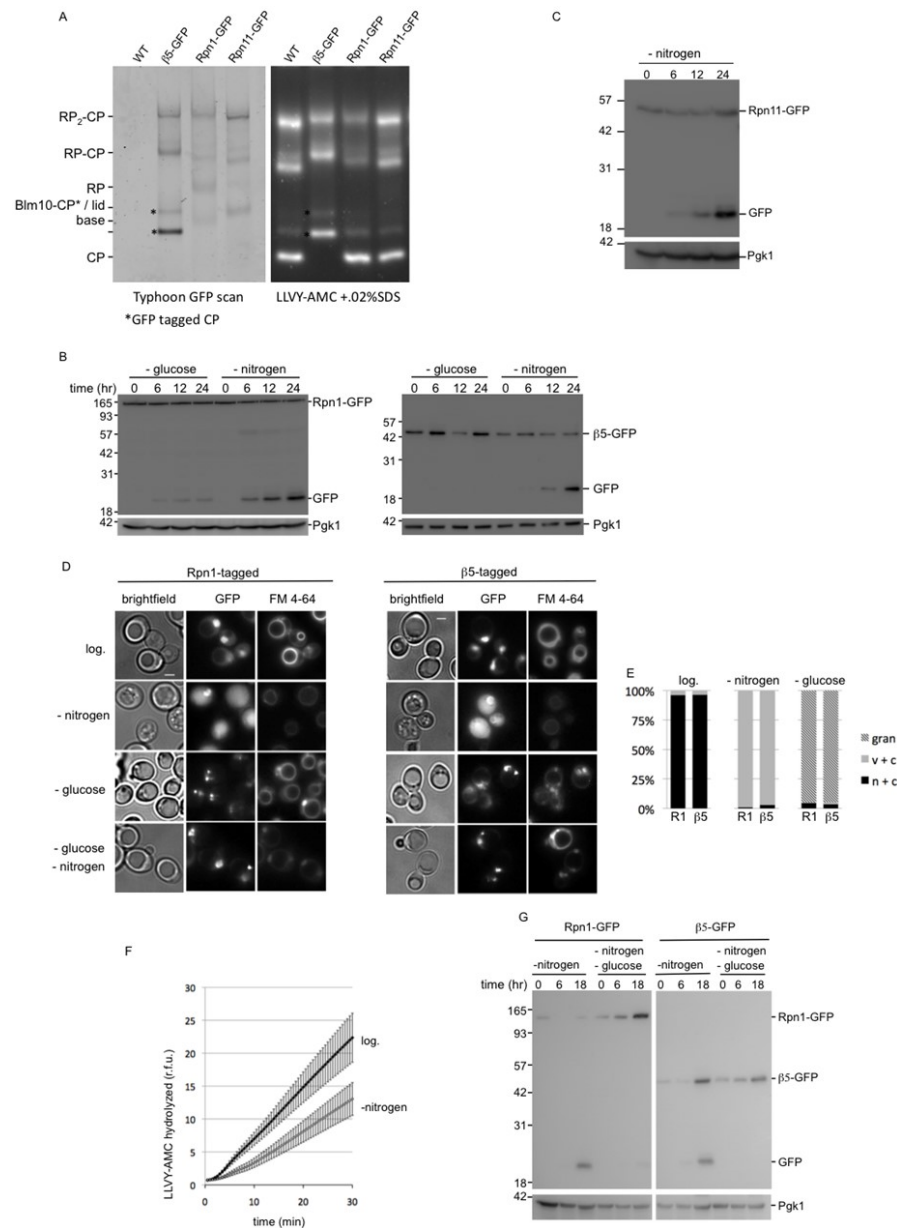
If nitrogen starvation results in vacuolar targeting of proteasomes, one would predict a reduced proteasome activity in lysates upon nitrogen starvation. We tested this by comparing peptidase activity in nitrogen-starved and non-starved cells using LLVY-AMC as substrate. Upon starvation, we observed strong reduction in LLVY-AMC peptidase activity (40–50%). For both starved and non-starved conditions, the addition of the proteasome inhibitor bortezomib (50 μ M) reduced the peptidase activity to less than 3.5% (data not shown), indicating that the activity represents proteasome peptidase activity. The reduction in peptidase activity upon starvation is consistent with previous observations and with proteasomal targeting to the vacuole (Fig. 2-1F) (10).

Next, we tested whether cells starved for both nitrogen and glucose showed vacuolar targeting of proteasomes (Fig. 2-1, D and G). Surprisingly, in the presence of both starvation conditions, proteasomes do not undergo vacuolar targeting, but appear to end up in PSGs (Fig. 2-1D). However, this process seems less efficient or somewhat delayed compared with only glucose starvation. Thus, nitrogen starvation does not always lead to vacuolar targeting of proteasomes, as it depends on the physiological state of the cells.

To confirm that the processing of GFP-tagged proteasomes upon nitrogen starvation occurs in the vacuole, we tested the dependence of this processing on acidic hydrolases using a strain deleted for *PEP4*. The deletion of *PEP4* in combination with the addition of the protease inhibitor PMSF cause a strong reduction in hydrolytic activity by vacuolar hydrolases, as many hydrolases depend on Pep4 for maturation (31). The strongly reduced levels of free GFP in these strains confirm that, indeed, the free GFP appears as a result of the activity of vacuolar acidic hydrolases (Fig. 2-2, A

and B). Microscopic analyses of strains deleted for *PEP4* showed that proteasomes are still targeted to the vacuole in these cells, and consequently, images are similar to those observed for the wild type strain after nitrogen starvation (Fig. 2-2C).

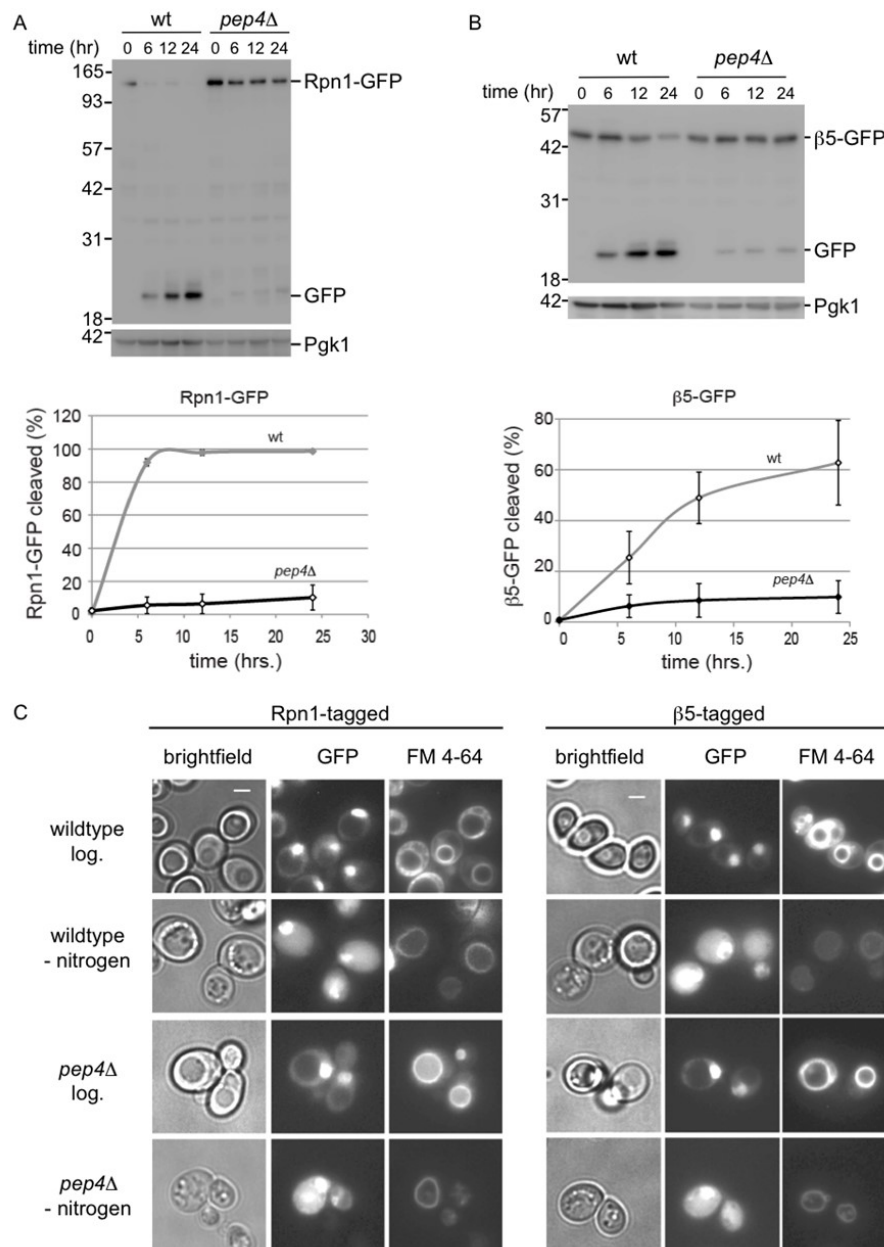
Figure 2-1. Nitrogen starvation targets proteasomes to the vacuole



(A) Strains were C-terminally tagged with GFP at the endogenous locus of the core particle subunits β 5, the regulatory particle subunit Rpn1, or the regulatory particle subunit Rpn11. Cell lysates of indicated strains were resolved on native gel and scanned to determine incorporation of GFP-tagged subunits in proteasome complexes. Next, gels were stained with proteasome fluorogenic substrate LLVY-AMC, to visualize proteasome peptidase

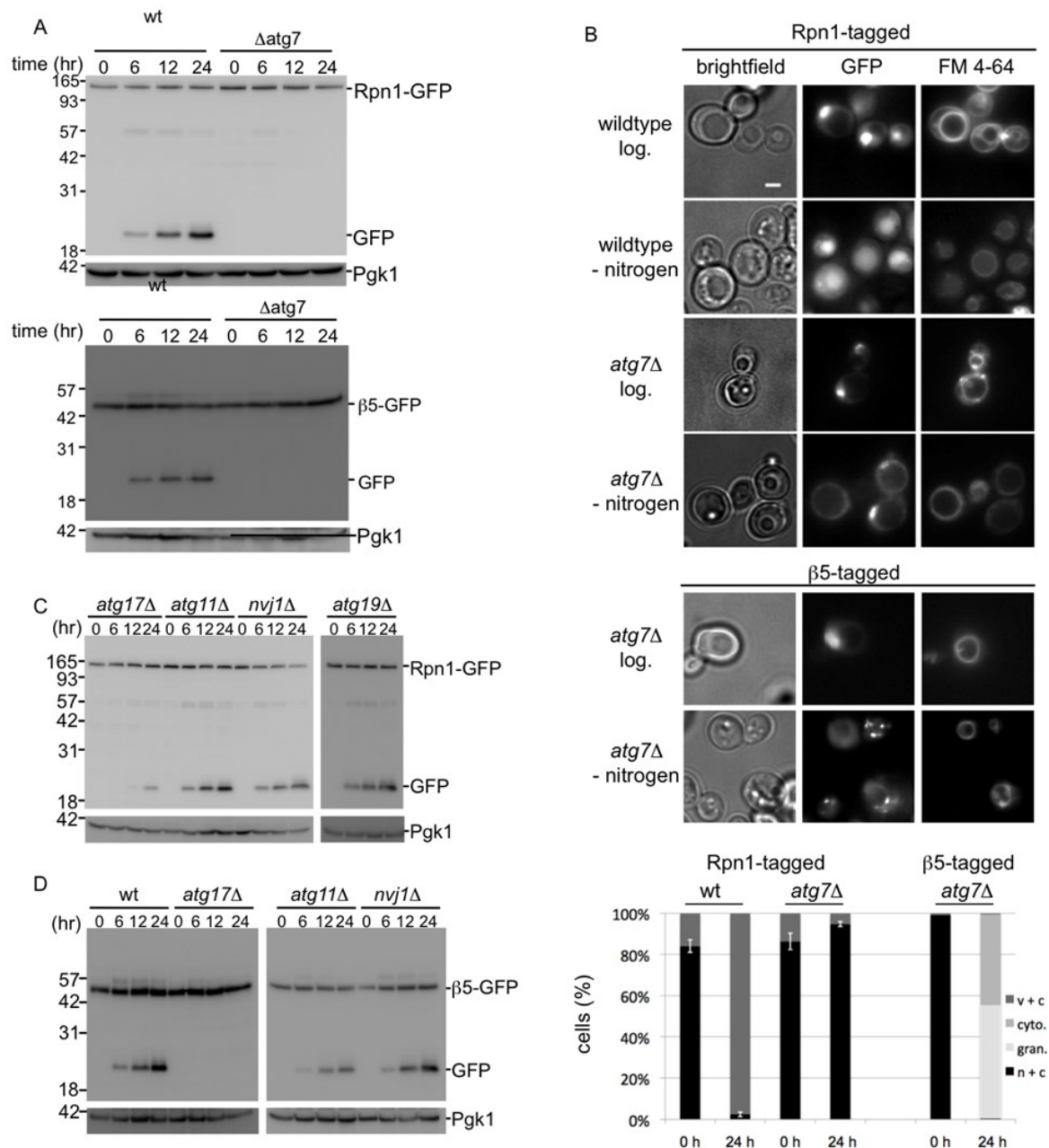
activity. * indicates CP with $\beta 5$ -GFP; GFP-tagged CP showed a migrational shift compared to untagged CP. The different subunits can be detected in assembled proteasomes and the appropriate subcomplexes (i.e $\beta 5$ in CP; Rpn1 in RP and base; Rpn11 in RP and lid). **(B)** Strains expressing Rpn1-GFP or $\beta 5$ -GFP were inoculated at an OD₆₀₀ of 0.5 and grown to log phase for 4 hrs in YPD to OD₆₀₀ 1.5, washed and transferred to minimal media lacking either glucose or nitrogen. Cells were collected and lysed at indicated time points. Samples were resolved on SDS-PAGE and immunoblotted for GFP and the loading control Pgk1. **(C)** Strain expressing Rpn11-GFP was analyzed upon nitrogen starvation as described in (B). **(D)** Strains expressing Rpn1-GFP or $\beta 5$ -GFP were analyzed by fluorescent microscopy for GFP localization prior to starvation or after incubation for 24 hours in minimal media lacking glucose or nitrogen. Cells were stained with FM 4-64 to visualize vacuoles. Representative images are shown and quantifications are shown in (E). **(E)** At least one hundred cells were used to quantify the localization as displayed in the stacked bar graph as percentage. n + c, nuclear and cytosolic staining with stronger signals in nucleus; c + v, little nuclear staining and clear vacuolar localization; gran, presence of distinct granular localization sometimes combined with weak nuclear and cytosolic staining, but no vacuolar staining. Three independent experiments showed similar amounts and localization results. **(F)** Cells were lysed before and after nitrogen starvation. Equal amounts of protein were assayed for hydrolysis of LLVY-AMC. Accumulation of fluorescent AMC over time is displayed as relative fluorescence units (r.f.u.). The average of six independent experiments are shown with SEM. Activity upon starvation is reduced to 58% and the difference was highly significant (paired student's t test $p = 0.01$, $n=6$). **(G)** Experiment as (B) only cells were starved in minimal media lacking nitrogen or nitrogen and glucose. Localization of the fluorescent signal in double starved cells (see **(D)**) resembled that of glucose starvation, showing granules and little to no vacuolar localization.

Figure 2-2. Vacuolar hydrolases are responsible for cleavage of tagged proteasome subunits



(A + B) Wild type or *pep4Δ* strains with gene fusions encoding Rpn1-GFP **(A)** or β5-GFP **(B)** were starved for nitrogen in the presence of 1 mM PMSF and lysed. Samples were analyzed as in Fig. 1C. Three independent immunoblots were quantified, corrected for input (Pgk1 amounts) and percentage cleaved (displayed on y-axis) was calculated as amount of Rpn1-GFP signal divided by the amount of Rpn1-GFP signal plus free GFP signal for each time point. **(C)** Cells were analyzed by fluorescent microscope to visualize proteasome subunits (GFP) and vacuoles (FM 4-64) prior to starvation or after incubation for 24 hours in minimal media lacking nitrogen.

Figure 2-3. Proteasomes are targeted to the vacuole by autophagy



(A) The wild type or $atg7\Delta$ strains with gene fusions encoding Rpn1-GFP or β5-GFP were starved for nitrogen and lysed. Samples were resolved on SDS-PAGE and immunoblotted for GFP and the loading control Pgk1. (B) Microscope images and quantification ($n > 100$) of cells as in (A). n + c, nuclear and cytosolic staining with stronger signals in nucleus; c + v, little nuclear staining and clear vacuolar localization; cyto, clear cytosolic staining without nuclear enrichment and no vacuolar staining; gran, presence of distinct granular localization sometimes combined with weak nuclear and cytosolic staining, but no vacuolar staining. Three independent experiments showed similar amounts and localization results. (C + D) Gene fusions encoding Rpn1-GFP (C) or β5-GFP (D) were introduced in strains deleted for indicated genes and analyzed as in (A).

Vacuolar Targeting of Proteasomes Depends on the Autophagy Pathway—Recent work in yeast suggests that glucose starvation inhibits autophagy and leads to endocytosis of substantial amounts of membrane proteins and lipids (32). Nitrogen starvation, however, is well known to induce autophagy (1). Thus, the most likely pathway for the proteasome to be targeted to the vacuole is autophagy. A common core of autophagy genes is required for all autophagy. However, there are several forms of selective autophagy, each requiring a specific subset of additional factors (1, 2). To test the involvement of autophagy genes in this process, we first deleted the *ATG7* gene from Rpn1-GFP- and β 5-GFP-tagged strains. Atg7 is crucial for macro- and microautophagy; it is a ubiquitin-activating enzyme homolog that activates Atg12 and Atg8, two polypeptides that ultimately are attached to Atg5 and phosphatidylethanolamine, respectively, in the autophagic process (1). No free GFP was detected, and the GFP remained covalently attached to Rpn1 and β 5 upon nitrogen starvation in the *atg7* cells (Fig. 2-3A). Microscopic analyses of these cells shows that, in the absence of *ATG7*, the fluorescent signal does not accumulate in the vacuole, indicating that proteasomes are not transported to the vacuole (Fig. 2-3B). Thus, the vacuolar targeting of proteasomes depends on a functional autophagy pathway.

Next, we tested the need for the macroautophagy core component Atg17 (Fig. 2-3, C and D) (33). The deletion of *ATG17* in the Rpn1-GFP background resulted in a strong reduction in the formation of free GFP after nitrogen starvation. For β 5-GFP, we were unable to detect any free GFP in this background. Thus, Atg17 plays an important role in autophagy of proteasomes. The small amount of free GFP observed in the Rpn1-GFP strain might indicate the presence of a minor Atg17- independent pathway, because we never observed any free GFP when analyzing Rpn1-GFP *atg7* strains. Atg17 is an early acting protein involved in the formation of the preautophagosomal structure (PAS), which is essential for the formation of autophagosomes upon starvation (33). Nevertheless, it has also been reported to play a role in piecemeal microautophagy (34). Piecemeal autophagy is a process in which nuclear envelope material as well as nuclear content are directly targeted to the vacuole for degradation. Considering the nuclear localization of a large portion of proteasomes under logarithmic growth (Fig. 2-1D) (27, 28), Atg17 might impact proteasome autophagy through this

pathway. The piecemeal autophagy pathway depends on the nuclear envelope receptor Nvj1 (35). However, the autophagy of neither CP nor RP was substantially reduced upon deletion of *NVJ1* (Fig. 2-3, C and D). Thus, proteasomes are not targeted for degradation through Nvj1-dependent piecemeal autophagy and are thus most likely exported out of the nucleus prior to autophagic packaging. Cytosolic protein material in yeast can be targeted to the vacuole through a specialized form of autophagy called the cytosol-to-vacuole targeting pathway (1). To test whether proteasome autophagy depends on the cytosol-to-vacuole targeting pathway, we deleted *ATG19* in a Rpn1-GFP-tagged strain, which impairs this process. Immunoblots from nitrogen-starved samples showed that proteasome autophagy still occurs at high efficiency in strains impaired in the cytosol-to-vacuole targeting pathway (Fig. 2-3C). Thus, proteasome autophagy does not depend on this yeast-specific pathway to transport proteasomes to the vacuole but requires *ATG7* and *ATG17*, genes that are conserved between yeast and humans.

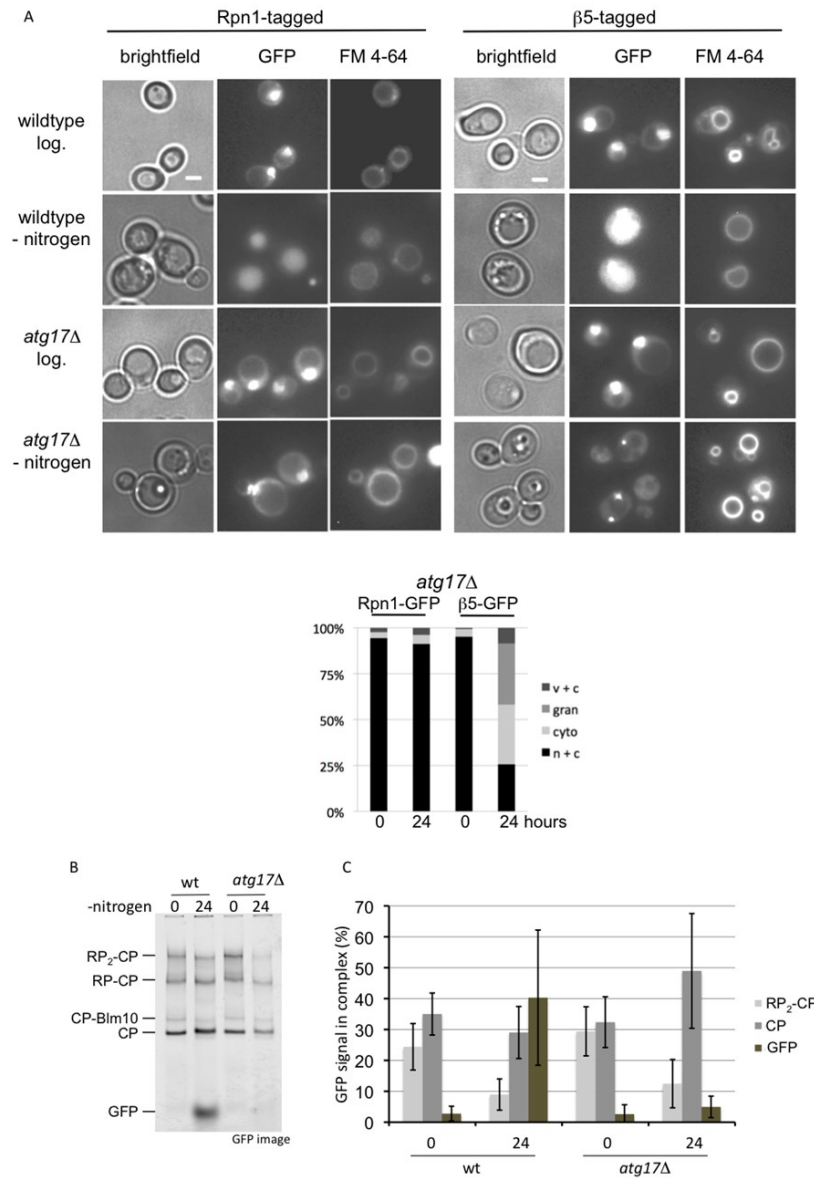
Many forms of selective autophagy depend on the adaptor protein Atg11, which interacts with various receptors (2, 36). Furthermore, Atg11 can interact with Atg17. To our surprise, *ATG11* deleted cells were still able to efficiently perform nitrogen-induced proteasome autophagy, as both Rpn1-GFP- and β 5-GFP-tagged strains showed free GFP upon nitrogen starvation in an *atg11* background (Fig. 2-3, C and D). Thus, this adaptor, which is common to many forms of selective autophagy, is not required for targeting this multi-subunit protein complex for autophagy. This might suggest that proteasome autophagy is not selective or reflects a more general difference between autophagy of organelles (many require Atg11) and protein complexes, as the autophagy of acetaldehyde dehydrogenase (and presumably ribosomes as well) also requires Atg17 but is independent of Atg11 (2, 37, 38). Autophagy of leucine aminopeptidase III (Lap3) depends on Atg17 and to a lesser extent on Atg11. However, Lap3 shows some Atg17-independent autophagy similar to Rpn1 (Fig. 3C) (37).

RP and CP Are Processed Independently for Proteaphagy— Microscopic analyses of the localization of Rpn1-GFP and β 5-GFP upon nitrogen starvation in strains deleted for *ATG7* or *ATG17* showed a remarkable difference; Rpn1-GFP remained enriched in the nucleus, although a substantial amount of β 5-GFP accumulated in the cytosol and

granules resembling PSGs (Figs. 2-3B and 2-4A). This suggests that nitrogen starvation leads to some dissociation of proteasomes into RP and CP complexes. To test this, we analyzed wild type and *atg17Δ* strains expressing β5-GFP on native gels before and after nitrogen starvation (Fig. 2-4B). As the protein content of cells under the different conditions might be different per cell, and we loaded equal amounts of proteins on the gel, we analyzed the distribution of β5-GFP among the different complexes as percentage of total β5-GFP for each sample, using the average results of three independent experiments (Fig. 2-4B). For wild type cells, nitrogen starvation results in a dramatic reduction in the percentage of assembled proteasomes (RP2-CP species went from 24 to 9%). Concurrent with a reduction in 26S proteasomes, there was a dramatic increase in free GFP (3–40%). Upon starvation of *atg17Δ* cells, the amount of assembled proteasomes also goes down (RP2-CP from 29 to 12%). However, instead of an increase in free GFP, now the levels of CP are increased (32–49%). These data show that β5-GFP is monitoring what happens to proteasomes and CP and does not show artifacts resulting from an unincorporated subunit. Furthermore, these data show that nitrogen starvation leads to a substantial amount of dissociation of proteasomes even if autophagy is blocked.

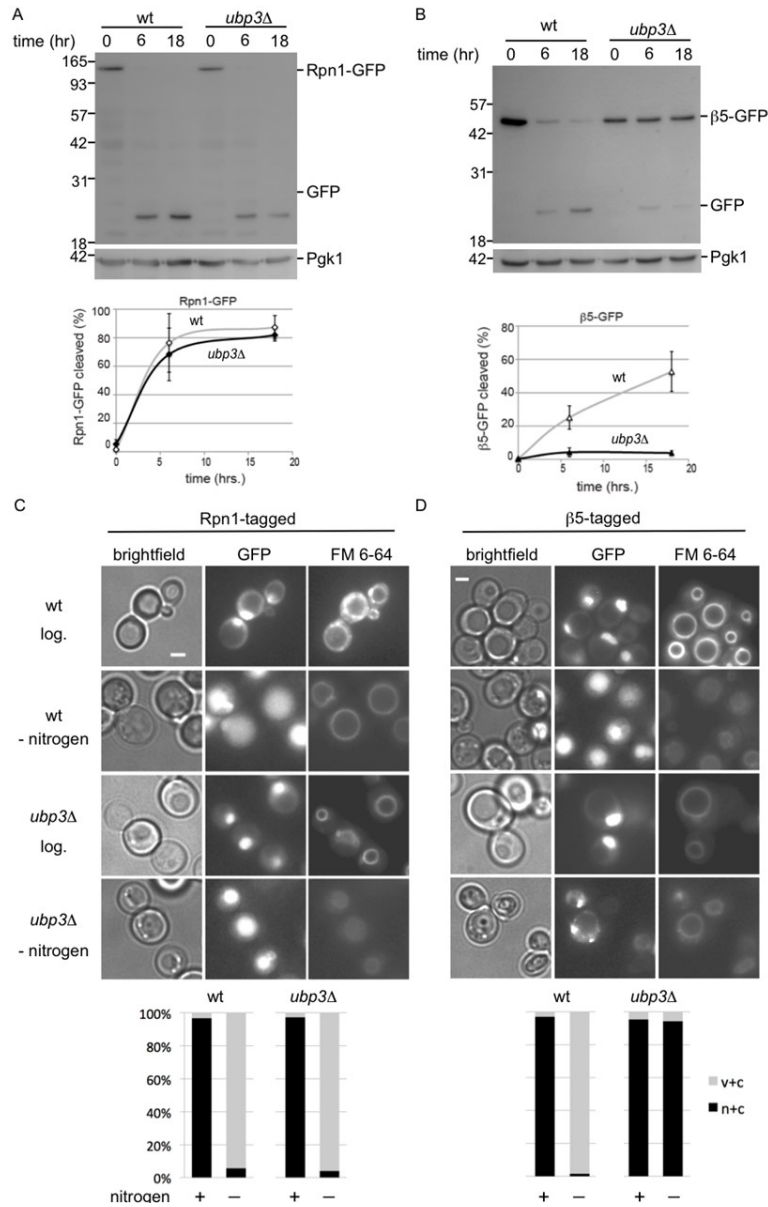
Additional data supporting the notion that CP and RP are targeted for autophagy separately came from our observation that RP and CP are affected differently by a deletion of *UBP3*. Ubp3 is a deubiquitinating enzyme important for ribophagy of the 60S ribosome but interestingly not the 40S ribosome (6). We studied how the deletion of *UBP3* impacts the vacuolar targeting of Rpn1-GFP and observed no difference in accumulated free GFP in *ubp3Δ* compared with wild type cells (Fig. 2-5A). This indicates that the deubiquitinating enzyme Ubp3 is not necessary for targeting Rpn1-GFP and presumably the complete RP subcomplex to the vacuole. However, β5-GFP showed strongly impaired vacuolar targeting, indicating the targeting of CP to the vacuole is dependent on Ubp3 (Fig. 2-5B). Consistent with the immunoblots, microscopic analyses showed the absence of vacuolar GFP signals for β5-GFP-expressing strains in the *ubp3Δ* background but not Rpn1-GFP-expressing strains (Fig. 2-5, C and D). Thus, the targeting of the proteasome core particle for autophagy depends on Ubp3.

Figure 2-4. Different processing of proteasome CP and RP autophagy



(A) Microscope images and quantification ($n > 100$) of *atg17Δ* cells. n + c, nuclear and cytosolic staining with stronger signals in nucleus; c + v, little nuclear staining and clear vacuolar localization; cyto, clear cytosolic staining with little to no vacuolar or nuclear staining; gran, presence of distinct granular localization sometimes combined with weak nuclear and cytosolic staining, but no vacuolar staining. Note that for Rpn1-GFP after nitrogen starvation in the *atg17Δ* cells the nuclear to cytosol ratio becomes somewhat smaller, but it remains enriched in the nucleus, hence no difference in our scoring. **(B)** wildtype and *atg17Δ* cells expressing $\beta 5$ -GFP were lysed and samples were resolved on native gel. Gels were scanned on Typhoon 9410 imager to identify the complexes $\beta 5$ -GFP was incorporated in. **(C)** Quantification of native gels from (B). For each condition the percentage of $\beta 5$ -GFP in a particular proteasome complex was determined. Displayed is the average of three independent experiments. Error bars indicate SEM.

Figure 2-5. Proteasomes core particles and regulatory particles depend differently on Ubp3 for vacuolar targeting



(A + B) The wild type or *ubp3Δ* strains with gene fusions encoding Rpn1-GFP **(A)** or β5-GFP **(B)** were analyzed for vacuolar targeting of RP and CP respectively upon nitrogen starvation. Samples were resolved on SDS-PAGE and immunoblotted for GFP and the loading control Pgk1. Lower panel shows quantifications as in Fig. 2. Shown are the averages from 3 to 5 experiments and error bars indicate SEM. **(C + D)** Microscope images and quantification ($n > 100$) of cells from **(A)** and **(B)** before and after 24 hours of starvation. c + v, little nuclear staining and clear vacuolar localization. n + c, nuclear and cytosolic staining with stronger signals in nucleus, except for β5-GFP *ubp3Δ* cells where there was clearly stronger signal in the cytosol. Irrespectively, all n + c all have little to no vacuolar staining.

Autophagy of proteasomes shows remarkable similarities with ribophagy; both occur upon nitrogen starvation and have one subcomplex that requires Ubp3 (CP and 60S), although the other does not (RP and 40S). To test whether the degradation of proteasomes and ribosomes is completely dependent on the same machinery, we analyzed whether proteaphagy required Ufd3 (also known as Doa1), a protein known to be necessary for ribophagy (7). Ufd3 is a cofactor of Cdc48/p97 that can bind ubiquitin. Neither CP nor RP required Ufd3 for efficient autophagy (Fig. 2-6, A and B), indicating that ribophagy and proteaphagy are distinct processes that share a subset of molecular components for targeting these complexes to the vacuole. A broader role of Ubp3 in autophagy beyond ribophagy is also suggested from our phenotypic analysis (Fig. 2-6C). Previously, it had been argued that sensitivity of *ubp3Δ* cells to nitrogen starvation and rapamycin indicates the importance of ribophagy for cell survival under starvation conditions. If true, *ufd3Δ*, which is also important for ribophagy (7), should show similar sensitivity to starvation as *ubp3Δ* cells. However, our analysis showed *ufd3Δ* resistance to rapamycin, although *ubp3Δ* was sensitive (Fig. 2-6C). Thus, the role of Ubp3 in starvation and rapamycin responses is likely to be more complex and expand beyond ribophagy. Our observations for a role of Ubp3 in proteasome autophagy is consistent with a broader role of Ubp3.

Because a majority of proteasomes are in the nucleus, it is interesting to analyze how disruption of the autophagy process affects the localization of proteasomes following nitrogen starvation. Here, as briefly mentioned earlier, we observed another difference between CP and RP. Upon disruption of the autophagy process in the Rpn1-GFP background, like the deletion of *ATG7* or *ATG17*, Rpn1-GFP remains largely nuclear upon nitrogen starvation (see *atg7* and *atg17* in Figs. 2-3B and 2-4A). For β 5-GFP-tagged strains disrupted in the autophagy process, the β 5-GFP signal does not remain largely nuclear and an accumulation of fluorescent signal outside the nucleus was observed (see *atg7*, *atg17*, and *ubp3* in Figs. 2-3B, 2-4A, and 2-5D). In the *atg7Δ* and *atg17Δ* backgrounds, a substantial amount of the β 5-GFP signal appears in granule-like structures that show similarity to PSGs (Fig. 2-4A). However, co-localization studies will have to confirm whether these are indeed PSGs. In the *ubp3Δ* background there is no nuclear enrichment upon nitrogen starvation, but granular accumulation is

observed more sporadically. In all, these data are consistent with the model that, upon nitrogen starvation, CP and RP dissociate in the nucleus and each is targeted to the lysosome using, at least partly, different pathways.

Figure 2-6. Proteaphagy does not depend on Ufd3 or Rpn10

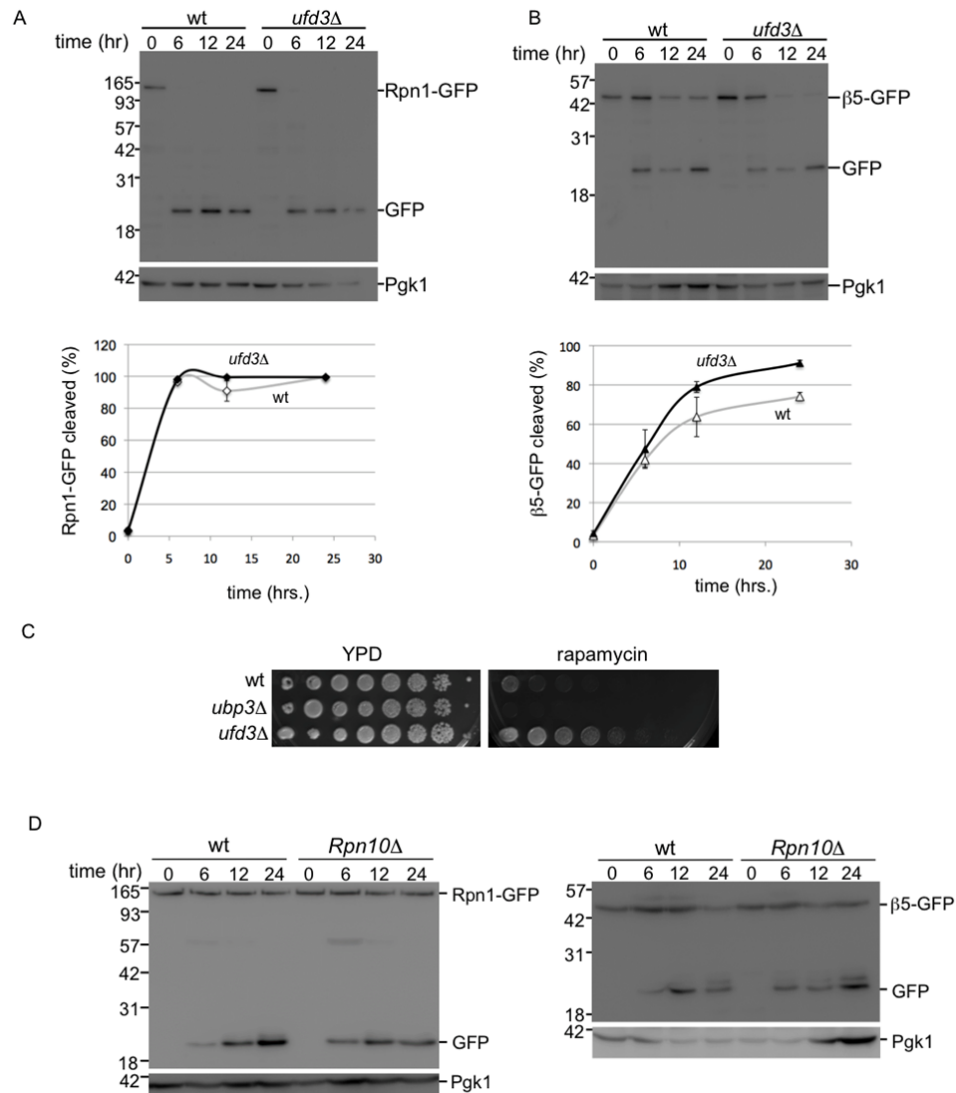
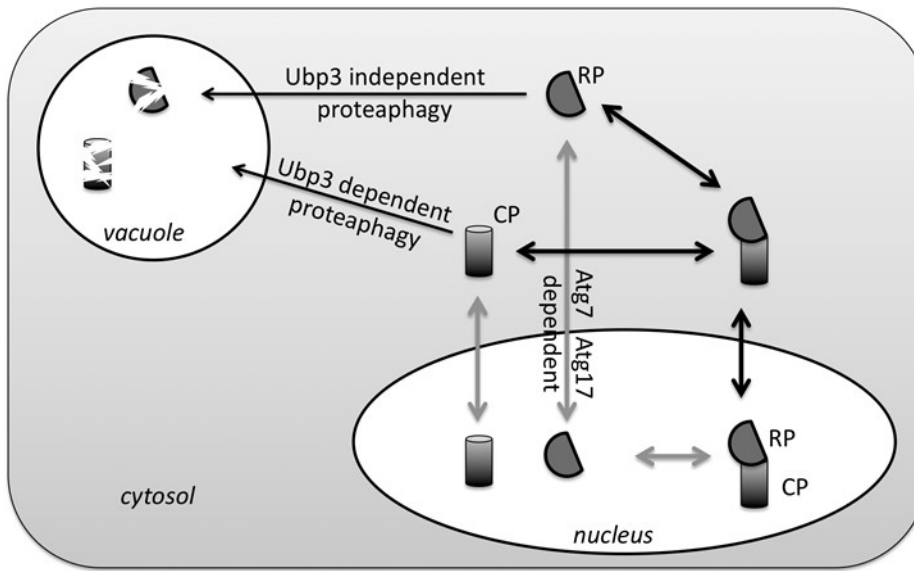


Figure 6. Proteaphagy does not depend on Ufd3 or Rpn10. (A + B) CP proteaphagy is distinct from 60S ribophagy as it does not depend on Ufd3. Strains expressing Rpn1-GFP (A) or β5-GFP (B), either in wild type or *UFD3* deletion background, were starved for nitrogen and lysed. Samples were resolved on SDS-PAGE and immunoblotted for GFP and the loading control Pgk1 (top panel). Lower panel shows quantification of three independent experiments. Error bars indicate SEM. (C) Strains were plated in four-fold serial dilution on indicated plates. (D) Nitrogen starvation induced proteaphagy does require Rpn10. Indicated strains expressing Rpn1-GFP (left) or β5-GFP (right) were analyzed as in (A).

Figure 2-7. Proposed model for proteaphagy



During logarithmic growth ~70% of yeast proteasomes are nuclear. Nitrogen starvation induces autophagy of proteasomes. However, similar to the observations for ribosomes, two subcomplexes (CP and RP) show a different requirement for components that target them to the vacuole as well as different fates upon disruption of autophagy. This indicates that CP and RP dissociate. It is currently not clear if this dissociation occurs prior to or following nuclear export resulting in two possible models of nitrogen starvation-induced proteaphagy.

Discussion

Proteasomes, like ribosomes, are very stable complexes, and little attention has been given to the process that removes them from cells. Nevertheless, cells need to be able to dispose of proteasomes and other large complexes for several reasons. First, cells need to specifically remove complexes that are not functioning optimally. Non-functional complexes can be present for various reasons. For example, functional complexes can become non-functional as a result of damage, like oxidation, or inhibitor treatment. Interestingly, in *Arabidopsis* it was recently shown that chemically inhibited proteasomes are removed through autophagy in a selective process coined proteaphagy (39). This pathway depended on the proteasome subunit Rpn10, a proteasome subunit not involved in nitrogen starvation-induced autophagy (Fig. 2-6D). Damaged ribosomes have also been proposed to be removed through autophagy (8, 40, 41). Non-functional complexes can also form during assembly. The assembly of large multi-subunit complexes is error-prone as efficient and accurate assembly

depends on a number of assembly chaperones (42– 46). Misassembled subcomplexes are sometimes recognized by traditional protein quality control machinery, as observed for mutant forms of the proteasome subunit Rpn5 (47). However, it is unlikely that traditional protein quality control mechanisms and the ubiquitin-proteasome system can always recognize and process all forms of misassembled complexes, particularly because they may be composed of properly folded subunits. Consistent with this, assembly of ribosomes and proteasomes has been shown to be under the control of complex-specific quality control mechanisms (40, 43– 45, 48). An important requirement for the removal of complexes that are not functioning optimally is the ability to specifically recognize such complexes and target only them for degradation. For proteasomes, the proteasome-associated protein Ecm29 has been shown to specifically bind to certain faulty proteasomes, and it is abundantly present on many mutants, suggesting it might play a role in targeting faulty proteasomes for autophagy (43, 44, 49–51).

A second reason to remove specific complexes from cells is to reduce the overall level of that complex. This can occur when cells encounter conditions that have reduced need for the complex. For example, ribosomes are degraded in the vacuole upon nitrogen starvation (6, 8). We show here that the same is true for proteasomes. Glucose starvation does not induce a similar response, instead proteasomes are transported to PSGs. This could be because the degradation of proteins is not the most efficient source for the energy required, hence the preference for endocytosis of glycosylated material and lipids (32). In contrast, proteins do provide a good source of amino acids and nitrogen compounds that become scarce upon nitrogen starvation. Alternatively, in evolution, glucose starvation might have been a more transient physiological state where cells could benefit from a quick recovery through an ability to retrieve functional proteasomes from the PSGs. Indeed, the addition of glucose to stationary cells has been shown to reverse the effects of glucose starvation with proteasomes going from PSG back to the nucleus (21, 29). If true, however, we would expect the nitrogen response to dominate glucose starvation. Because we observe the opposite, there are likely other factors to consider. Nevertheless, our data presented here show that proteasomes are removed through autophagy upon nitrogen starvation. Here, to reduce

proteasome levels, normally functioning proteasomes are likely targeted for degradation, and there is no need to recognize a specific subset of proteasomes.

It is unclear whether nitrogen starvation-induced proteaphagy is a form of selective autophagy, like the proteaphagy of inhibited proteasomes (39). Our data show that nuclear proteasomes are targeted for autophagy upon nitrogen starvation and show that starvation induces a dissociation of CP and RP, indicating that there must be signaling events in the cell that initiate this process. This does not necessarily imply selective autophagy, as the signaling could simply result in nuclear export, after which bulk autophagy engulfs the cytosolic proteasomes for degradation. However, we think this is unlikely for two reasons. First, the blocking of autophagy results in nuclear RP, suggesting there is a link between autophagy and nuclear export. Second, the autophagy of CP depends on Ubp3, a factor known to be dispensable for bulk autophagy. Furthermore, our data show that CP is cytosolic in a *ubp3Δ* strain and thus would be readily available for bulk autophagy. Nevertheless, without the identification of a receptor that links the proteasomes to the PAS, alternative interpretations are possible. For example, it could be that proteasomes are subject to bulk autophagy but that the presence of ubiquitinated CP subunits prevents the CP from being incorporated into autophagosomal structures. A similar mechanism might be at play for ribosomes. Here, no autophagy receptor has been identified, and the deletion of the ubiquitin ligase gene *LTN1* can rescue the ribophagy defects of *ubp3Δ* cells. The latter suggests that the removal of ubiquitin by Ubp3 is not involved in an active signaling event for selective autophagy but removes some inhibitory signal.

Even if nitrogen starvation-induced proteaphagy is not a form of selective autophagy, the process must involve several specific signaling events, because the majority of proteasomes in yeast are nuclear and not readily available for bulk autophagy. The removal of nuclear components either involves nucleophagy or export from the nucleus prior to autophagy. Based on our observation that *NVJ1*, a gene required for piecemeal autophagy, is not required for proteaphagy, we propose a model where proteasomes are exported from the nucleus prior to autophagy (Fig. 2-7). Nevertheless, our observation that RP is localized in the nucleus upon blocking of autophagy could indicate some form of nucleophagy is responsible for targeting RP for

autophagy. Interestingly, Atg39 has recently been identified as a receptor in a nucleophagy pathway that did not depend on *NVJ1* (52). However, it is unlikely this pathway is responsible for RP autophagy because Atg39 interacts with Atg11, and RP autophagy is not dependent on *ATG11* (Fig. 2-3) (52). Thus, unless other forms of nucleophagy exist or are involved (53), RP needs to be exported out of the nucleus prior to autophagic packaging. The nuclear localization of Rpn1-GFP could then indicate a process where RP export and autophagy are linked.

For a long time, proteasomes have been considered too large for transport through the nucleopore complex as they enter the nucleus as subcomplexes (22, 54, 55). This suggests that nitrogen starvation (as well as glucose starvation) requires the induction of a signal that leads to RP-CP dissociation prior to nuclear export. This is supported by our observations that a substantial fraction of CP is found in the cytosol in nitrogen-starved *atg7Δ* and *atg17Δ* cells, although RP remains largely nuclear. However, it has recently been shown that 26S proteasomes can be transported through the nuclear pore (23). Thus, proteasomes could be exported as fully assembled complexes and dissociate in the cytosol. In either case, there must be a nitrogen starvation-induced signal that leads to nuclear export and a signal that leads to dissociation of proteasomes. Phosphorylation is a likely candidate as nitrogen starvation is well known to regulate specific kinases (56). Furthermore, ubiquitination seems an obvious candidate. It has been shown to play a role in mitophagy, ribophagy, and proteasome inhibitor-induced proteaphagy among others (39, 57). In proteasome inhibitor induced proteaphagy, the ubiquitination appears to induce autophagy (39). In contrast, in nitrogen starvation-induced CP proteaphagy, the need for a deubiquitinating enzyme might suggest an opposite role for ubiquitination. However, the deubiquitination might also be important in a late stage to recycle the ubiquitin at the PAS, similar to how Doa4 removes ubiquitin at the multivesicular body (58). Until we identify the modified residue(s) or the E3 enzyme involved, we cannot determine whether an initial ubiquitination followed by a deubiquitination is important for CP autophagy or whether ubiquitination only serves as an inhibitory signal for CP autophagy.

In strains where CP autophagy is blocked, we observed CP in the cytosol upon nitrogen starvation (*ubp3Δ*, *atg7Δ*, and *atg17Δ*) and also a substantial amount in more

granule-like structures (*atg7Δ* and *atg17Δ*). It will be interesting to determine whether these structures are reminiscent of PSGs observed after glucose starvation. If so, this could indicate that the initial pathways leading to proteaphagy and PSG formation are shared with respect to proteasome dissociation and nuclear export. This is a strong possibility because there is substantial cross-talk between signaling pathways that regulate autophagy, nitrogen starvation, and glucose starvation (like target of rapamycin complex 1, protein kinase A, and AMP-activated kinase) (56, 59). Interestingly, yeast in a stationary phase, presumably starved for carbon as well as nitrogen, also show proteasomes targeted to PSGs, consistent with our observation during combined starvation (29).

Considering the numerous pathways and stress responses that rely on the degradation by proteasomes, the cellular levels of proteasomes are very important. A number of studies have shown the ability of cells to up-regulate proteasome levels under specific conditions, through Rpn4 in yeast or Nrf1 and Nrf2 in mammalian cells (60– 64). In this study, we characterized how nitrogen starvation, but not glucose starvation, induces autophagy of proteasomes in yeast. Starvation-induced proteaphagy has been observed in plants and humans as well, indicating the process is conserved throughout eukaryotic evolution (15, 39). However, our study provides the first indication that an excess of functional proteasomes, e.g. as a result of starvation, can be selectively removed through the process of regulated proteaphagy in yeast. This provides a new dynamic component to the regulation of this normally very stable multi-subunit complex.

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References-Chapter 2

1. Reggiori, F., and Klionsky, D. J. (2013) Autophagic processes in yeast: mechanism, machinery and regulation. *Genetics* 194, 341–361
2. Suzuki, K. (2013) Selective autophagy in budding yeast. *Cell Death Differ.* 20, 43–48
3. Finley, D. (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu. Rev. Biochem.* 78, 477–513
4. Inobe, T., and Matouschek, A. (2014) Paradigms of protein degradation by the proteasome. *Curr. Opin. Struct. Biol.* 24, 156–164
5. Warner, J. R. (1999) The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* 24, 437–440
6. Kraft, C., Deplazes, A., Sohrmann, M., and Peter, M. (2008) Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat. Cell Biol.* 10, 602–610
7. Ossareh-Nazari, B., Bonizec, M., Cohen, M., Dokudovskaya, S., Delalande, F., Schaeffer, C., Van Dorsselaer, A., and Dargemont, C. (2010) Cdc48 and Ufd3, new partners of the ubiquitin protease Ubp3, are required for ribophagy. *EMBO Rep.* 11, 548–554
8. Ossareh-Nazari, B., Nin˜o, C. A., Bengtson, M. H., Lee, J. W., Joazeiro, C. A., and Dargemont, C. (2014) Ubiquitylation by the Ltn1 E3 ligase protects 60S ribosomes from starvation-induced selective autophagy. *J. Cell Biol.* 204, 909–917
9. Tanaka, K., and Ichihara, A. (1989) Half-life of proteasomes (multiprotease complexes) in rat liver. *Biochem. Biophys. Res. Commun.* 159, 1309–1315
10. Athane´, A., Buisson, A., Challier, M., Beaumatin, F., Manon, S., BhatiaKisˇsˇova´, I., and Camougrand, N. (2015) Insights into the relationship between the proteasome and autophagy in human and yeast cells. *Int. J. Biochem. Cell Biol.* 64, 167–173
11. Korolchuk, V. I., Menzies, F. M., and Rubinsztein, D. C. (2010) Mechanisms of cross-talk between the ubiquitin-proteasome and autophagylysosome systems. *FEBS Lett.* 584, 1393–1398
12. Pandey, U. B., Nie, Z., Batlevi, Y., McCray, B. A., Ritson, G. P., Nedelsky, N. B., Schwartz, S. L., DiProspero, N. A., Knight, M. A., Schuldiner, O., Padmanabhan, R., Hild, M., Berry, D. L., Garza, D., Hubbert, C. C., et al. (2007) HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. *Nature* 447, 859–863

13. Suraweera, A., Mu'inch, C., Hanssum, A., and Bertolotti, A. (2012) Failure of amino acid homeostasis causes cell death following proteasome inhibition. *Mol. Cell* 48, 242–253
14. Wang, X. J., Yu, J., Wong, S. H., Cheng, A. S., Chan, F. K., Ng, S. S., Cho, C. H., Sung, J. J., and Wu, W. K. (2013) A novel crosstalk between two major protein degradation systems: regulation of proteasomal activity by autophagy. *Autophagy* 9, 1500–1508
15. Cuervo, A. M., Palmer, A., Rivett, A. J., and Knecht, E. (1995) Degradation of proteasomes by lysosomes in rat liver. *Eur. J. Biochem.* 227, 792–800
16. Vabulas, R. M., and Hartl, F. U. (2005) Protein synthesis upon acute nutrient restriction relies on proteasome function. *Science* 310, 1960–1963
17. Finley, D., Ozkaynak, E., and Varshavsky, A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell* 48, 1035–1046
18. Goldstein, A. L., and McCusker, J. H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15, 1541–1553
19. Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and Knop, M. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* 21, 947–962
20. Elsasser, S., Schmidt, M., and Finley, D. (2005) Characterization of the proteasome using native gel electrophoresis. *Methods Enzymol.* 398, 353–363
21. Weberruss, M. H., Savulescu, A. F., Jando, J., Bissinger, T., Harel, A., Glickman, M. H., and Enenkel, C. (2013) Bim10 facilitates nuclear import of proteasome core particles. *EMBO J.* 32, 2697–2707
22. Wendler, P., Lehmann, A., Janek, K., Baumgart, S., and Enenkel, C. (2004) The bipartite nuclear localization sequence of Rpn2 is required for nuclear import of proteasomal base complexes via karyopherin and proteasome functions. *J. Biol. Chem.* 279, 37751–37762
23. Pack, C. G., Yukii, H., Toh-e, A., Kudo, T., Tsuchiya, H., Kaiho, A., Sakata, E., Murata, S., Yokosawa, H., Sako, Y., Baumeister, W., Tanaka, K., and Saeki, Y. (2014) Quantitative live-cell imaging reveals spatio-temporal dynamics and cytoplasmic assembly of the 26S proteasome. *Nat. Commun.* 5, 3396
24. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast. *Nature* 425, 686–691

25. Klionsky, D. J., Abdalla, F. C., Abeliovich, H., Abraham, R. T., AcevedoArozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., AguirreGhiso, J. A., Ahn, H. J., Ait-Mohamed, O., Ait-Si-Ali, S., Akematsu, T., Akira, S., et al. (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 8, 445–544
26. Shintani, T., and Klionsky, D. J. (2004) Cargo proteins facilitate the formation of transport vesicles in the cytoplasm to vacuole targeting pathway. *J. Biol. Chem.* 279, 29889–29894
27. Enenkel, C., Lehmann, A., and Kloetzel, P. M. (1999) GFP-labelling of 26S proteasomes in living yeast: insight into proteasomal functions at the nuclear envelope/rough ER. *Mol. Biol. Rep.* 26, 131–135
28. Wilkinson, C. R., Wallace, M., Morpew, M., Perry, P., Allshire, R., Javerzat, J. P., McIntosh, J. R., and Gordon, C. (1998) Localization of the 26S proteasome during mitosis and meiosis in fission yeast. *EMBO J.* 17, 6465–6476
29. Laporte, D., Salin, B., Daignan-Fornier, B., and Sagot, I. (2008) Reversible cytoplasmic localization of the proteasome in quiescent yeast cells. *J. Cell Biol.* 181, 737–745
30. Peters, L. Z., Hazan, R., Breker, M., Schuldiner, M., and Ben-Aroya, S. (2013) Formation and dissociation of proteasome storage granules are regulated by cytosolic pH. *J. Cell Biol.* 201, 663–671
31. Parr, C. L., Keates, R. A., Bryksa, B. C., Ogawa, M., and Yada, R. Y. (2007) The structure and function of *Saccharomyces cerevisiae* proteinase A. *Yeast* 24, 467–480
32. Lang, M. J., Martinez-Marquez, J. Y., Prosser, D. C., Ganser, L. R., Buelto, D., Wendland, B., and Duncan, M. C. (2014) Glucose starvation inhibits autophagy via vacuolar hydrolysis and induces plasma membrane internalization by down-regulating recycling. *J. Biol. Chem.* 289, 16736–16747
33. Suzuki, K., Kubota, Y., Sekito, T., and Ohsumi, Y. (2007) Hierarchy of Atg proteins in pre-autophagosomal structure organization. *Genes Cells* 12, 209–218
34. Krick, R., Muehe, Y., Prick, T., Bremer, S., Schlotterhose, P., Eskelinen, E. L., Millen, J., Goldfarb, D. S., and Thumm, M. (2008) Piecemeal microautophagy of the nucleus requires the core macroautophagy genes. *Mol. Biol. Cell* 19, 4492–4505
35. Pan, X., Roberts, P., Chen, Y., Kvam, E., Shulga, N., Huang, K., Lemmon, S., and Goldfarb, D. S. (2000) Nucleus-vacuole junctions in *Saccharomyces cerevisiae* are formed through the direct interaction of Vac8p with Nvj1p. *Mol. Biol. Cell* 11, 2445–2457
36. Okamoto, K. (2014) Organellophagy: eliminating cellular building blocks via selective autophagy. *J. Cell Biol.* 205, 435–445

37. Kageyama, T., Suzuki, K., and Ohsumi, Y. (2009) Lap3 is a selective target of autophagy in yeast, *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* 378, 551–557
38. Onodera, J., and Ohsumi, Y. (2004) Ald6p is a preferred target for autophagy in yeast, *Saccharomyces cerevisiae*. *J. Biol. Chem.* 279, 16071–16076
39. Marshall, R. S., Li, F., Gemperline, D. C., Book, A. J., and Vierstra, R. D. (2015) Autophagic degradation of the 26S proteasome is mediated by the dual ATG8/ubiquitin receptor RPN10 in arabidopsis. *Mol. Cell* 58, 1053–1066
40. Hinnebusch, A. G. (2009) Active destruction of defective ribosomes by a ubiquitin ligase involved in DNA repair. *Genes Dev.* 23, 891–895
41. Cebollero, E., Reggiori, F., and Kraft, C. (2012) Reticulophagy and ribophagy: regulated degradation of protein production factories. *Int. J. Cell Biol.* 2012, 182834
42. Bedford, L., Paine, S., Sheppard, P. W., Mayer, R. J., and Roelofs, J. (2010) Assembly, structure, and function of the 26S proteasome. *Trends Cell Biol.* 20, 391–401
43. De La Mota-Peynado, A., Lee, S. Y., Pierce, B. M., Wani, P., Singh, C. R., and Roelofs, J. (2013) The proteasome-associated protein Ecm29 inhibits proteasomal ATPase activity and in vivo protein degradation by the proteasome. *J. Biol. Chem.* 288, 29467–29481
44. Lehmann, A., Niewianda, A., Jechow, K., Janek, K., and Enenkel, C. (2010) Ecm29 fulfils quality control functions in proteasome assembly. *Mol. Cell* 38, 879–888
45. Karbstein, K. (2013) Quality control mechanisms during ribosome maturation. *Trends Cell Biol.* 23, 242–250
46. Chari, A., and Fischer, U. (2010) Cellular strategies for the assembly of molecular machines. *Trends Biochem. Sci.* 35, 676–683
47. Peters, L. Z., Karmon, O., David-Kadoch, G., Hazan, R., Yu, T., Glickman, M. H., and Ben-Aroya, S. (2015) The protein quality control machinery regulates its misassembled proteasome subunits. *PLoS Genet.* 11, e1005178
48. Tomko, R. J., Jr., and Hochstrasser, M. (2013) Molecular architecture and assembly of the eukaryotic proteasome. *Annu. Rev. Biochem.* 82, 415–445
49. Lee, S. Y., De la Mota-Peynado, A., and Roelofs, J. (2011) Loss of Rpt5 protein interactions with the core particle and Nas2 protein causes the formation of faulty proteasomes that are inhibited by Ecm29 protein. *J. Biol. Chem.* 286, 36641–36651

50. Panasenko, O. O., and Collart, M. A. (2011) Not4 E3 ligase contributes to proteasome assembly and functional integrity in part through Ecm29. *Mol. Cell Biol.* 31, 1610–1623
51. Park, S., Kim, W., Tian, G., Gygi, S. P., and Finley, D. (2011) Structural defects in the regulatory particle-core particle interface of the proteasome induce a novel proteasome stress response. *J. Biol. Chem.* 286, 36652–36666
52. Mochida, K., Oikawa, Y., Kimura, Y., Kirisako, H., Hirano, H., Ohsumi, Y., and Nakatogawa, H. (2015) Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus. *Nature* 522, 359–362
53. Mijaljica, D., Prescott, M., and Devenish, R. J. (2012) A late form of nucleophagy in *Saccharomyces cerevisiae*. *PLoS ONE* 7, e40013
54. Lehmann, A., Janek, K., Braun, B., Kloetzel, P. M., and Enenkel, C. (2002) 20 S proteasomes are imported as precursor complexes into the nucleus of yeast. *J. Mol. Biol.* 317, 401–413
55. Isono, E., Nishihara, K., Saeki, Y., Yashiroda, H., Kamata, N., Ge, L., Ueda, T., Kikuchi, Y., Tanaka, K., Nakano, A., and Toh-e, A. (2007) The assembly pathway of the 19S regulatory particle of the yeast 26S proteasome. *Mol. Biol. Cell* 18, 569–580
56. Rødkaer, S. V., and Faergeman, N. J. (2014) Glucose- and nitrogen sensing and regulatory mechanisms in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 14, 683–696
57. Wani, W. Y., Boyer-Guittaut, M., Dodson, M., Chatham, J., Darley-Usmar, V., and Zhang, J. (2015) Regulation of autophagy by protein post-translational modification. *Lab. Invest.* 95, 14–25
58. Luhtala, N., and Odorizzi, G. (2004) Bro1 coordinates deubiquitination in the multivesicular body pathway by recruiting Doa4 to endosomes. *J. Cell Biol.* 166, 717–729
59. Stephan, J. S., and Herman, P. K. (2006) The regulation of autophagy in eukaryotic cells: do all roads pass through Atg1? *Autophagy* 2, 146–148
60. Mannhaupt, G., Schnall, R., Karpov, V., Vetter, I., and Feldmann, H. (1999) Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. *FEBS Lett.* 450, 27–34
61. Radhakrishnan, S. K., Lee, C. S., Young, P., Beskow, A., Chan, J. Y., and Deshaies, R. J. (2010) Transcription factor Nrf1 mediates the proteasome recovery pathway after proteasome inhibition in mammalian cells. *Mol Cell* 38, 17–28

62. Sha, Z., and Goldberg, A. L. (2014) Proteasome-mediated processing of Nrf1 is essential for coordinate induction of all proteasome subunits and p97. *Curr. Biol.* 24, 1573–1583
63. Xie, Y., and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3056–3061
64. Pickering, A. M., Linder, R. A., Zhang, H., Forman, H. J., and Davies, K. J. (2012) Nrf2-dependent induction of proteasome and Pa28 regulator are required for adaptation to oxidative stress. *J. Biol. Chem.* 287, 10021–10031
65. Kleijnen, M. F., Kirkpatrick, D. S., and Gygi, S. P. (2007) The ubiquitin proteasome system regulates membrane fusion of yeast vacuoles. *EMBO J.* 26, 275–287

Chapter 3 - Induction of Proteasome Autophagy Occurs Only Under Specific Conditions and Requires Factors Involved in Selective Autophagy

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Abstract

The proteasomal and autophagic degradation systems function to degrade proteins under specific cellular conditions. Proteasomes degrade short-lived signaling or misfolded proteins, whereas autophagic degradation clears larger substrates and is upregulated in response to various stresses. Proteasomes themselves are degraded through autophagy upon nitrogen starvation or inhibition. However, not all general autophagy-inducing stimuli result in proteasome autophagy, suggesting that proteasomes are only recognized for degradation under specific conditions. As this and other data suggests specificity in the process, we sought to determine when proteasome autophagy occurs and how this is regulated. We show that inhibition of TORC1 using rapamycin induces a bi-phasic response where proteasomes are initially upregulated and then degraded through autophagy. Other TORC1 inhibitors, namely nitrogen starvation and caffeine, failed to induce proteasome upregulation but did induce proteasome autophagy. Other general autophagy inducing conditions, including amino acid starvation and mitochondrial inhibition, failed to induce proteasome degradation, suggesting proteasome autophagy is distinct from general autophagy. Consistently, we found that Atg11, a specific autophagy receptor, plays a role in autophagy of a subset of proteasomes. The map kinase Mpk1 and upstream factors Mkk1 and Mkk2 also regulate proteasome autophagy. Finally, it is well established that starvation for carbon induces proteasome storage granules (PSGs). We have found that proteasome inhibitors prevent the formation of PSGs, likely by restricting proteasomes to the nucleus. This was not only apparent with PSG inducers, but also when proteasomes were improperly localized to other cytosolic granules

Introduction

There are two major pathways for eukaryotic cells to recycle proteins, the ubiquitin proteasome system (UPS) and autophagy. Material can be channeled into the UPS, where an enzyme cascade recognizes and labels substrates for degradation by post-translational modification of lysine residues with ubiquitin or ubiquitin chains. These marked substrates are recognized, unfolded and digested into short peptides by proteasomes. The peptides are further processed by peptidases into amino acids and become intermediates for various metabolic processes (1, 2). In the second pathway, substrates are recycled into amino acids by acid hydrolyses in the lysosome (or vacuoles in yeast and plants). The lysosome receives substrates from endocytosis or from various intracellular locations through autophagy. The targeting of substrates to autophagic vesicles is, like in the UPS, often initiated by ubiquitination of target proteins. Ubiquitinated targets are recognized by autophagy adapters, such as p62 (in mammals) or Atg11 (in yeast). These adapters contain ubiquitin interacting motifs (UIM's) that facilitate substrate interactions, as well as Atg8 interacting motifs (AIM's) which bind Atg8 found on expanding pre-autophagosomes (known as phagophores) (3, 4). Double membrane autophagosomes develop upon closure of phagophores and capture substrates. Once autophagosomes fuse with lysosomes/vacuoles, the inner membrane and phagosome contents are exposed to hydrolases and degraded. Amino acids resulting from this process are exported out of the vacuole via transporters, and replenish the cytosolic amino acid pool (5).

The ability of both the UPS and lysosomal degradation to contribute to the free amino acid pool in cells becomes particularly important during physiological conditions that reduce free amino acids. For example, nitrogen starvation halts de novo synthesis of amino acids, and autophagy becomes essential (6, 7). Both autophagy and the UPS replenish the amino acid pool and utilize ubiquitination as a degradation signal, suggesting there is some level of redundancy. The upregulation of one pathway can to some extent compensate for the impairment of the other (8–10). 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 (11, 12). Further, an increase in proteasome activity was reported in yeast and mammalian cells following deletion of the essential autophagy gene *ATG5*, or chemical inhibition of autophagy (13). Similarly, proteasome activity was shown to be significantly increased in an *ATG32* knock out but not wild type cells upon treatment with antimycin A, a drug that targets complex III of the mitochondrial respiratory chain and induces non-selective autophagy (13, 14). Here, proteasomes seem to

compensate for the inability of *atg32Δ* cells to induce non-selective autophagy upon antimycin A treatment (13).

Any compensation between autophagy and the UPS is facilitated by both utilizing the conjugation of ubiquitin to substrates and the subsequent recognition by ubiquitin binding proteins as part of their mechanism of action (10). This is exemplified by the mammalian protein p62/SQSTM1, which can bind ubiquitinated substrates and deliver them to either the proteasome or autophagic membranes depending on the cellular condition (15). Nevertheless, some functions are clearly unique to each pathway. Proteasomes are, in large, responsible for the degradation of short-lived proteins. For examples, I κ B during NF κ B signaling and cyclins during cell cycle progression are two such proteasome substrates (16, 17). Autophagy, on the other hand, can dispose of aggregated proteins or defective (parts of) organelles directly through the extension of membrane structures that vary in number and size. The formation of these de novo cytoplasmic vesicles is tightly regulated and requires the concerted actions of various autophagy related genes (ATGs) (18). The process is induced by a number of cellular stresses such as nutrient starvation, mitochondrial stress, or viral and bacterial infections (5, 19, 20). Induced autophagy appears to capture cytosolic material non-specifically, while other types of autophagy, for example mitophagy, pexophagy, and ER-phagy are selective and recognize specific cargo. These selective pathways require unique receptors such as Atg32, Pex14, and Atg39/40 that ensure specific and efficient engulfment of particular substrates into autophagosomes (21–24). Besides organelles or parts of organelles, selective degradation has also been observed for ribosomes, a process named ribophagy. The deubiquitinating complex of Ubp3/Bre5 as well as Cdc48 and a ubiquitin binding co-factor Ufd3 have been shown to be required for ribophagy in yeast (23, 25). These factors are not required for general autophagy, suggesting ribophagy is selectively coordinated through ubiquitination events. More recently, NUFIP1 was identified as a selective ribophagy receptor in mammalian cells (26).

It has recently emerged that proteasomes themselves can be targeted to the autophagic machinery under certain physiological conditions or upon treatment with proteasome inhibitor (27–30). This process is conserved throughout evolution as it was observed in human cells, yeast, and *Arabidopsis*. Here, as opposed to bulk cargo, proteasomes appear to be selectively targeted. For example, upon amino acid starvation of mammalian cells, proteasomes are recognized by p62 and targeted to autophagosomes (30). Inhibited *Arabidopsis* proteasomes bind Atg8 via ubiquitinated Rpn10 for efficient degradation, and several factors that are dispensable for bulk autophagy are required for proteasome autophagy upon nitrogen starvation in yeast (27, 28, 31). Thus, proteasome localization and targeting for degradation seem to be

tightly regulated, suggesting the involvement of specific signaling pathways that are linked to cellular metabolic state.

The target of rapamycin complex 1 (TORC1) is a master regulator controlling cell growth and metabolic activity-based signals e.g. derived from the cells nutrient state. Under nutrient-rich conditions, TORC1 is active and general autophagy is inhibited through the phosphorylation and inactivation of Atg13 and Atg1/Ulk1 (32–34). Interestingly, treatment of cells with the TORC1 inhibitor rapamycin has been shown to increase the transcript levels of proteasomal genes in yeast, as well as proteasome levels and activity in yeast and mammalian cells (32, 35, 36). Further, an increase in K48-linked ubiquitinated substrates was reported along with an increase in proteasomal proteolysis in mammalian cells treated with mTOR inhibitors. Apparently, the capacity of protein degradation by the proteasome becomes important under conditions where TORC1 is not active. The notion that inhibition of TORC1 results in upregulation of proteasomes is surprising, because proteasomes undergo autophagic degradation in yeast, plants, and mammalian cells upon nitrogen starvation, another condition well-known to cause TORC1 inhibition. (27, 30, 31). To better understand the response of proteasomes to autophagic stimuli, we characterized the regulation of proteasome autophagy using different chemical treatments and physiological conditions known to induce autophagy. Our data indicate a biphasic response upon TORC1 inhibition by rapamycin, where an initial increase in proteasome abundance and activity lasted for approximately 4hrs. After this, proteasomes were targeted for vacuolar degradation. However, nitrogen starvation, where TORC1 is also inhibited, did not give a similar response. Under various other stimuli that are known to inhibit TORC1 and induce autophagy, we observed little to no induction of proteasome autophagy. This, together with several additional genes we identified to be required for proteasome autophagy, further strengthens the model that proteasome autophagy does not always coincide with general autophagy.

Materials and Methods

Yeast strains – All strains used in this study are reported in supplementary table 1. Our background strains are the W303 derived SUB61(Mat α) and Sub62(MatA);(*lys2-801 leu2-3, 2-112 ura3-52 his3- Δ 200 trp1-1*) that arose from a dissection of DF5 (37). Standard PCR based procedures (primers and plasmids presented in supplementary table 2) were used to delete specific genes from the genome or introduce sequence at the endogenous locus that resulted in the expression of C-terminal fusions of GFP or mCherry (38–40). GFP-atg8 expressing strains

were generated by transformation with BS-Ura3-GFP-Atg8, a gift from Zhiping Xie (Addgene plasmid #69194). To express GFP from a GPD promotor, or GFP with nuclear localization or exclusion signals, plasmids pJR763, pJR792 and pJR796 were digested with Sac1/Sal1, transformed into yeast and selected for crossover at the *URA3-TIM9* region by selection for growth on histidine lacking plates.

Yeast Growth Conditions – Overnight cultures of yeast were diluted to an OD₆₀₀ of 0.5 and grown in YPD medium to an OD₆₀₀ 1.5 (approximately 4 hrs). Cultures were then treated with drugs for indicated time periods and harvested by centrifugation. Rapamycin was used at 200 nM final concentration, caffeine at 10 mM, tunicamycin at 6 μ M, and the proteasome inhibitor PS341 (a.k.a. bortezomib) at 100 μ M. To induce starvation, cultures growing logarithmically in YPD (dextrose) or YPR (raffinose) were centrifuged, washed with the respective starvation medium, re-inoculated at an OD₆₀₀ of 1.5, and incubated at 30 °C with constant shaking.

Protein lysates and electrophoresis – For native gel analyses, 50 ODs of cells were pelleted, washed in ddH₂O, and resuspended in 50 μ 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 Diagnostics (41). After cryogrinding, lysates were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM ATP, 5 mM MgCl₂, 1 mM EDTA). For native gel analyses, equal volumes of lysates were loaded on gel and separated by electrophoresis (90 V, 2.5 to 3 hrs., 4 °C). Gels were scanned on Typhoon 9410 or Trio imager (excitation at 488 nm and a 526SP emission filter). Gels were then incubated with suc-LLVY-AMC and imaged using a Syngene G-Box (UV excitation, 440 nm band-pass emission filter) to visualize peptidase activity of 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, 60 mM DTT, 345 mM Tris-HCl [ph. 6.8], 0.005% bromophenol blue). For experiments not involving native analysis, 2 OD's of cells were collected at indicated timepoint and stored at -80 °C. Lysis was completed using previously establish methods (42). Following electrophoreses, samples were transferred to PVDF membranes and immuno-blotted using antibodies against indicated proteins or tags followed by the appropriate horseradish-peroxidase conjugated secondary antibodies. Antibodies used were anti-GFP (1:500; Roche, #11814460001) and anti-Pgk1 (1:5000; Invitrogen, #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 were fluorescently tagged (Rpn1-GFP, α 1-GFP, α 6-GFP, or β 5-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 μ L of PBS, and 3 μ L mounted on 1% soft agar slides as described by E. Muller (43)(<https://www.youtube.com/watch?v=ZrZVbFg9NE8>). All imaging by fluorescence microscopy was done within 10 mins following wash to avoid the effects of prolonged incubation on slides. We have observed that proteasome granules can be lost over time if cells are left on slides under these conditions for extended time periods. Routinely, yeast vacuoles were assigned based on phase contrast images. However, we regularly stained vacuolar membranes with the dye FM464 (Invitrogen; microscope settings: excitation filter 555/28 nm, emission filter of 617/73 nm) or stained the vacuolar lumen using the dye CMAC-Arg (Invitrogen; microscope settings: excitation filter 350/50 nm, emission filter of 457/50 nm). To confirm nuclear localization, DNA was stained using Hoechst (Invitrogen; microscope settings: excitation filter 350/50 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 R3tm camera (QImaging). Images were collected using Metamorph software (Molecular Devices) and analyzed using ImageJ.

Table 3-1 Strain List

Strain	Genotype (<i>lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1</i>)	Figure	Source
SUB61	MAT α	S2	(1)
SUB62	MATA		(1)
sJR288	MATA <i>rpn4::G418</i>	S2	
sJR786	MAT α <i>atg7::CloNat rpn1::RPN1-GFP (HIS3)</i>	1a,b 2f	(3)
sJR858	MAT α <i>pre2::PRE2-GFP (HIS3)</i>	3g S2	(3)
sJR861	MAT α <i>rpn1::RPN1-GFP (HIS3)</i>	1a,b 2c,f 3a,c,d,h,i,j 4b 5b,c,d,e,f S3 S5	(3)
sJR880	MAT α <i>pre2::PRE2-GFP (HIS3) ubp3::HYG</i>	3g	(3)
sJR882	MAT α <i>rpn1::RPN1-GFP (HIS3) atg17::HYG</i>	3d	(3)
sJR900	MAT α <i>rpn1::RPN1-GFP (HIS3) atg11::G418</i>	3d, 4b	(3)
sJR923	MAT α <i>rpn1::RPN1-GFP (HIS3) atg39::HYG</i>	2g	(4)
sJR925	MAT α <i>pre2::PRE2-GFP (HIS3) atg7::CloNat</i>	3e,f	(3)
sJR992	MAT α <i>GFP-atg8 (from pJR683) ura</i>	3a,c 4a,c 5a	(4)
sJR1035	SUB61 with Pgp stable GFP (HIS) (pJR763 (Sac1/Sal1))	3c 4a,c 5a S4 S5	(4)
sJR1073	SUB61 with GFP-2xNLS (HIS) (pJR792 (Sac1/Sal1))	3b	(4)
sJR1074	SUB61 with NES-GFP (HIS) (pJR796 (Sac1/Sal1))	3b	(4)
sJR1084	MAT α <i>scl1::SCL1-GFP (HIS3)</i>	1a,b 2a,b,d,e 3c,g,i, 4a,c 5a,c,f S1 S2 S4	(4)
sJR1085	MAT α <i>pre5::PRE5-GFP (HIS3)</i>	3g S2	(4)
sJR1086	MATA <i>atg7::CloNat scl1::SCL1-GFP (HIS3)</i>	1a,b 3e S1	(4)
sJR1087	MATA <i>atg7::CloNat pre5::PRE5-GFP (HIS3)</i>	3e	(4)
sJR1088	MAT α <i>pre2::SCL1-GFP (HIS3) ubp3::HYG</i>	3g	(4)
sJR1089	MAT α <i>pre2::PRE5-GFP (HIS3) ubp3::HYG</i>	3g	(4)
sJR1091	MAT α <i>rpn1::RPN1-GFP (HIS3) atg11::G418 atg17::Ura</i>	3d	(4)
sJR1151	MAT α <i>rpn1::RPN1-GFP (HIS3) mpk1::G418</i>	3h	(4)
sJR1155	MAT α <i>pre6::PRE6-GFP (HIS3)</i>	S2	(4)
sJR1160	MAT α <i>pre2::PRE2-GFP (HIS3) atg7::CloNat snx4::G418</i>	3f	(4)
sJR1197	MATA <i>rpn4::G418 pre2::PRE2-GFP (HIS3)</i>	S2	(4)
sJR1216	MAT α <i>scl1::SCL1-GFP (HIS3) mpk1::HYG</i>	3h	(4)
sJR1243	MAT α <i>rpn1::RPN1-GFP (HIS3) atg40::G418</i>	2g	(4)
sJR1244	MAT α <i>rpn1::RPN1-GFP (HIS3) atg39::HYG atg40::G418</i>	2g	(4)
sJR1326	MATA <i>rpn4::G418 scl1::SCL1-GFP (HIS3)</i>	S2	(4)
sJR1360	MATA <i>pdr5::G418 rpn1::RPN1-GFP (HIS3)</i>	5b,g	(4)
sJR1361	MATA <i>pdr5::G418 scl1::SCL1-GFP (HIS3)</i>	5g	(4)
sJR1387	MAT α <i>rpn1::RPN1-GFP (HIS3) mkk2::HYG</i>	3j	(4)
sJR1390	MAT α <i>rpn1::RPN1-GFP (HIS3) mkk1::G418</i>	3j	(4)
sJR1392	MAT α <i>rpn1::RPN1-GFP (HIS3) mkk2::HYG mkk1::G418</i>	3ij	(4)
sJR1393	MAT α <i>scl1::SCL1-GFP (HIS3) mkk2::HYG mkk1::G418</i>	3i	(4)
sJR1422	MAT α <i>pre2::PRE2-GFP (HIS3) atg7::CloNat rpn1::RPN1-mCherry (G418)</i>	3e	(4)
sJR1423	MATA <i>atg7::CloNat scl1::SCL1-GFP (HIS3) rpn1::RPN1-mCherry (G418)</i>	3e	(4)
sJR1424	MATA <i>atg7::CloNat pre5::PRE5-GFP (HIS3) rpn1::RPN1-mCherry (G418)</i>	3e	(4)
a) All strains have the DF5 background genotype (<i>lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1</i>) 1. Finley, D., Ozkaynak, E., and Varshavsky, A. (1987) Cell 48, 1035-1046 2. Leggett, D. S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R. T., Walz, T., Ploegh, H., and Finley, D. (2002) Mol Cell 10, 495-507 3. Waite, K.A., De La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2015) JBC M115.699124 4. This study			

Table 3-2 Primer List

Primer	Genotype	Template	Sequence (5' to 3')
rvrs/rpn1	<i>rpn1::rpn1-mCherry (G418)</i>	pBS34 ³	TTTGAATTTTCTATTCTGGTTGATATTGCCAAAAG CTATTCA GTTTAATCGATGAATTCGAGCTCG
pRL36	<i>scl1::SCL1-GFP (HIS3)</i>	pYM28 ²	GTGTTGACGCGTGTGATTTTCACATTATGTTGTGCGAGGA AGATCGATGAATTCGAGCTCG
pRL236	<i>atg7::CloNAT</i>	pAG25(38)	TTCATTATATTTCAACAAATATAAGATAATCAAGAAT AAACGTACGCTGCAGGTCGACG
pRL237	<i>atg7::CloNAT</i>	pAG25(38)	CGGAAAGTGGCACCACAATATGTACCAATGCTATTAT ATGCAATCGATGAATTCGAGCTCG
pRL281	<i>ubp3::HYG</i>	pFA6a- hphNT1 ²	CCATCATCCAGGTACCGCTTTCCTTCCATCATCATTA AACGTACGCTGCAGGTCGAC
pRL282	<i>ubp3::HYG</i>	pFA6a- hphNT1 ²	TTATTTTATGATTTTGTCTATAATACCAACCCCGG TCATCGATGAATTCGAGCTCG
pRL293	<i>atg17::HYG</i>	pFA6a- hphNT1 ²	ATTCGATACTGCGAGGATATTCAACGTATTTAACA CCTCGTACGCTGCAGGTCGAC
pRL294	<i>atg17::HYG</i>	pFA6a- hphNT1 ²	GATACAATTATTGAATCTTTGTACCGTATCCTTTTT TCCTATCGATGAATTCGAGCTCG
pRL340	<i>atg11::HYG</i>	pFA6a- hphNT1 ²	GTTGTTGCGAAAGTACTTCTTTATTTCTTTTATAC ATCCGTACGCTGCAGGTCGAC
pRL341	<i>atg11::HYG</i>	pFA6a- hphNT1 ²	ACATAATTAATCTTGTCAATTTGTGACAAACGTTT AGCACATCGATGAATTCGAGCTCG
pRL371	<i>atg39::HYG</i>	pFA6a- hphNT1 ²	TAATAGAGACTAGTAAACAGTCGAGTTGTCGGACC TAAACGTACGCTGCAGGTCGAC
pRL372	<i>atg39::HYG</i>	pFA6a- hphNT1 ²	CTTTGTTAATTTTCACTTCTCATGCTGGGTTTGGAT GATATCGATGAATTCGAGCTCG
pRL401	<i>rpn1::rpn1-mCherry (G418)</i>	pBS34 ³	AGTAATTTTAAAGAAGAACCCTGACTATCGTGAAGA GGAGGGTCGACGGATCCCCGGG
pRL451	<i>mpk1::G418</i>	pFA6a-kanMX6	GTAGAAATAATTGAAGGCGTGATATAACAATTCTGG GAGCGTACGCTGCAGGTCGAC
pRL452	<i>mpk1::G418</i>	pFA6a-kanMX6	GCTTACATCTATGGTGATTCTATACTCCCCGTTAC TTATAGATCGATGAATTCGAGCTCG
pRL589	<i>scl1::SCL1-GFP (HIS3)</i>	pYM28 ²	TGCTGAGAACATCGAAGAAAGGCTAGTAGCAATTGCTGA ACAAGATCGTACGCTGCAGGTCGAC
pRL590	<i>pre5::PRE5-GFP (HIS3)</i>	pYM28 ²	CTTTTACAATTTACGATGGAGAAGCTGTTGCTAAATACATC CGTACGCTGCAGGTCGAC
pRL591	<i>pre5::PRE5-GFP (HIS3)</i>	pYM28 ²	CTTTGACAATTGCTTAGTGAAACGATACTTTGTGTCCAG ATCGATGAATTCGAGCTCG
pRL596	<i>snx4::G418</i>	pFA6a-kanMX6	ATTTACGGTATACCACAATACTGCTCTTTTGTGAG GATCGTACGCTGCAGGTCGACG
pRL597	<i>snx4::G418</i>	pFA6a-kanMX6	AGGTATTATCAGTAGTAATGGGAAACATTAGAG CACCAATCGATGAATTCGAGCTCG
pRL602	<i>pre6::PRE6-GFP (HIS3)</i>	pYM28 ²	AGAGCAGCAAGAGCAGGACAAAAAGAAAAATCTAACCAT CGTACGCTGCAGGTCGAC
pRL603	<i>pre6::PRE6-GFP (HIS3)</i>	pYM28 ²	TATTTTATATAGGTTTTATGCCCAATATATATCGCCGTTTATC GATGAATTCGAGCTCG
pRL690	<i>atg40::G418</i>	pFA6a-kanMX6	ACGTTCTTTCTGCTGTCTTCACTCCACCATAGAAAA CTACGTACGCTGCAGGTCGACG
pRL691	<i>atg40::G418</i>	pFA6a-kanMX6	CTTCATAGACTACCATTATGGTAAAAATGGAAAACT ATTCATCGATGAATTCGAGCTCG
pRL792	<i>mkk2::HYG</i>	pFA6a- hphNT1 ²	GTTATCATATCTACAAAATACCAATTATATACACAGG ATACGTACGCTGCAGGTCGACG
pRL793	<i>mkk2::HYG</i>	pFA6a- hphNT1 ²	AAAAAGTCAGTTCTGGTTACGAGAGGAAAAATGTTGG AAGTATCGATGAATTCGAGCTCG
pRL796	<i>mkk1::G418</i>	pFA6a-kanMX6	CCAAATTAACCTATCCTTCCATTGCACAATTGCCCA GTCGTACGCTGCAGGTCGACG
pRL797	<i>mkk1::G418</i>	pFA6a-kanMX6	AAAATAAACTTAATCATGTTGCCAAAAAATGCTTAT TGAATCGATGAATTCGAGCTCG
1.	Goldstein, A. L. & McCusker, J. H. Three new dominant drug resistance cassettes for gene disruption in <i>Saccharomyces cerevisiae</i> . <i>Yeast</i> 15, 1541–53 (1999).		
2.	Janke, C. et al. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. <i>Yeast</i> 21, 947–62 (2004).		
3.	Hailey DW, Davis TN, Muller EG. Fluorescence resonance energy transfer using color variants of green fluorescent protein. <i>Methods Enzymol.</i> 351:34-49 (2002).		

Results

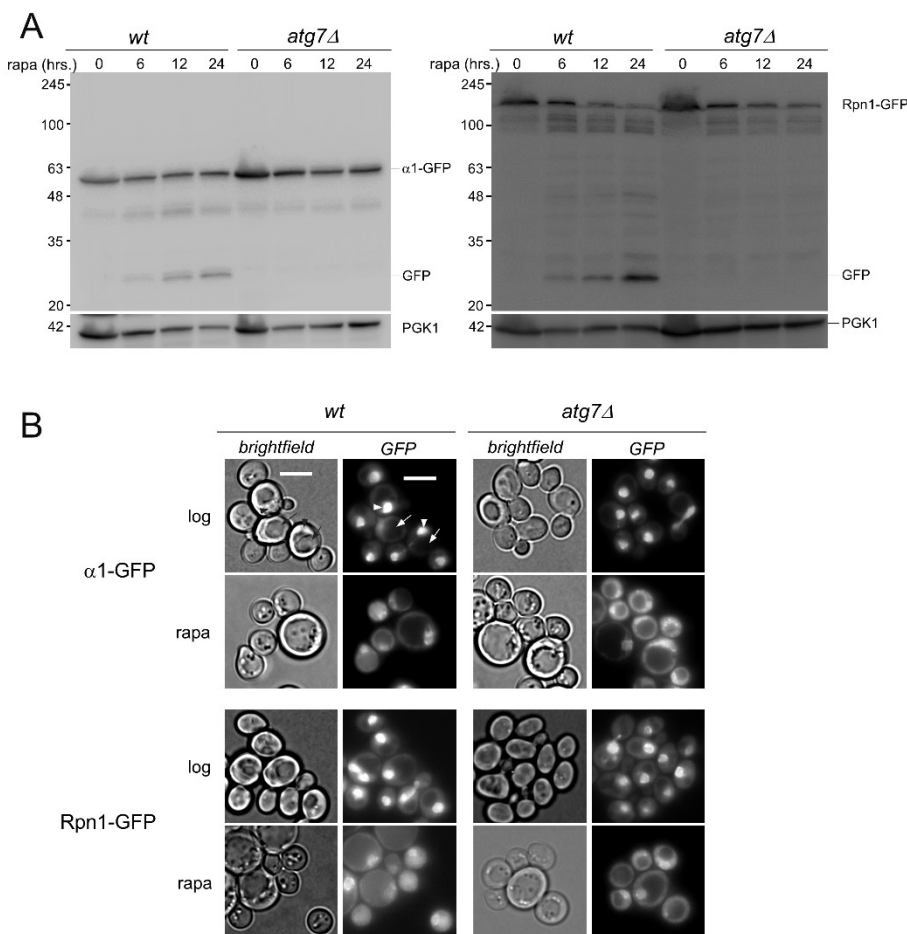
Rapamycin Induces Proteasome Autophagy—Upon nitrogen starvation, proteasomes are targeted for vacuolar degradation via an autophagy-dependent pathway. The extent of this degradation appears to vary (27, 28, 31). To elucidate some of the signaling pathways involved, we sought to determine the role of TORC1 inhibition in this process. We monitored targeting of proteasomes to the vacuole using different strains with GFP-tagged proteasome subunits. Specific subunits were tagged at their endogenous locus to ensure expression at physiological levels and the absence of untagged copies. We have previously shown that Rpn1-GFP is incorporated into proteasomes using native gel analyses (27). Here, we show α 1-GFP is also incorporated into functional 20S proteasomes (see supplementary figure 1). While β 5-GFP has been used previously by us and others (27, 44, 45), we observed a tag-induced phenotype for β 5-GFP, but not α 1, when combined with a deletion of the transcription factor *RPN4* (see supplementary figure 2). As Rpn4 is responsible for upregulation of proteasome subunits under conditions of reduced proteasome activity, any compromised proteasome function resulting from manipulating subunits is often masked by Rpn4-dependent transcriptional upregulation of proteasome genes (46–48). Therefore, to reduce the risk of tag-induced artifacts we used an α 1-GFP core particle tag in place of β 5-GFP.

To monitor vacuolar targeting, we analyzed strains by fluorescence microscopy. Here, vacuolar localization was regularly confirmed by co-staining of vacuoles using the vacuolar dye FM464 (to stain membrane) or CMAC derivatives (to stain lumen) (see supplementary figure 3). In parallel, we monitored the vacuolar targeting biochemically by immunoblotting of lysates. Here, the appearance of a low molecular weight band when immunoblotting for GFP is generally indicative of vacuolar targeting. Vacuolar hydrolysis digests the linker between GFP and the tagged proteasome subunit resulting in an ~25 kDa “free GFP” species that maintains stability in yeast vacuoles (27, 49). When cells were treated with the TORC1 inhibitor rapamycin, we observed the accumulation of “free GFP” derived from cleavage of either Rpn1-GFP or α 1-GFP (Fig. 3-1A). This indicates that both the proteasome regulatory particle (RP), as represented by Rpn1, and core particle (CP), represented by α 1, are targeted to the lysosome upon inhibition of TORC1. Targeting was dependent on the autophagy pathway, as it was abrogated in a strain defective for autophagy as a result of *ATG7* deletion. Fluorescence microscopy analysis under the same conditions showed that, prior to treatment, most proteasomes in logarithmically growing cells localize to the nucleus as previously reported (50, 51). At 4-6 hrs after rapamycin addition, we observed a reduction in nuclear localization and the appearance of GFP in yeast

vacuoles. After 24 hrs, we observed the appearance of a predominantly perinuclear and vacuolar localization of GFP fluorescence (Fig. 3-1B).

These results are similar to what we have reported previously for nitrogen starvation (27); however, a subset of often larger cells showed poor induction of proteasome autophagy, with little GFP-fluorescence in their vacuoles. For example, the larger rapamycin treated WT cells in figure 3-1B showed less vacuolar GFP than smaller cells. When we quantified the number of WT cells displaying vacuolar GFP localization, we observed that a little over fifty percent of cells showed vacuolar localization following rapamycin treatment (55% for Rpn1, 53% for $\alpha 1$). This is in contrast to what we have previously reported for nitrogen starvation, where more than 90% of cells showed vacuolar GFP (27). We did not detect vacuolar localization in *atg7 Δ* cells. In sum, the inhibition of TORC1 using rapamycin leads to proteasome autophagy in yeast, but to a lesser extent than nitrogen starvation.

Figure 3-1 Rapamycin induces proteasome autophagy



(A) WT and *atg7Δ* yeast expressing $\alpha 1$ -GFP and *rpn1*-GFP, were inoculated in YPD medium at OD=0.5 and grown to log phase (~ 4hrs) before treatment with 200nM rapamycin. $\alpha 1$ -GFP, *rpn1*-GFP, and free GFP were monitored at indicated times by immunoblotting for GFP. PGK1 was used as a loading control. (B) Yeast collected at the 0 and 24 hr timepoints from Fig. 1A were used to collect brightfield and GFP images. Arrow heads point to nuclei and arrows to vacuoles. Scale bars represent 0.5 μ m.

Proteasome Autophagy Induced by Rapamycin Occurs Following an Initial Upregulation—

Our observation of proteasome autophagy upon rapamycin treatment appears to contradict previous data that report a rapamycin-induced increase in proteasome levels and activity (36, 52). Therefore, we monitored the response of cells over a more detailed range of time points and included native gel analyses which allowed us to monitor native proteasome complexes and peptidase activity. Here, approximately 0.5 to 4 hours following rapamycin addition, yeast cells showed an upregulation of proteasome levels as determined by $\alpha 1$ -GFP signal detected on native gel (Fig. 3-2A). Coinciding with the increased levels, we observed more hydrolysis of the suc-LLVY-amc fluorogenic substrate (Fig. 3-2A). Following this upregulation, we observed a reduction in proteasome activity and proteasome levels on native gels. This was accompanied by the appearance of a faster migrating GFP species on native gel. The accumulation of this species showed kinetics similar to the accumulation of the “free GFP” observed on immunoblots (Fig. 3-2B) suggesting this band is derived from “free GFP.” Furthermore, the species migrated on native gel where GFP migrates when it is not tagged to proteasome subunits (Fig. 3-2A and supplementary figure 4). Thus, the GFP released from tagged subunits by acid hydrolase activity can be observed accumulating on native gels. To determine if the upregulation of assembled proteasomes required *de novo* protein synthesis, we tested the effect of cycloheximide, a translation inhibitor. We detected only a slight increase in proteasome activity following rapamycin addition in the presence of cycloheximide (CHX) (Fig. 3-2C). Thus, to a large extent, proteasome upregulation upon rapamycin treatment depended on protein synthesis. The slight increase in the presence of CHX may represent the formation of proteasomes from preexisting subcomplexes. Overall, our data show that upon rapamycin treatment, proteasomes undergo a bi-phasic response, where an initial upregulation in proteasome levels through protein synthesis is followed by an overall reduction via autophagy.

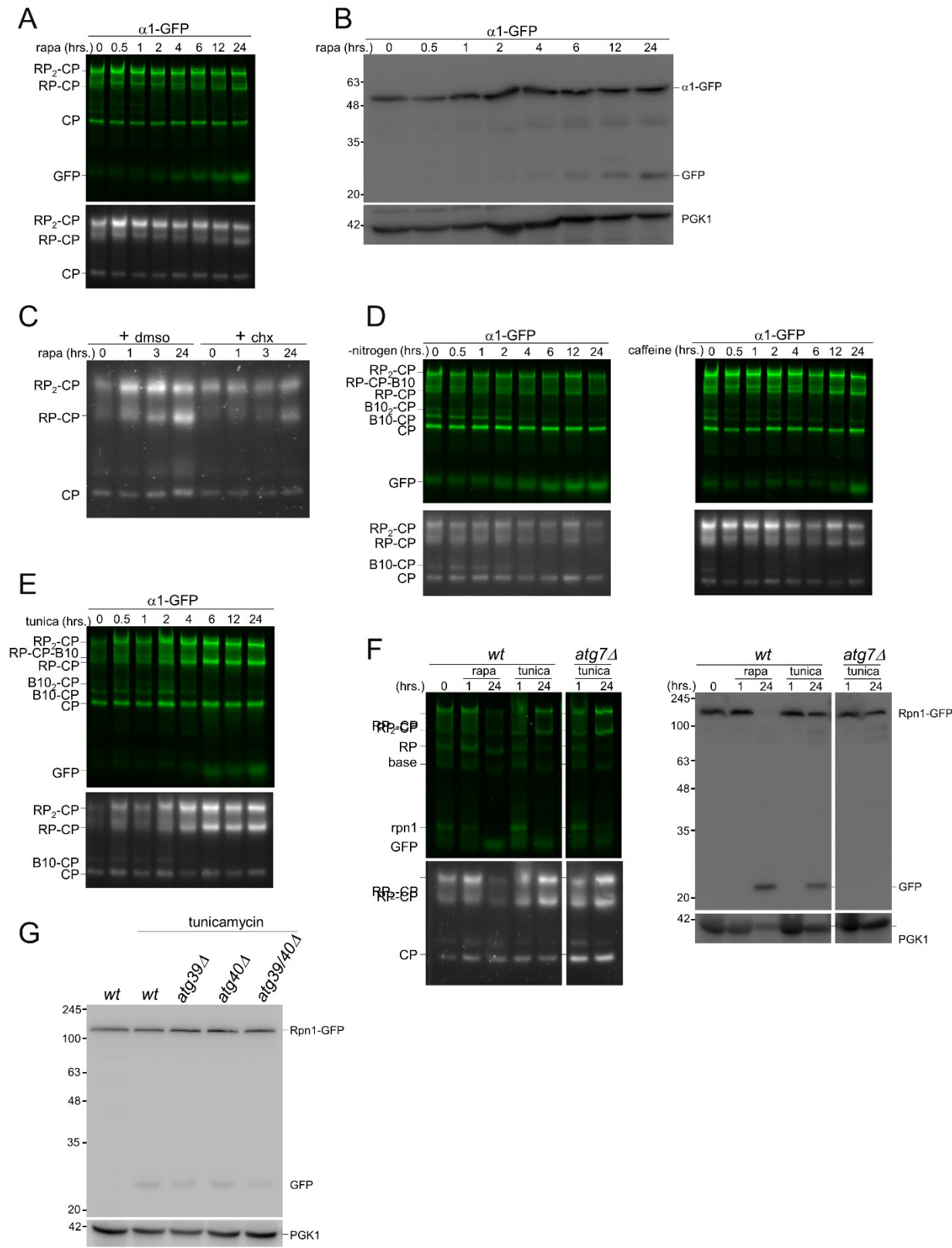
Nitrogen starvation and rapamycin treatment both lead to the inactivation of TORC1 with various downstream effects, such as nuclear translocation of the nitrogen responsive transcription factor GLN3 (32, 53). Thus, rapamycin and nitrogen starvation elicit, at least in part, a similar response through TOR inhibition. However, we only observed proteasome

upregulation with rapamycin and not upon nitrogen starvation (Fig. 3-2D). Therefore, we tested another inhibitor of TORC1, caffeine (54, 55). As with nitrogen starvation, we did not detect an upregulation of proteasome levels or activity when caffeine was added to growing cells, though proteasome autophagy was detected (Fig. 3-2D). Free GFP derived from proteasomes was detected at later timepoints after caffeine treatment compared to rapamycin or nitrogen starvation, perhaps because caffeine is a less potent inhibitor of TORC1 (54, 55). Thus, rapamycin, unlike caffeine and nitrogen starvation, showed an upregulation of proteasome levels and activity prior to induction of proteasome autophagy. As protein synthesis is required for proteasome upregulation upon rapamycin addition (Fig. 3-2B), the fact that proteasomes are not upregulated upon nitrogen starvation could result from a general reduction in protein synthesis. This is supported by our observation that cells do not show much, if any, growth in nitrogen starvation media and it is well documented that general translation is inhibited and a specific subset of mRNAs is translated (53, 56). In the presence of rapamycin, cells continued to proliferate. This is consistent with previous reports that show yeast cells are able to grow in the presence of rapamycin, even at concentrations that inhibit TORC1 effectively and induce autophagy (54, 57).

Another drug that was shown to induce proteasome upregulation was tunicamycin, which induces ER stress and the unfolded protein response (UPR) (36). To test if this was followed by an induction of proteasome autophagy, we treated cells with tunicamycin and analyzed the effect on proteasome levels and activity using native gels. Indeed, we observed a bi-phasic response similar to that of rapamycin (Fig. 3-2E). However, less proteasome autophagy occurred compared to rapamycin treatment as native gels showed a higher ratio of Rpn1-GFP: free GFP and more peptidase activity 24 hours post tunicamycin addition (Fig. 3-2F). The accumulation of free GFP was dependent on Atg7, confirming that tunicamycin induced autophagy of proteasomes (Fig. 3-2F). Since tunicamycin induces both ER autophagy and general autophagy (58), we wondered if the autophagy of proteasomes we observed resulted from ER-associated proteasomes that traffic to the vacuole via ER-phagy. To address this, we deleted genes known to be specifically required for ER/nucleophagy in yeast, *ATG39* and *ATG40*. Neither gene product was required for proteasome autophagy under conditions of nitrogen starvation (28, 29). We observed no reduction in the amount of free GFP generated upon tunicamycin treatment when *ATG39*, *ATG40* or both genes were deleted (Fig. 3-2G). Thus, proteasome autophagy observed upon tunicamycin treatment is distinct from ER-phagy.

(A) Yeast expressing $\alpha 1$ -GFP were grown in YPD medium and treated with rapamycin as in Fig.1. Lysates were run on native gels and imaged for GFP as described in materials and methods. Proteasome peptidase activity was assayed using the proteasome substrate suc-LLVY-AMC. **(B)** Samples from Fig. 2A were denatured and used for western blot. $\alpha 1$ -GFP and free GFP were monitored by immunoblotting as in Fig.1. PGK1 was used as a loading control. **(C)** Yeast were treated with rapamycin as above but including DMSO as a control or cycloheximide at a final concentration of 50 μ g/ml. Proteasome peptidase activity was monitored using suc-LLVY-AMC. **(D)** Yeast expressing $\alpha 1$ -GFP were nitrogen starved and treated with caffeine as described in materials and methods. Cells were collected at indicated time points and native gel analysis was carried out as described in materials and methods. **(E)** Yeast expressing $\alpha 1$ -GFP were treated with tunicamycin (5 μ g/ml) and samples collected at indicated timepoints. native gel analysis was carried out as described in materials and methods. **(F)** Yeast strains expressing Rpn1-GFP were treated with rapamycin or tunicamycin. Native gel analysis was carried out as described in materials and methods. Peptidase activity was monitored as described in Fig. 2A. **(G)** *WT*, *atg39 Δ* , *atg40 Δ* , and *atg39 Δ atg40 Δ* yeast expressing Rpn1-GFP were treated with tunicamycin (5 μ g/ml). 2 ODs of cells were harvested and lysed as described in materials and methods. Immunoblotting for GFP and PGK1 were performed as described above.

Figure 3-2 Rapamycin induces a bi-phasic proteasome response



Proteasome Autophagy is Distinct from General Autophagy—We have shown above that proteasomes are delivered to the yeast vacuoles under several conditions that are known to inhibit TORC1. However, prior to degradation, there is an upregulation of proteasomes after treatment with rapamycin or tunicamycin, suggesting autophagy of proteasomes might be delayed. This is in contrast to general autophagy, which is rapid and utilizes the constitutively expressed Atg1 and Atg13 (34, 59). Proteasome autophagy has been detected at around 6 hrs of nitrogen starvation (27, 28), while general autophagic bodies are detected in vacuoles as early as 2 hrs following nitrogen starvation (60). We hypothesized based on these observations that proteasome autophagy is slow compared to general (non-selective) autophagy. To compare the timing of proteasome autophagy with that of other autophagy substrates, we utilized the widely used GFP-atg8 marker. However, Atg8 is part of the core autophagy machinery and not a genuine substrate. Therefore, we also generated forms of free cytosolic GFP (containing a nuclear export signal). We compared the accumulation of GFP from proteasomes as well as these other substrates over time. The accumulation of free GFP signal from the cytosolic GFP reporter could be detected early on, at approximately 2 hrs. This is similar to the dynamics observed for GFP-Atg8 (Fig. 3-3A and B, supplementary Fig. 5). Quantifications show that GFP was cleaved from Atg8 earlier than from Rpn1 upon nitrogen starvation. This trend was consistent following rapamycin treatment as free GFP was detected from GFP-Atg8 earlier than proteasomes (Fig. 3-3C, supplementary Fig. 5). When comparing the RP reporter Rpn1 and the CP reporter $\alpha 1$, we detect proteasome autophagy for the RP earlier than CP with both nitrogen starvation and rapamycin treatment (Fig. 3-3B). These data support the notion that RP and CP dissociate prior to autophagy and undergo different pathways, a model we have previously proposed and has been supported by subsequent data (27, 28)

The above discussed conditions all induce general autophagy and the delayed autophagy we observed indicates proteasomes are not subject to general autophagy but undergo a selective process. However, it could also reflect a time lag for the targeting of nuclear proteins to general autophagy due to the nuclear export requirement, since the majority of proteasomes are nuclear. We therefore generated a GFP reporter with nuclear localization signals. This reporter indeed showed slower accumulation of free GFP which resembled proteasome dynamics. Similarly, expressing GFP from a strong promotor (GPD) with both NLS and NES resulted in localization to both the cytoplasm and nucleus, and we observed dynamics similar to proteasomes (Fig. 3-3C). It appears that autophagy of nuclear-localized material occurs slower than for cytosolic cargo. This could partly explain why proteasomes did not

appear as autophagic cargo until later timepoints. Consistent with this, we see a small amount of free GFP early for Rpn1, with a large increase at later timepoints (Fig. 3-3A, C) (27). This could reflect the cytosolic portion of proteasomes undergoing earlier autophagy.

If proteasome autophagy is selective it should not solely depend on factors that are required for general autophagy. A hallmark that distinguishes general autophagy from selective autophagy is that the latter pathway depends on specific receptors that recognize and sequester substrates into autophagosomes. For example, selective autophagy of peroxisomes utilizes the receptor Atg30, ER-phagy utilizes Atg39 and/or Atg40, and mitophagy Atg32 or Atg33 (5, 22). All of these factors can interact with the selective autophagy scaffolding factor Atg11 (4, 61). General autophagy, on the other hand, does not depend on Atg11 (62). We have previously reported that autophagy of an Rpn1-GFP fusion strain upon nitrogen starvation was not entirely abolished by a deletion of *ATG17* (27). Interestingly, the residual autophagy of proteasomes in the *atg17Δ* strain was abolished following deletion of *ATG11* (Fig. 3-3D). With respect to rapamycin, we were unable to detect free GFP in an *ATG17* mutant. This suggests that Atg11 is not important for proteasome autophagy induced by rapamycin. However, less proteasome autophagy is induced with this drug generally, 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, (28, 30, 31), further support the notion that proteasome autophagy, at least in part, occurs through a selective pathway.

We have previously reported that autophagy of the CP and the RP occur through different pathways. This was based on our observations that RP largely remained nuclear when autophagy was blocked, for example in an *ATG7* or *ATG17* mutant, while CP appeared in cytosolic punctate structures. Further, we reported the requirement for the deubiquitinating enzyme Ubp3 for autophagy of CP but not RP (27). These phenotypes were apparent when the $\beta 5$ subunit of the proteasome CP was tagged with GFP. Unexpectedly, we recently observed that $\alpha 1$ and $\alpha 6$ GFP fusions did not recapitulate these phenotypes to the same extent (Fig. 3-3D). Upon deletion of *ATG7* in $\alpha 1$ or $\alpha 6$ GFP-tagged strains, fluorescence remained largely nuclear upon nitrogen starvation. To further investigate this, we also generated doubly tagged RP and CP strains. In $\alpha 1$ or $\alpha 6$ GFP-tagged strains, Rpn1-mCherry signal remained nuclear as we had previously reported for strains with only Rpn1 tagged (27) (Fig. 3-3E). The presence of $\beta 5$ -GFP, on the other hand, changed the localization of Rpn1-mCherry. In a $\beta 5$ -GFP background, Rpn1-mCherry colocalized with $\beta 5$ -GFP in cytosolic puncta upon nitrogen starvation of *atg7Δ* cells. This shows that C-terminal tagging of $\beta 5$ can influence proteasome

localization under these conditions. Therefore, we conclude $\alpha 1$ and $\alpha 6$ data represent physiological CP localization while a tag on $\beta 5$ creates a mis-localized Rpn1, suggesting 26S proteasomes are present in granules under some conditions. This would distinguish $\beta 5$ -tag-dependent proteasome granules from previously defined proteasome storage granules (PSGs). Further, similar to what was reported by Nemec et. al., for $\alpha 4$ -GFP granules, these $\beta 5$ -GFP specific granules were dependent on the sorting nexin Snx4 (ATG24). Upon *SNX4* and *ATG7* deletion, $\beta 5$ -GFP localized to the nucleus following nitrogen starvation in a large proportion of cells compared to the granule localization observed in *ATG7* deleted cells (Fig. 3-3F). Interestingly, PSGs induced by carbon starvation were not dependent on *SNX4* (supplementary Fig. 6). This further distinguishes between PSGs and $\beta 5$ -tag-dependent granules.

We have previously reported that autophagy of CP using a $\beta 5$ -GFP tagged strain was reduced upon deletion of *UBP3*. A role for Ubp3 in proteasome CP autophagy has also been reported by Marshall et. al. following carbon starvation in a *BLM10* knock out (63). When we deleted *UBP3* in $\alpha 1$ -GFP and $\alpha 6$ -GFP tagged strains, we found autophagy induced by nitrogen starvation was substantially less affected than when *UBP3* was deleted in the $\beta 5$ -GFP tagged strain (Fig. 3-3G). We detected only a small reduction in the amount of free GFP from $\alpha 1$ and $\alpha 6$ compared to $\beta 5$. Microscopically, after nitrogen starvation we observed more nuclear GFP in $\alpha 1$ and $\alpha 6$ tagged strains compared to $\beta 5$ -tagged strains when *UBP3* was deleted. As previously reported, in $\beta 5$ -GFP *ubp3 Δ* strains starved for nitrogen, GFP signal primarily localizes to the cytoplasm with some in granules (Fig. 3-3G) (27). As discussed previously, the $\beta 5$ -GFP tagged strain showed growth defects in combination with a deletion of *RPN4* (see supplementary figure 2). The slight reduction in autophagy for $\alpha 1$ and $\alpha 6$ suggests a role for Ubp3 in CP autophagy, however, we cannot exclude a modest tag induced effect or related to a more general role for *UBP3* when the CP is compromised with a GFP tag. Why deletion of this de-ubiquitinating enzyme causes proteasomes (likely 26S) to fail autophagy or form granules is being investigated further.

To identify other factors involved in proteasome autophagy, we focused on a key MAPK signaling pathway in yeast which utilizes the kinase Mpk1. Mpk1 is not involved in general autophagy (64), but regulates proteasome abundance upon rapamycin addition (36). Deletion of *MPK1* in our GFP-tagged proteasome strains resulted in an approximately 40 % $\pm$ 7.2 (SEM) reduction in free GFP released from Rpn1 and 32 % $\pm$ 2.5 (SEM) reduction from $\alpha 1$ after nitrogen starvation (Fig. 3-3H). This indicates a role for Mpk1 in efficient proteasome autophagy 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 two kinases redundant in function and strains deleted of either *MKK1* or *MKK2* displayed normal proteasome autophagy (Fig. 3-3J). However, we did observe a reduction in proteasome autophagy following nitrogen starvation for the *MKK1* and *MKK2* double deletion mutant (Fig. 3-3I and J). An approximately $48\% \pm 8.0$ (SEM) reduction in free GFP was observed for Rpn1 and $36\% \pm 4.9$ (SEM) for $\alpha 1$. This indicates that Mkk1 and Mkk2 play a redundant role and, like Mpk1, are important for efficient proteasome autophagy. Bck1 which acts upstream of Mkk1 and Mkk2, however, did not show a reduction in proteasome autophagy following nitrogen starvation suggesting activation does not go through the standard cell wall integrity pathway (not shown). The identification of roles for signal transduction (MPK1, MKK1/2) and selective autophagy (Atg11) in proteasome autophagy reinforces the model that a portion of proteasomes are specifically targeted for lysosomal degradation.

Next, we sought to determine if proteasome autophagy always occurs with general autophagy. Starvation for nutrients such as nitrogen, phosphate, amino acids, zinc, and carbon are reported inducers of autophagy (65–68). Furthermore, the mitochondrial inhibitors antimycin A and potassium cyanide (KCN) have also been shown to induce autophagy (14). We sought to determine if proteasome autophagy was induced by these various stimuli. As reported previously, amino acid starvation induced general autophagy (66). We found both GFP-atg8 and pGPD-GFP expressed in yeast underwent autophagy upon amino acid starvation (Fig. 3-4A) to a similar extent as nitrogen starvation. This indicates general autophagic flux. Proteasome autophagy, on the other hand, was not induced to a similar extent under this condition as indicated by the reduction in free and vacuolar GFP (Fig. 3-4A). We next sought to determine whether phosphate starvation induced proteasome autophagy. Phosphate starvation has been reported to induce general autophagy, albeit to a lesser extent than nitrogen starvation (67). Consistent with this, we found that GFP-Atg8 is targeted to vacuoles upon phosphate starvation. However, we however did not detect autophagy for pGPD-GFP (Fig. 3-4A). This suggests there is little general autophagy occurring under phosphate starvation in our strain, and the GFP-Atg8 processing we observed is mainly derived from selective autophagy. This is consistent with the reported requirement for *ATG11* in phosphate starvation induced autophagy (67), as Atg11 is a protein normally involved in selective autophagy. Proteasomes also appeared to undergo phosphate starvation induced autophagy, albeit to a lesser extent than under nitrogen starvation. A role for *ATG11* in nitrogen starvation induced autophagy of RP was shown above (Fig. 3-3C). We sought to determine if this protein was required for proteasome autophagy in phosphate starvation. However, compared to wildtype, we did not

detect a reduction in the amount of free GFP for the RP in an *atg11* Δ strain (Fig. 3-4B). As our pGPD-GFP construct was not degraded under this condition, this suggests that, while phosphate starvation is selective, factors other than *ATG11* likely play roles in this selectivity. Other autophagy inducing stimuli failed to induce proteasome autophagy while we observed general autophagy. Inhibiting mitochondrial respiration using the drugs antimycinA or KCN induced general autophagy (14). When we treated our GFP-Atg8 expressing strains or strains expressing pGPD-GFP, we observed the accumulation of free GFP, indicating vacuolar targeting of these factors upon the inhibition of mitochondrial respiration (Fig. 3-4C). We tested if these conditions that induce general autophagy also induce proteasome autophagy by following the fate of α 1-GFP upon treatment of cells with KCN or antimycinA. No increase in free GFP levels was observed under these conditions, indicating proteasomes are not targeted to vacuoles (Fig. 3-4C). In all, our data show that proteasomes are not simply targeted to vacuoles by general autophagy, but that their fate appears to be specifically regulated under various cellular conditions.

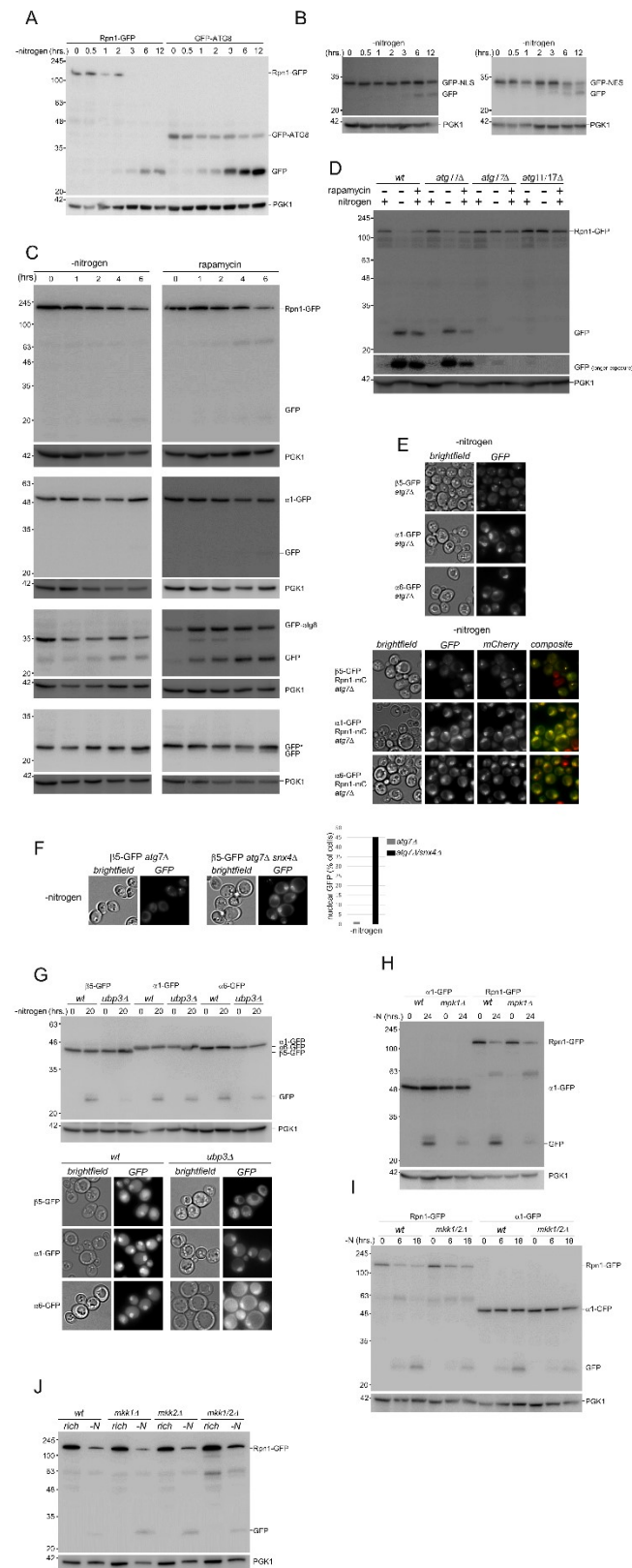
Proteasome Inhibitors Prevent Proteasome Storage Granule Formation—Carbon starvation has been shown to induce general autophagy (68). However, proteasome autophagy is not observed under this condition. Instead, proteasomes re-localize to cytoplasmic puncta termed proteasome storage granules (PSGs) (Fig. 3-5A) (27, 69). Signaling to re-localize to granules is also dominant over the vacuolar targeting signal that is induced by nitrogen starvation, as combined nitrogen and carbon starvation leads to PSG formation (27, 63). This suggests proteasomes might be protected from autophagic degradation by the formation of proteasome storage granules (PSGs). In fact, it was reported that when Nat3, Spg5, Blm10 or Ubp3 are deleted, proteasome storage granules fail to form, and proteasomes instead are degraded through autophagy (63, 70, 71). Since proteasome inhibitor treatment has also been reported to induce proteasome autophagy, we were curious if carbon starvation provided a dominant signal over this targeting pathway as well. When we treated cells with proteasome inhibitor, we observed vacuolar targeting of proteasomes in a *PDR5* knockout, which is more sensitive to proteasome inhibitor because the multidrug resistant pump Pdr5 is absent (Fig. 3-5B). These results are consistent with observations by Marshall et. al. (29) who used *erg6* Δ strains to sensitize their background to proteasome inhibitor. However, the levels of proteasome inhibitor-induced autophagy we observed, even at high inhibitor concentrations, were substantially lower and resembled those reported by Nemec et. al. (28). Interestingly, when we treated cells with proteasome inhibitors and starved for carbon, PSGs failed to form, and proteasomes did not undergo autophagy. Instead, proteasomes appeared to be enriched near

the nuclear envelope (Fig. 3-5C). Quantifications show an approximately 70% reduction in granule formation (Fig. 3-5D). This might indicate inhibited proteasomes fail to undergo export and therefore are unable to form cytoplasmic granules. When we quantify the intensity of fluorescent signal for the nucleus and the cytoplasm, we found that the nuclear GFP signal, as well as the fraction of proteasomes in the nucleus increased, in particular for the $\alpha 1$ -GFP reporter (Fig. 3-5C). The smaller number of cytosolic proteasomes in these conditions might be insufficient to induce PSGs or too weak to detect. Despite the absence of PSGs, we did not detect proteasome autophagy.

In yeast, quiescence also induces PSG formation (44). Similarly, in quiescence, general autophagy is normally upregulated (72, 73). Using a *PDR5* mutant strain, we found that proteasome inhibition under quiescence conditions also restrict PSG formation at 24, 48, and 72 hours with no detectable proteasome autophagy (Fig. 3-5E). At the 72 hr. timepoint, the proteasome inhibitor treated cells developed granules. However, at all time points tested, the level of proteasomes in the nucleus and cytoplasm, as measured by fluorescence intensity, was higher in proteasome inhibitor treated cells (Fig. 3-5E). This may indicate that stabilization of *RPN4* is generally responsible for proteasome upregulation. However, when we compared the fraction of GFP signal resulting from nuclear proteasomes, we again observed an increase in the presence of inhibitors, as seen when cells were starved for carbon (Fig. 3-5E). Thus, proteasome inhibitors caused an upregulation of proteasomes as well as an increase in the nuclear fraction, corresponding with a reduction in PSG formation.

Next, we tested how proteasome inhibition affects proteasome autophagy induced by nitrogen starvation. We analyzed Rpn1-GFP following inhibitor treatment using immunoblots and fluorescence microscopy. We observed little to no difference in the accumulation of free GFP as a result of proteasome inhibitor addition for this RP-subunit in wild type cells or *pdr5* Δ cells (Figs. 3-5F and 3-5G). It appeared Rpn1-GFP was more stable in the *pdr5* Δ mutant compared to wildtype cells following nitrogen starvation in the presence of proteasome inhibitor (Fig. 3-5G). Furthermore, microscopic analyses showed increased fluorescence intensity following inhibitor treatment (Figs. 3-5E and 3-5G). This increase in Rpn1-GFP likely reflects a transcriptionally induced upregulation of proteasome subunits (as opposed to a defect in proteasome autophagy) as it was most apparent when *PDR5* was deleted.

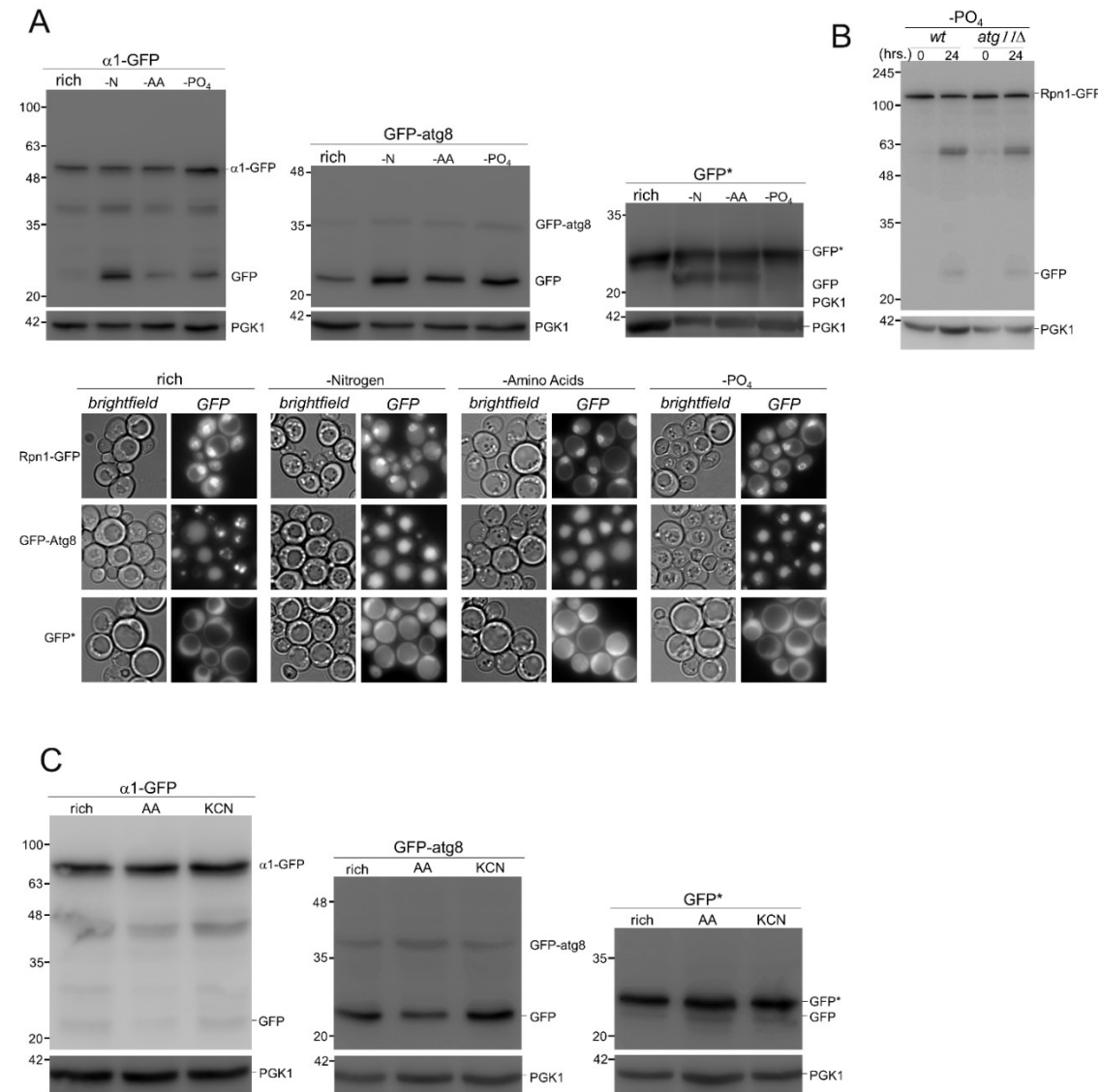
Figure 3-3 Proteasome autophagy is distinct from general autophagy



(A) Rpn1-GFP and GFP-Atg8 expressing yeast were starved for nitrogen. 2 ODs of cells were harvested at indicated timepoints and lysed as in Fig.2G. Immunoblotting for GFP and PGK1 were performed as described above. (B) Yeast expressing GFP with a nuclear localization signal (GFP-NLS) or GFP with a nuclear exclusion signal (GFP-NES) were starved for nitrogen and 2 ODs were collected at indicated timepoints. Immunoblotting for GFP and PGK1 were performed as described above. (C) Rpn1-GFP, α 1-GFP, GFP-Atg8, and free GFP that localizes to the cytoplasm and nucleus were starved for nitrogen or treated with rapamycin. 2 ODs of cells were harvested at indicated timepoints and lysed as in Fig.2G. Immunoblotting for GFP and PGK1 were performed as described above. (D) *WT*, *atg11 Δ* , *atg17 Δ* , *atg11/17 Δ* yeast expressing Rpn1-GFP were starved for nitrogen or treated with rapamycin for 24hrs. Protein lysates were obtained as in Fig. 2G. Immunoblotting for GFP and PGK1 were performed as described above. (E) Atg7 Δ strains expressing β 5-GFP, α 1-GFP or α 6-GFP (on left), or GFP tagged strains also expressing Rpn1-mCherry (on right) were starved for nitrogen. Microscopy was performed as described in materials and methods. (F) β 5-GFP expressing yeast with *atg7 Δ* or *atg7 Δ snx4 Δ* were starved for nitrogen.

Quantification of number of cells with nuclear GFP was carried out using FIJI. (G) β 5-GFP, α 1-GFP and α 6-GFP expressing yeast with *ubp3 Δ* were starved for nitrogen. Protein lysates were obtained as in Fig. 3A. Microscopy was performed as described in materials and methods. (H) *WT* and *mpk1 Δ* cells expressing Rpn1-GFP and α 1-GFP were starved for nitrogen and 2 ODs harvested at indicated time points. Immunoblotting for GFP and PGK1 were performed as described above. 40% percent reduction in free GFP from Rpn1 ($p=0.0231$) and a 32% reduction if free GFP from α 1 ($p=.00075$). Protein lysates produced as in Fig. 3A. (I) *WT* and *Mkk1/2 Δ* cells expressing Rpn1-GFP and α 1-GFP were starved for nitrogen and 2 ODs harvested at indicated time points. Immunoblotting for GFP and PGK1 were performed as described above. 48% percent reduction in free GFP from Rpn1 ($p=0.012$) and a 36% reduction if free GFP from α 1 ($p=.013$). Protein lysates produced as in Fig. 3A. (J) *WT*, *Mkk1 Δ* , *Mkk2 Δ* and *Mkk1/2 Δ* cells expressing Rpn1-GFP and α 1-GFP were starved for nitrogen and 2 ODs harvested at indicated time points. Protein lysates produced as in Fig. 3A.

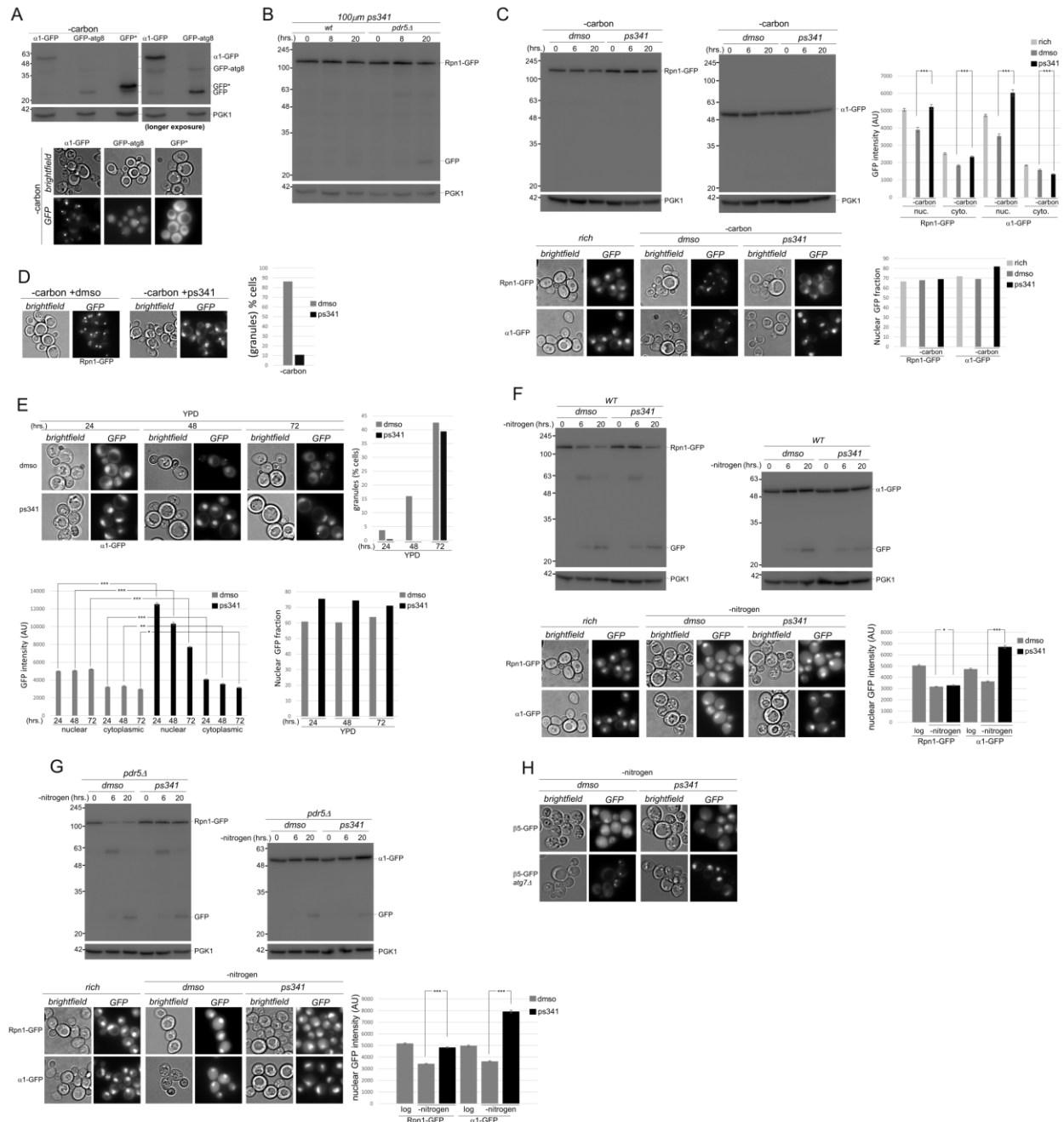
Figure 3-4 Proteasome autophagy is distinct from general autophagy



(A) Yeast strains expressing $\alpha 1$ -GFP, GFP-atg8, or free nuclear and cytosolic GFP were grown in YPD and starved for nitrogen, amino acids, or phosphate. 50 ODs of cells were collected at 24hrs. Lysates were produced by cryogrinding and equal volumes were blotted for GFP and PGK1. Microscopy was performed at the same time points. **(B)** *WT* and *Atg11Δ* yeast were grown to log phase in rich medium then switched to SD medium lacking phosphate. 2 ODs of cells were harvested at indicated timepoints and lysed as in Fig. 2G. **(C)** Yeast strains expressing $\alpha 1$ -gfp, GFP-Atg8, or nuclear and cytoplasmic GFP were grown to log phase in rich medium then treated with potassium cyanide (KCN) or Antimycin A (AA) at 1mM and 2 μ g/ml respectively. Protein lysates were obtained as in Fig. 2G.

The inhibition of proteasomes leads to the stabilization of the Rpn4 transcription factor that controls transcription of proteasome genes (46, 74, 75). Interestingly, for α 1-GFP tagged strains (both wildtype and *pdr5* Δ) we observed a reduction in free GFP accumulation in cells treated with proteasome inhibitor in nitrogen starvation media compared to nitrogen starvation media alone. Furthermore, microscopic analyses of these strains showed little vacuolar fluorescence and more nuclear fluorescence in the presence of inhibitors (Figs. 3-5F and 3-5G microscopy and quantification). This indicates that proteasome inhibition, under these conditions, affect RP and CP differently. Considering the current models that indicate proteasome autophagy involves dissociation of proteasome sub subcomplexes (27–29, 63) and that inhibitors bind specifically to CP, this may explain these observations. However, this does not explain why inhibitors prevent PSGs for both RP and CP reporters upon carbon starvation (Fig. 3-5C). Nevertheless, it appears that the nuclear retention of proteasomes can affect their vacuolar targeting. Consistent with this idea, other approaches to retain proteasomes in the nucleus have also been shown to prevent proteasome autophagy (28). As discussed above, we have previously used β 5-GFP and Rpn1-GFP expressing yeast to characterize proteasome autophagy following nitrogen starvation (27) (Fig. 3-3E). Upon deletion of *ATG7*, β 5-GFP signal appeared predominantly cytoplasmic and in granular structures. These structures were dependent on the GFP being on the β 5 subunit as α 1 and α 6 GFP strains did not show a similar phenotype (Fig. 3-3E). Furthermore, it appeared that having the GFP tag on β 5 induced mis-localization of the regulatory particle. We tested if proteasome inhibitors could prevent the nuclear export of these β 5-GFP specific granules. Like PSGs, these granules were indeed prevented by the addition of proteasome inhibitors (Fig. 3-5H). Overall, proteasome inhibitors appear to prevent proteasome re-localization under different conditions.

Figure 3-5 Proteasome inhibitors prevent proteasome storage granule formation



(A) Yeast strains expressing α1-GFP, GFP-Atg8, or nuclear and cytoplasmic GFP were grown to log phase in rich medium then switched to SD medium lacking carbon sources for 20 hrs. Protein lysates were obtained as in Fig. 2G. Microscopy was performed with cells taken from the same timepoint. (B) *WT* and *pdr5Δ* yeast were grown in rich medium to log phase and treated with the proteasome inhibitor ps341 (100μM final concentration). 2 ODs of cells were harvested at indicated times and lysed as in Fig. 2G. (C) Rpn1-GFP and α1-GFP expressing cells were starved for carbon as described above with or without ps341 (100μM). 2 ODs of cells were harvested at indicated timepoints and lysed as in Fig. 2G. Microscopy was performed at 20hrs in rich media or carbon starvation with or without

ps341. Quantifications of nuclear and cytoplasmic GFP intensity were done using FIJI and are represented in arbitrary units (AU). Error bars represent standard error of the mean (SEM) and p-values were determined using an unpaired, two-tailed Students t-test (*<0.01, **<0.001, ***<0.0001) **(D)** Rpn1-GFP expressing cells were starved for carbon with or without ps341 as described above. Quantification of the percentage of cells with granules was done using FIJI. **(E)** α 1-GFP expressing cells were grown in rich medium for the indicated times with and without ps341. Microscopy was performed as described above and quantification of granules as well as nuclear and cytosolic fluorescence intensity was done using FIJI. Error bars represent SEM and p values were determined as in C. **(F)** Rpn1-GFP and α 1-GFP expressing cells were starved for nitrogen as described above with or without ps341 (100 μ M). 2 ODs of cells were harvested at indicated timepoints and lysed as in Fig. 2G. Microscopy was performed as described above and quantification of nuclear fluorescence intensity was done using FIJI. Error bars represent SEM and p values were determined as in C **(G)** *Pdr5 Δ* cells expressing rpn1-GFP and α 1-GFP were starved for nitrogen as described above with or without ps341 (100 μ M). 2 ODs of cells were harvested at indicated timepoints and lysed as in Fig. 2G. Microscopy was performed as described above and quantification of nuclear fluorescence intensity was done using FIJI. Error bars represent SEM and p values were determined as in C **(H)** *WT* and *atg7 Δ* cells expressing β 5-GFP were starved for nitrogen with and without ps341 (100 μ M) for 20 hrs.

Discussion

As an important regulator of many cellular processes, including cell cycle regulation, UPS function is essential. Similarly, the ability to degrade proteins via autophagy is crucial during mammalian development (76). However, individual cells, such as MEFs, grow and multiply when autophagy is blocked (77). Consistent with this, yeast strains deleted for the various autophagy genes can grow at wild type rates under optimal conditions. Only upon exposure to certain stress conditions, like starvation, does autophagy become essential (78). While this indicates distinct functions for the UPS and autophagy, it has become clear in recent years that proteasomal and autophagic protein degradation can be synergistic, where one system can, to a certain extent, compensate for the impairment of the other (10, 13). However, inconsistent with this model, recent observations show proteasomes themselves are substrates for autophagic degradation (27–31). While such degradation makes sense under conditions where proteasomes are damaged or not functional (e.g. as a result of inhibition), it is less clear why proteasomes are degraded upon nitrogen starvation. Here, protein degradation through the UPS as well as autophagy could contribute to replenishing amino acid pools that become depleted. One possible explanation is that translation is largely blocked during nitrogen starvation and ribosomes are targeted for autophagy (23, 79). Thus, less proteasome activity would be required to degrade newly synthesized proteins. Another possibility is that cells use highly abundant complexes such as ribosomes and proteasomes to provide a valuable pool of

resources that can be utilized to survive nitrogen starvation. Approximately 70% of proteasomes in yeast are nuclear and direct autophagy of nuclear material does not seem to be responsible for vacuolar targeting of proteasomes (27, 28) suggesting specific signaling is responsible for their re-localization to the cytosol. Proteasomes require nuclear export for their efficient degradation through autophagy (28). We also report that proteasome autophagy is slow compared to general autophagy of cytosolic protein. This suggests that these complexes are retained in the nucleus early during starvation. A master regulator of metabolic signal 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 (36). Here, our extended analyses showed that, following TORC1 inhibition by rapamycin, proteasomes are indeed initially upregulated via de novo synthesis; however, following upregulation, proteasomes are degraded through autophagy.

A similar bi-phasic response was also observed with tunicamycin. This drug inhibits N-linked glycosylation, induces the unfolded protein response at the ER, and has previously been reported to induce proteasome upregulation (36). Since tunicamycin elicits the unfolded protein response (UPR), the upregulation of proteasomes seen with this drug seems logical as proteasomes are important for clearance of misfolded proteins. Indeed, the proteasome transcriptional regulator Rpn4 promotes cell survival after inducing ER stress with tunicamycin (80). Perhaps proteasomal upregulation by rapamycin also reflects a cellular response that benefits cellular survival under specific conditions. For example, proteasomes may contribute to replenishment of amino acid pools early in starvation (36). On the other hand, starvation for nitrogen and treatment with caffeine, while also leading to depleted amino acid pools (55), failed to elicit a similar biphasic response. Instead, proteasomes levels remained steady initially and autophagy was observed 6 hrs. following treatment, which is slower as compared to the targeting of many cytosolic autophagy factors. The delay in proteasome degradation under various conditions described as well as the upregulation upon rapamycin treatment indicate a requirement for proteasome activity early after autophagy induction, potentially to facilitate reprogramming of the cellular response. In support of this idea, TORC1 inhibition upon nutrient deprivation has recently been reported to lead to the proteasomal degradation of proteins required for DNA replication (81). Further, degradation of translation and RNA turnover factors by the proteasome, like Pop2, a de-adenylase subunit, and Dcp2, a de-capping enzyme, has been reported upon both nitrogen starvation and rapamycin treatment (82). Other factors related to ribosome function were shown to be degraded through autophagy under starvation

conditions (82). Thus, it seems that the UPS and autophagy work synergistically in response to stress by facilitating the appropriate cellular re-reprogramming.

Apart from the degradation of specific components, proteasomes may be required in yeast to provide an initial source of recycled amino acids when TORC1 is inhibited. This is consistent with the observation that proteasomes in yeast, unlike mammalian cells (30), do not undergo robust autophagy following amino acid starvation compared to nitrogen starvation. While our inability to detect upregulation of proteasomes with nitrogen starvation or caffeine might indicate that the upregulation of proteasomes in response to rapamycin is unique to this TORC1 inhibitor, the relatively slow degradation of proteasomes following autophagic stimuli suggest there might be an initial need for these proteases early in autophagy.

The delayed targeting of proteasomes is similar to what has been reported for ribosomes and inconsistent with a simple targeting of proteasomes to vacuoles by bulk autophagy (83). More direct evidence for specificity in proteasome autophagy comes from the identification of specific factors that are required for proteasome autophagy but dispensable for bulk autophagy. Examples are the requirement for Rpn10 in plants, Atg24 and Cue5 in yeast, and p62 in mammalian cells (28–31). Here, we present further evidence in support of specific proteasome autophagy upon nitrogen starvation. We observed a requirement for Atg11 for a subset of proteasomes to undergo autophagic degradation. We had previously reported that a subset of regulatory particle was still degraded in an *ATG17* mutant (27). Here, we show a deletion of *ATG11* in an *atg17* Δ strain completely blocked autophagy. Atg11 is known to be required for the specific recognition of autophagic cargo in yeast, for example during mitophagy, pexophagy, and the CVT pathway (4, 61, 84, 85). Whether or not these proteasomes directly interact with Atg11 via a selective autophagy receptor remains to be determined.

We have previously reported that blocking autophagy, for example by deleting *ATG7* or *ATG17*, resulted in nuclear retention of regulatory particles, while CP localized to granules (27). While the latter occurred for β 5 GFP-tagged proteasomes in our strain background, when we conducted similar experiments with α 1 or α 6 GFP-tagged strains, we found that GFP signal remained largely nuclear. Furthermore, strains tagged with both β 5 and Rpn1 under these conditions, showed Rpn1 colocalized with β 5, indicating a dominant tag-induced effect of β 5 under this particular condition. In sum, it appears proteasome holoenzymes remain largely nuclear when autophagy is blocked. This raises the question of how proteasome nuclear export/import may be linked to autophagy flux. When autophagy is blocked by knocking out *ATG7*, autophagosomes fail to form (21, 86). However, cellular metabolic conditions and

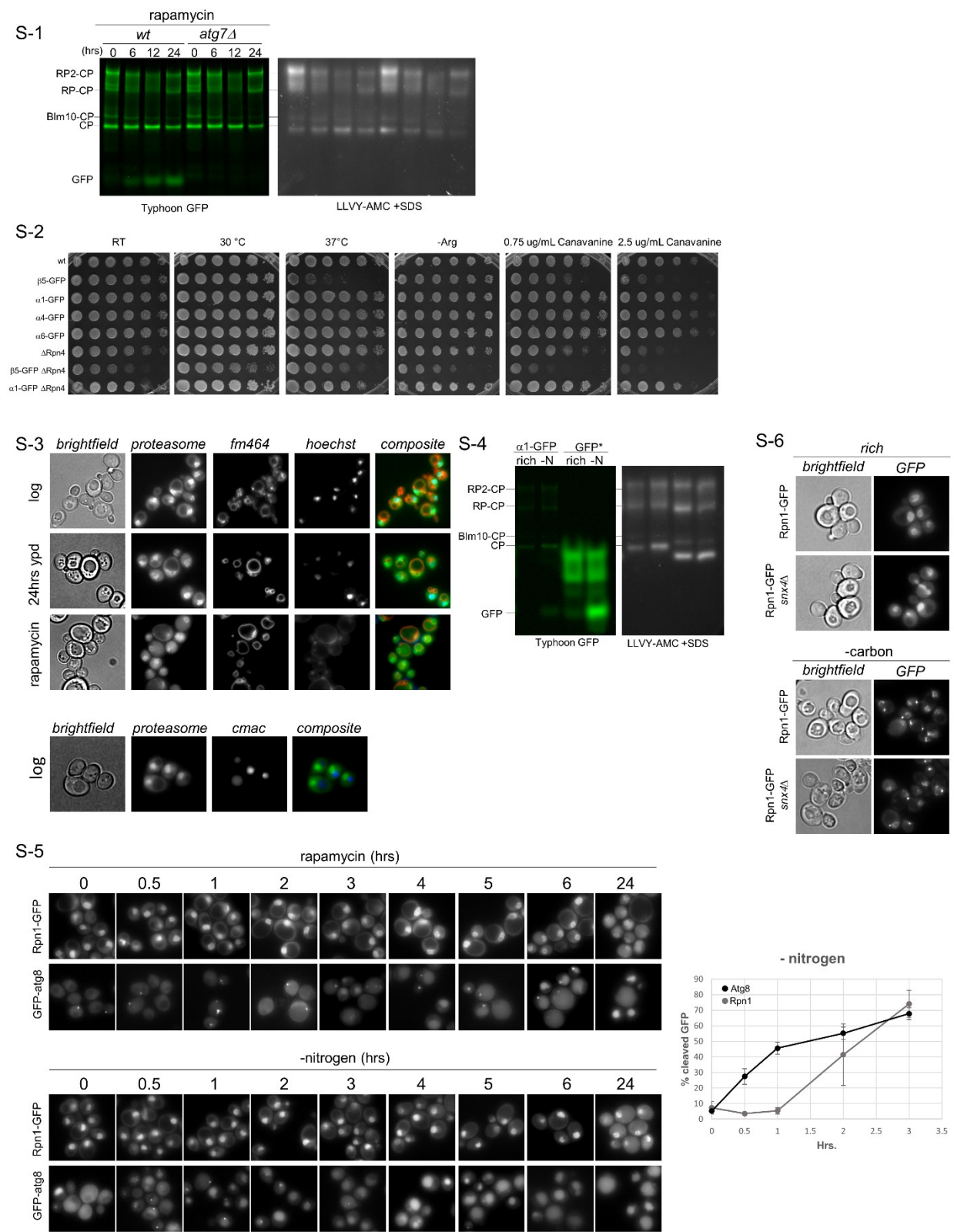
signals that initiate autophagy are still present in nitrogen starved or rapamycin treated cells. Potentially, proteasome nuclear export does not depend on autophagy induction but instead, autophagic flux. This might be related to maintaining a certain equilibrium of proteasomes between the cytoplasm and nucleus. Here, cytosolic proteasomes would be degraded before nuclear material is exported and becomes available. Consistent with this, conditions where autophagy is blocked result in nuclear localization of proteasomes (Fig. 3-1A). Further, very little proteasome autophagy is observed early in starvation which increases substantially at later timepoints (Fig. 3-3A, C). Similarly, we observed that a nuclear localized autophagy reporter displayed dynamics similar to proteasome autophagy whereas a cytosolic reporter was degraded sooner (Fig. 3B)

In addition to Atg11, we also observed an important role for the MAP kinase Mpk1 in proteasome autophagy, with an approximately 30-40% reduction in its absence. Consistent with other work, we did not observe a substantial dependence on Mpk1 for general autophagy (64). Currently it is unclear if Mpk1 facilitates proteasome autophagy through direct phosphorylation of proteasomes or through its function in a signaling cascade that leads to proteasome autophagy. Intriguingly, this kinase is required for proteasome upregulation following rapamycin addition (36). The role of Mpk1 in rapamycin induced proteasome autophagy could not be determined as these cells die approximately 4hrs following rapamycin addition, prior to detectable robust proteasome autophagy. In terms of up-regulation, Mpk1 works to signal proteasome assembly through the chaperone Adc17. We did not detect a role for Adc17 in proteasome autophagy or upregulation during nitrogen starvation (not shown). Understanding the molecular relationship between MAP kinase signaling and proteasome autophagy will be subject of future studies.

Proteasome autophagy is induced only under some autophagy inducing conditions. We did not observe proteasome autophagy under amino acid starvation, antimycin A, or potassium cyanide treatment. Furthermore, there are several unique conditions that induce proteasome autophagy, including proteasome inhibitor or phosphate starvation, that do not induce general autophagy. This indicates proteasome autophagy is subject to specific regulation and targeting of complexes to vacuoles. We propose that proteasomes are selectively recognized as autophagic cargo. However, recent work suggest that proteasomes might also be selectively excluded from bulk autophagy (63). For example, carbon starvation induces general autophagy that largely excludes proteasomes. Proteasomes instead re-localize to cytosolic foci termed proteasome storage granules (PSGs). Intriguingly, we found that proteasome inhibitors largely prevented the formation of proteasome granules in carbon starvation as well as stationary

phase. Under these conditions, we did not observe proteasome autophagy. Instead, proteasomes remained largely nuclear. This is to some extent inconsistent with the notion that proteasome inhibitors induce proteasome autophagy (29). In our hands, we did not detect robust proteasome autophagy upon treatment with proteasome inhibitors alone, or in combination with carbon starvation. When we tested how vacuolar targeting of proteasomes upon nitrogen starvation was impacted by inhibitors, we observed no increase in autophagy. Here, proteasome inhibitors appeared to have different effects on the RP and CP, where RP was largely unaffected while CP appeared to be retained in the nucleus to a certain extent. This supports the notion that proteasomes dissociate prior to autophagic degradation. In sum, it appears proteasome inhibitors can restrict proteasome nuclear export and targeting to granules. The notion that proteasome inhibitors, which are used and extensively studied as anticancer drugs, might change cellular localization of proteasomes under certain physiological conditions has not been reported or evaluated before to the best of our knowledge.

Figure 3-6 Supplementary figures



S-1. Native gel corresponding to samples obtained in Fig.1A showing $\alpha 1$ -GFP incorporates into proteasomes and these proteasomes are active. Native gels were performed as described in materials and methods.

S-2. WT, $\beta 5$ -GFP, $\alpha 1$ -GFP $\alpha 4$ -GFP and $\alpha 6$ -GFP expressing yeast as well as *Rpn4 Δ* mutants were grown to log phase in YPD medium, 1 OD was collected, and 4-fold dilutions were carried out. Dilutions were either spotted on YPD to test temperature phenotypes, or plates lacking arginine with 0.75 μ g/ml or 2.5 μ g/ml canavanine. All plates were incubated for 24hrs at 30°C unless otherwise indicated.

S-3. Rpn1-GFP expressing yeast were incubated with the vacuole membrane marker FM464 and the nuclear lumen marker Hoechst. Microscopy was performed at log phase, 24hrs growth in YPD and 24hrs after rapamycin treatment. Rpn1-GFP expressing yeast growing logarithmically were incubated with the vacuole lumen marker CMAC-arg. Images were processed using FIJI.

S-4. $\alpha 1$ -GFP and pGPD-GFP expressing cells were grown in rich medium then starved for nitrogen to monitor the migration of free GFP on native gels.

S-5. Rpn1-GFP and GFP-Atg8 expressing cells were grown to log phase in YPD and either starved for nitrogen or treated with rapamycin as described in materials and methods. Microscopy was performed at indicated timepoints. Graph shows quantification of the percentage of free GFP generated from Atg8 and Rpn1. Error bars indicate SEM.

S-6. *WT* and *snx4 Δ* yeast expressing rpn1-GFP were starved for carbon as described above. Images were processed using FIJI.

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References-Chapter 3

1. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–79
2. Tomko, R. J., and Hochstrasser, M. (2013) Molecular architecture and assembly of the eukaryotic proteasome. *Annu. Rev. Biochem.* **82**, 415–45
3. Noda, N. N., Ohsumi, Y., and Inagaki, F. (2010) Atg8-family interacting motif crucial for selective autophagy. *FEBS Lett.* **584**, 1379–1385
4. Suzuki, H., and Noda, N. N. (2018) Biophysical characterization of Atg11, a scaffold protein essential for selective autophagy in yeast. *FEBS Open Bio.* **8**, 110–116
5. Feng, Y., He, D., Yao, Z., and Klionsky, D. J. (2014) The machinery of macroautophagy. *Cell Res.* **24**, 24–41
6. Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., Kominami, E., Tanaka, K., and Chiba, T. (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol.* **169**, 425–434
7. Onodera, J., and Ohsumi, Y. (2005) Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *J. Biol. Chem.* **280**, 31582–6
8. Nam, T., Han, J. H., Devkota, S., and Lee, H.-W. (2017) Emerging Paradigm of Crosstalk between Autophagy and the Ubiquitin-Proteasome System. *Mol. Cells.* **40**, 897–905
9. Ji, C. H., and Kwon, Y. T. (2017) Crosstalk and Interplay between the Ubiquitin-Proteasome System and Autophagy. *Mol. Cells.* **40**, 441–449
10. Dikic, I. (2017) Proteasomal and Autophagic Degradation Systems. *Annu. Rev. Biochem.* **86**, 193–224
11. Cha-Molstad, H., Sung, K. S., Hwang, J., Kim, K. A., Yu, J. E., Yoo, Y. D., Jang, J. M., Han, D. H., Molstad, M., Kim, J. G., Lee, Y. J., Zakrzewska, A., Kim, S.-H., Kim, S. T., Kim, S. Y., Lee, H. G., Soung, N. K., Ahn, J. S., Ciechanover, A., Kim, B. Y., and Kwon, Y. T. (2015) Amino-terminal arginylation targets endoplasmic reticulum chaperone BiP for autophagy through p62 binding. *Nat. Cell Biol.* **17**, 917–929
12. Harada, M., Hanada, S., Toivola, D. M., Ghorri, N., and Omary, M. B. (2008) Autophagy activation by rapamycin eliminates mouse Mallory-Denk bodies and blocks their proteasome inhibitor-mediated formation. *Hepatology.* **47**, 2026–2035
13. Athané, A., Buisson, A., Challier, M., Beaumatin, F., Manon, S., Bhatia-Kiššová, I., and Camougrand, N. (2015) Insights into the relationship between the proteasome and autophagy in human and yeast cells. *Int. J. Biochem. Cell Biol.* **64**, 167–173
14. Deffieu, M., Bhatia-Kissova, I., Salin, B., Klionsky, D. J., Pinson, B., Manon, S., and Camougrand, N. (2013) Increased levels of reduced cytochrome b and mitophagy components are required to trigger nonspecific autophagy following induced mitochondrial dysfunction. *J. Cell Sci.* **126**, 415–

15. Liu, W. J., Ye, L., Huang, W. F., Guo, L. J., Xu, Z. G., Wu, H. L., Yang, C., and Liu, H. F. (2016) p62 links the autophagy pathway and the ubiquitin-proteasome system upon ubiquitinated protein degradation. *Cell. Mol. Biol. Lett.* **21**, 29
16. Nemeth, Z. H., Wong, H. R., Odoms, K., Deitch, E. A., Szabó, C., Vizi, E. S., and Haskó, G. (2004) Proteasome Inhibitors Induce Inhibitory B (I B) Kinase Activation, I B Degradation, and Nuclear Factor B Activation in HT-29 Cells. *Mol. Pharmacol.* **65**, 342–349
17. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature.* **349**, 132–138
18. Jin, M., and Klionsky, D. J. (2014) Regulation of autophagy: modulation of the size and number of autophagosomes. *FEBS Lett.* **588**, 2457–63
19. Baba, M., Osumi, M., Scott, S. V., Klionsky, D. J., and Ohsumi, Y. (1997) Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome. *J. Cell Biol.* **139**, 1687–95
20. Mizushima, N. (2005) The pleiotropic role of autophagy: from protein metabolism to bactericide. *Cell Death Differ.* **12**, 1535–1541
21. Kim, J., Dalton, V. M., Eggerton, K. P., Scott, S. V., and Klionsky, D. J. (1999) Apg7p/Cvt2p Is Required for the Cytoplasm-to-Vacuole Targeting, Macroautophagy, and Peroxisome Degradation Pathways. *Mol. Biol. Cell.* **10**, 1337–1351
22. Mochida, K., Oikawa, Y., Kimura, Y., Kirisako, H., Hirano, H., Ohsumi, Y., and Nakatogawa, H. (2015) Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus. *Nature.* **522**, 359–362
23. Kraft, C., Deplazes, A., Sohrmann, M., and Peter, M. (2008) Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat. Cell Biol.* **10**, 602–610
24. Kraft, C., Reggiori, F., and Peter, M. (2009) Selective types of autophagy in yeast. *Biochim. Biophys. Acta - Mol. Cell Res.* **1793**, 1404–1412
25. Ossareh-Nazari, B., Bonizec, M., Cohen, M., Dokudovskaya, S., Delalande, F., Schaeffer, C., Van Dorsselaer, A., and Dargemont, C. (2010) Cdc48 and Ufd3, new partners of the ubiquitin protease Ubp3, are required for ribophagy. *EMBO Rep.* **11**, 548–554
26. Wyant, G. A., Abu-Remaileh, M., Frenkel, E. M., Laqtom, N. N., Dharamdasani, V., Lewis, C. A., Chan, S. H., Heinze, I., Ori, A., and Sabatini, D. M. (2018) NUFIP1 is a ribosome receptor for starvation-induced ribophagy. *Science.* **360**, 751–758
27. Waite, K. A., De La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2015) Starvation Induces Proteasome Autophagy with Different Pathways for Core and Regulatory Particle. *J. Biol. Chem.* 10.1074/jbc.M115.699124

28. Nemec, A. A., Howell, L. A., Peterson, A. K., Murray, M. A., and Tomko, R. J. (2017) Autophagic clearance of proteasomes in yeast requires the conserved sorting nexin Snx4. *J. Biol. Chem.* **292**, 21466–21480
29. Marshall, R. S., McLoughlin, F., and Vierstra, R. D. (2016) Autophagic Turnover of Inactive 26S Proteasomes in Yeast Is Directed by the Ubiquitin Receptor Cue5 and the Hsp42 Chaperone. *Cell Rep.* **16**, 1717–1732
30. Cohen-Kaplan, V., Livneh, I., Avni, N., Fabre, B., Ziv, T., Kwon, Y. T., and Ciechanover, A. (2016) p62- and ubiquitin-dependent stress-induced autophagy of the mammalian 26S proteasome. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E7490–E7499
31. Marshall, R. S., Li, F., Gemperline, D. C., Book, A. J., Vierstra Correspondence, R. D., and Vierstra, R. D. (2015) Autophagic Degradation of the 26S Proteasome Is Mediated by the Dual ATG8/Ubiquitin Receptor RPN10 in Arabidopsis. *Mol. Cell.* **58**, 1053–1066
32. Crespo, J. L., and Hall, M. N. (2002) Elucidating TOR signaling and rapamycin action: lessons from *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **66**, 579–91, table of contents
33. Kamada, Y., Yoshino, K. -i., Kondo, C., Kawamata, T., Oshiro, N., Yonezawa, K., and Ohsumi, Y. (2010) Tor Directly Controls the Atg1 Kinase Complex To Regulate Autophagy. *Mol. Cell. Biol.* **30**, 1049–1058
34. Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biol.* **150**, 1507–13
35. Zhang, N., Quan, Z., Rash, B., and Oliver, S. G. (2013) Synergistic effects of TOR and proteasome pathways on the yeast transcriptome and cell growth. *Open Biol.* **3**, 120137
36. Rousseau, A., and Bertolotti, A. (2016) An evolutionarily conserved pathway controls proteasome homeostasis. *Nature.* **536**, 184–189
37. Finley, D., Özkaynak, E., and Varshavsky, A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell.* **48**, 1035–1046
38. Goldstein, A. L., and McCusker, J. H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast.* **15**, 1541–53
39. Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and Knop, M. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast.* **21**, 947–962
40. Hailey, D. W., Davis, T. N., and Muller, E. G. D. (2002) Fluorescence resonance energy transfer using color variants of green fluorescent protein. *Methods Enzymol.* **351**, 34–49
41. Roelofs, J., Suppahia, A., Waite, K. A., and Park, S. (2018) Native Gel Approaches in Studying Proteasome Assembly and Chaperones. in *Methods in molecular biology (Clifton, N.J.)*, pp. 237–260, **1844**, 237–260

42. Kushnirov, V. V. (2000) Rapid and reliable protein extraction from yeast. *Yeast*. **16**, 857–860
43. Sundin, B. A., Chiu, C.-H., Riffle, M., Davis, T. N., and Muller, E. G. D. (2004) Localization of proteins that are coordinately expressed with Cln2 during the cell cycle. *Yeast*. **21**, 793–800
44. Gu, Z. C., Wu, E., Sailer, C., Jando, J., Styles, E., Eisenkolb, I., Kuschel, M., Bitschar, K., Wang, X., Huang, L., Vissa, A., Yip, C. M., Yedidi, R. S., Friesen, H., and Enenkel, C. (2017) Ubiquitin orchestrates proteasome dynamics between proliferation and quiescence in yeast. *Mol. Biol. Cell*. **28**, 2479–2491
45. Enenkel, C. (2012) Using Native Gel Electrophoresis and Phosphofluoroimaging to Analyze GFP-Tagged Proteasomes, pp. 339–348, Humana Press, 10.1007/978-1-61779-474-2_23
46. Xie, Y., and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3056–61
47. Roelofs, J., Park, S., Haas, W., Tian, G., McAllister, F. E., Huo, Y., Lee, B.-H., Zhang, F., Shi, Y., Gygi, S. P., and Finley, D. (2009) Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature*. **459**, 861–5
48. Dohmen, R. J., Willers, I., and Marques, A. J. (2007) Biting the hand that feeds: Rpn4-dependent feedback regulation of proteasome function. *Biochim. Biophys. Acta - Mol. Cell Res.* **1773**, 1599–1604
49. Kimura, S., Fujita, N., Noda, T., and Yoshimori, T. (2009) Chapter 1 Monitoring Autophagy in Mammalian Cultured Cells through the Dynamics of LC3. *Methods Enzymol.* **451**, 1–12
50. Wilkinson, C. R. M., Wallace, M., Morpew, M., Perry, P., Allshire, R., Javerzat, J.-P., McIntosh, J. R., and Gordon, C. (1998) Localization of the 26S proteasome during mitosis and meiosis in fission yeast. *EMBO J.* **17**, 6465–6476
51. Enenkel, C., Lehmann, A., and Kloetzel, P.-M. (1999) GFP-labelling of 26S proteasomes in living yeast: insight into proteasomal functions at the nuclear envelope/rough ER. *Mol. Biol. Rep.* **26**, 131–135
52. Zhao, J., Zhai, B., Gygi, S. P., and Goldberg, A. L. (2015) mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 15790–7
53. Beck, T., and Hall, M. N. (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature*. **402**, 689–692
54. Wanke, V., Camerini, E., Uotila, A., Piccolis, M., Urban, J., Loewith, R., and De Virgilio, C. (2008) Caffeine extends yeast lifespan by targeting TORC1. *Mol. Microbiol.* **69**, 277–285
55. Kuranda, K., Leberre, V., Sokol, S., Palamarczyk, G., and Francois, J. (2006) Investigating the caffeine effects in the yeast *Saccharomyces cerevisiae* brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. *Mol. Microbiol.* **61**, 1147–1166

56. Onodera, J., and Ohsumi, Y. (2005) Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *J. Biol. Chem.* **280**, 31582–6
57. Evans, S. K., Burgess, K. E. V, and Gray, J. V (2014) Recovery from Rapamycin DRUG-INSENSITIVE ACTIVITY OF YEAST TARGET OF RAPAMYCIN COMPLEX 1 (TORC1) SUPPORTS RESIDUAL PROLIFERATION THAT DILUTES RAPAMYCIN AMONG PROGENY CELLS *. 10.1074/jbc.M114.589754
58. Schuck, S., Gallagher, C. M., and Walter, P. (2014) ER-phagy mediates selective degradation of endoplasmic reticulum independently of the core autophagy machinery. *J. Cell Sci.* **127**, 4078–4088
59. Abeliovich, H., Dunn, W. A., Kim, J., and Klionsky, D. J. (2000) Dissection of autophagosome biogenesis into distinct nucleation and expansion steps. *J. Cell Biol.* **151**, 1025–34
60. Baba, M., Takeshige, K., Baba, N., and Ohsumi, Y. (1994) Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. *J. Cell Biol.* **124**, 903–13
61. Delorme-Axford, E., and Klionsky, D. J. (2015) A missing piece of the puzzle: Atg11 functions as a scaffold to activate Atg1 for selective autophagy. *Autophagy.* **11**, 2139–2141
62. Kim, J., Kamada, Y., Stromhaug, P. E., Guan, J., Hefner-Gravink, A., Baba, M., Scott, S. V, Ohsumi, Y., Dunn, W. A., and Klionsky, D. J. (2001) Cvt9/Gsa9 functions in sequestering selective cytosolic cargo destined for the vacuole. *J. Cell Biol.* **153**, 381–96
63. Marshall, R. S., and Vierstra, R. D. (2018) Proteasome storage granules protect proteasomes from autophagic degradation upon carbon starvation. *Elife.* 10.7554/eLife.34532
64. Manjithaya, R., Jain, S., Farré, J.-C., and Subramani, S. (2010) A yeast MAPK cascade regulates pexophagy but not other autophagy pathways. *J. Cell Biol.* **189**, 303–10
65. Davie, E., Forte, G. M. A., and Petersen, J. (2015) Nitrogen Regulates AMPK to Control TORC1 Signaling. *Curr. Biol.* **25**, 445–454
66. Ecker, N., Mor, A., Journo, D., and Abeliovich, H. (2010) Induction of autophagic flux by amino acid deprivation is distinct from nitrogen starvation-induced macroautophagy. *Autophagy.* **6**, 879–890
67. Yokota, H., Gomi, K., and Shintani, T. (2017) Induction of autophagy by phosphate starvation in an Atg11-dependent manner in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **483**, 522–527
68. Adachi, A., Koizumi, M., and Ohsumi, Y. (2017) Autophagy induction under carbon starvation conditions Autophagy induction under carbon starvation conditions is negatively regulated by carbon catabolite repression. Autophagy induction under carbon starvation conditions. *JBC Pap. Press. Publ. Oct.* 10.1074/jbc.M117.817510
69. Enenkel, C. (2018) The paradox of proteasome granules. *Curr. Genet.* **64**, 137–140

70. van Deventer, S., Menendez-Benito, V., van Leeuwen, F., and Neefjes, J. (2014) N-terminal acetylation and replicative age affect proteasome localization and cell fitness during aging. *J. Cell Sci.* **128**, 109–117
71. Weberruss, M. H., Savulescu, A. F., Jando, J., Bissinger, T., Harel, A., Glickman, M. H., and Enenkel, C. (2013) Bim10 facilitates nuclear import of proteasome core particles. *EMBO J.* **32**, 2697–707
72. Noda, T., and Ohsumi, Y. (1998) *Tor, a Phosphatidylinositol Kinase Homologue, Controls Autophagy in Yeast**, [online] <http://www.jbc.org/> (Accessed January 10, 2019)
73. Gray, J. V, Petsko, G. A., Johnston, G. C., Ringe, D., Singer, R. A., and Werner-Washburne, M. (2004) “Sleeping beauty”: quiescence in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **68**, 187–206
74. Wang, X., Xu, H., Ju, D., and Xie, Y. (2008) Disruption of Rpn4-induced proteasome expression in *Saccharomyces cerevisiae* reduces cell viability under stressed conditions. *Genetics*. **180**, 1945–53
75. Ju, D., Wang, L., Mao, X., and Xie, Y. (2004) Homeostatic regulation of the proteasome via an Rpn4-dependent feedback circuit. 10.1016/j.bbrc.2004.06.105
76. Tsukamoto, S., Kuma, A., Murakami, M., Kishi, C., Yamamoto, A., and Mizushima, N. (2008) Autophagy Is Essential for Preimplantation Development of Mouse Embryos. *Science* (80-.). **321**, 117–120
77. Pyo, J.-O., Nah, J., Kim, H.-J., Lee, H.-J., Heo, J., Lee, H., and Jung, Y.-K. (2008) Compensatory activation of ERK1/2 in Atg5-deficient mouse embryo fibroblasts suppresses oxidative stress-induced cell death. *Autophagy*. **4**, 315–21
78. Tsukada, M., and Ohsumi, Y. (1993) Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* **333**, 169–74
79. Kelly, S. P., and Bedwell, D. M. (2015) Both the autophagy and proteasomal pathways facilitate the Ubp3p-dependent depletion of a subset of translation and RNA turnover factors during nitrogen starvation in *Saccharomyces cerevisiae*. *RNA*. **21**, 898–910
80. Schmidt, R. M., Schessner, J. P., Borner, G. H., and Schuck, S. (2019) The proteasome biogenesis regulator Rpn4 cooperates with the unfolded protein response to promote ER stress resistance. *Elife*. 10.7554/eLife.43244
81. Yamamoto, K., Makino, N., Nagai, M., Honma, Y., Araki, H., and Ushimaru, T. (2018) TORC1 signaling regulates DNA replication via DNA replication protein levels. *Biochem. Biophys. Res. Commun.* 10.1016/j.bbrc.2018.10.018
82. Kelly, S. P., and Bedwell, D. M. (2015) Both the autophagy and proteasomal pathways facilitate the Ubp3p-dependent depletion of a subset of translation and RNA turnover factors during nitrogen starvation in *Saccharomyces cerevisiae*. *RNA*. **21**, 898–910
83. Ossareh-Nazari, B., Niño, C. A., Bengtson, M. H., Lee, J.-W., Joazeiro, C. A. P., and Dargemont, C. (2014) Ubiquitylation by the Ltn1 E3 ligase protects 60S ribosomes from starvation-induced

- selective autophagy. *J. Cell Biol.* **204**, 909–917
84. Mao, K., Wang, K., Liu, X., and Klionsky, D. J. (2013) The Scaffold Protein Atg11 Recruits Fission Machinery to Drive Selective Mitochondria Degradation by Autophagy. *Dev. Cell.* **26**, 9–18
 85. Farré, J.-C., Burkenroad, A., Burnett, S. F., and Subramani, S. (2013) Phosphorylation of mitophagy and pexophagy receptors coordinates their interaction with Atg8 and Atg11. *EMBO Rep.* **14**, 441–9
 86. Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. (1999) Apg7p/Cvt2p: A Novel Protein-activating Enzyme Essential for Autophagy. *Mol. Biol. Cell.* **10**, 1367–1379

Chapter 4 - Proteasome Localization is Regulated Through Mitochondrial Function and Proteasome Quality Control

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Abstract

Active 26S proteasomes are responsible for the specific recognition and degradation of most short-lived polypeptides. As a result, these ATP-dependent proteases are essential for regulating various cellular events. While under optimal conditions proteasomes mainly reside in the nucleus, stress conditions can change this localization. For instance, carbon starvation in yeasts is known to lead to the re-localization of proteasomes from cell nuclei to cytosolic proteasome storage granules (PSGs). However, how PSG formation is regulated remains poorly understood. Our hypothesis was that the drop in energy level during carbon starvation is an important trigger in PSG formation. To test this, we interfered with mitochondrial function and ATP maintenance using chemical inducers of mitochondrial stress or phosphate starvation. Our data show that starvation for phosphate, or induction of mitochondrial stress, results in PSG formation independent of carbon starvation. As removal of ATP results in proteasome dissociation into sub-complexes, we next tested if factors known to stabilize the RP-CP interaction, namely overexpression of the proteasome-associated protein Ecm29, or proteasome inhibitor treatment, would interfere with PSG formation. Our data show that proteasome inhibitors and Ecm29 both limit PSG formation. Additionally, we found that mutants with increased levels of Ecm29, including a previously characterized *NAT3* mutant, fail to form PSGs in part due to Ecm29. Further, we show that the Mpk1 kinase cascade is important for PSG formation. Our working model, based on these data, is that PSG-inducing stimuli provide a signal that induces proteasome dissociation. This allows efficient nuclear export and targeting to granules. Proteasome inhibitors and Ecm29 stabilize the RP-CP interaction thereby preventing granule formation.

Introduction

Protein degradation is a tightly regulated process that governs numerous cellular events. Two major pathways in eukaryotes carry out this degradation (1–3). Macroautophagy (hereafter referred to as autophagy) is a cellular process of self-eating involving the targeting of proteins via the de novo synthesis of double membrane autophagosomes that engulf cytosolic cargo (1, 3, 4). These engulfed cargoes subsequently fuse with vacuoles (lysosomes in mammals) and are degraded. Autophagy facilitates cell survival by clearing, among others, misfolded/aggregated proteins, damaged organelles, viruses, and multi-subunit complexes (5–12). Autophagy can occur selectively or non-selectively depending on the stimulus and substrate. The second pathway, proteasome-dependent degradation, is largely selective. This selectivity is achieved through a cascade of enzymes that together with proteasomes forms the ubiquitin proteasome system (UPS). In a number of sequential steps, the enzymes activate ubiquitin and ultimately ligate it to a substrate (2, 13, 14). Ubiquitination of substrates can serve various biological functions, but is best known for its role in targeting substrates to the proteasome for degradation (15–18). The regulatory particle (RP) of the proteasome holoenzyme has receptors for ubiquitin and upon substrate binding, the substrate is de-ubiquitinated, unfolded, and translocated into the core particle (CP). The CP is composed of subunits α 1–7 that form two outer heptameric rings, and β 1–7 which form two inner heptameric rings, thus producing a cylindrical structure with a central channel (19, 20). Three catalytic subunits within the CP serve to cleave polypeptides passing through the channel (21, 22). Examples of substrates include proteins involved in cell cycle regulation, regulation of stress responses, cell differentiation and immune regulation (13, 23–26). Regulated protein degradation through both autophagy and the UPS are essential for cellular health and survival. In accordance with this, it has become clear in recent years that autophagy and the UPS can function synergistically (1, 27, 28). When one pathway is blocked, either pharmacologically or through genetic mutation, it has been shown that the other can compensate for the loss of function. Further, factors that regulate autophagy have

been found to be substrates of the UPS, and proteasomes themselves are substrates for autophagy (7, 8, 29–31).

Whereas proteasomal degradation is highly selective, autophagic degradation can occur non selectively. Evidence from our lab and others point to selective regulation of proteasome autophagy (7, 32–34). In addition to the requirement for proteins not involved in general autophagy, proteasome autophagy does not occur under all conditions that induce general autophagy (Chapter 3, submitted). The most striking of these is carbon starvation where proteasomes are not only excluded from autophagic degradation, but are re-localized to cytosolic punctate structures termed proteasome storage granules (PSGs) (35–37). The mechanisms that govern proteasome localization under these conditions remains to be fully elucidated. In the present work, we have identified additional conditions that result in the formation of proteasome containing granules in yeast. We have found that mitochondrial function plays a role in PSG regulation. Inhibiting mitochondria at different electron transport chain (ETC) complexes induced PSGs. We further report that factors known to stabilize RP-CP interactions largely restrict proteasome granules. In particular, the proteasome chaperone Ecm29 appears to restrict proteasome granule formation under various conditions. We have also found that map kinase signaling plays a role in proteasome granule formation. Intriguingly, this was linked to mitochondrial function. In all, our data point to a model of proteasome disassembly followed by nuclear export to facilitate granule formation under conditions of impaired mitochondrial function.

Material and Methods

Yeast Strains – All strains used in this study are reported in supplementary table 1. Our background strains are the W303 derived SUB61(Mat α) or Sub62(MatA);(*lys2-801 leu2-3, 2-112 ura3-52 his3- Δ 200 trp1-1*) that arose from a dissection of DF5 (15). Standard PCR based procedures (primers and plasmids presented in supplementary table 2) were used to delete specific genes from the genome, change the promoters, or introduce sequences at the endogenous locus that resulted in the expression of C-terminal fusions of GFP or mCherry (38–40).

Yeast Growth Conditions – Overnight cultures of yeast were diluted to an OD₆₀₀ of 0.5 and grown in YPD medium to an OD₆₀₀ 1.5 (approximately 4hrs). To induce

starvation, cultures growing logarithmically in YPD (dextrose), YPR (raffinose) or YPG (glycerol) were centrifuged, washed with respective starvation medium, re-inoculated at an OD₆₀₀ of 1.5, and incubated at 30 °C with constant shaking. Starvation media lacking carbon or phosphate was made as previously described and using established methods (Chapter 3, submitted) For drug treatments, cultures were grown to an OD₆₀₀ of 1.5 as above, then treated with drugs. PS341 (bortezomib) and Mg132 were used at a final concentration of 100 µM. Sodium azide, antimycin A and oligomycin A, were used at final concentrations of 0.5 µM, 0.1 mM, and 2.5 µM respectively. CCCP was used at a final concentration of 10 µM and potassium cyanide was used at concentrations shown in Fig. 2E. Anoxic growth was performed by transferring 1.8mL of logarithmically growing culture to a sealed 2 mL culture tube and incubating without shaking for 24 hrs. at 30 °C. Data shown are representative of consistently observed trends from at least three independent biological replicates

Yeast Phenotype Assays – 1 OD of logarithmically growing cells were pelleted then resuspended in 133 µL of ddH₂O, and 1 in 4 serial dilutions were performed. Plates were spotted with diluted cultures and grown for 24hrs at 30 °C unless otherwise indicated. Viability was determined by harvesting 1 OD of culture after indicated treatments. Sample was diluted by a factor of 10⁵, plated on YPD agar, and grown at 30 °C. Colonies were then counted and CFUs/mL determined. Optical density was used to compare growth rate over time.

ATP Measurements – 1 OD of cell was collected following indicated treatments and frozen in liquid nitrogen. Pellets were resuspended in 50 µL of 2.5% trichloro-acetic-acid (TCA) and boiled for 3 mins. Sample was centrifuged (13,000XG) for 1 min and 2 µL supernatant was added to 98 µL of 25 mM Tris-HCL [pH 8.8]. 10 µL of this mixture was combined with 40 µL of 25 mM Tris-HCL [pH 8.8] in a white 96 well plate. 50 µL of rLuciferase/Luciferin reagent (Promega, ENLITEN® ATP Assay System) was added to each well and luminescence was monitored using a Wallac Victor 2 plate reader.

Protein Lysates and Electrophoresis – For western blots, 2 ODs of cells were collected at indicated timepoints and stored at -80 °C. Lysis was completed using previously established methods (41). Following electrophoreses, samples were transferred to PVDF membranes and immuno-blotted using antibodies against indicated proteins or

tags followed by the appropriate horseradish-peroxidase conjugated secondary antibodies. Antibodies used were anti-GFP (1:500; Roche, #11814460001), anti-Ecm29 (1:1000; a generous gift from the Finley lab), anti-mCherry (1:1000; Abcam, ab183628), and anti-Pgk1 (1:5000; Invitrogen, #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. Native gel analyses were performed as previously described (42). Briefly, 50 ODs of cells were pelleted, washed in ddH₂O, and resuspended in 50 μ 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 Diagnostics. After cryogrinding, lysates were resuspended in 50 μ L lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM ATP, 5 mM MgCl₂, 1 mM EDTA). For native gel analyses, equal volumes of lysate were loaded on gel and separated by electrophoresis (90 V, 2.5 to 3 hrs., 4 °C). Gels were scanned on Typhoon 9410 or Trio imager (excitation at 488 nm and a 526SP emission filter for GFP and excitation at 532 nm and a 560LP or 610 BP30 emission for mCherry). Gels were then incubated with suc-LLVY-AMC and imaged using the G-box imaging system (Syngene) with GeneSnap software; (UV excitation, 440 nm band-pass emission filter) to visualize peptidase activity of proteasome complexes

Fluorescence Microscopy – All microscopy was performed with live yeast where proteasome subunits (Rpn1-GFP and α 1-GFP) or Ecm29 (mCherry) were fluorescently tagged at their endogenous locus with expression driven by the endogenous promoter. GFP-Atg8 was produced as previously described (43) After indicated treatments, approximately 2 ODs of cells were pelleted, washed with PBS, then resuspend in 30 μ L of PBS. 3 μ L of sample was then mounted on 1% soft agar slides as described by E. Muller (<https://www.youtube.com/watch?v=ZrZVbFg9NE8>) (44). All imaging by fluorescence microscopy was done within 10 mins following wash to avoid the effects of prolonged incubation on slides as previously described (Chapter 3, submitted). 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 R3tm

camera (QImaging). Images were collected using Metamorph software (Molecular Devices) and analyzed using FIJI. All quantification was performed using FIJI.

Table 4-1. Strain List

Strain	Genotype (<i>lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1</i>)	Figure	Source
SUB61	MAT α	2f-i 3g,f	(1)
SUB62	MATA		(1)
sJR502	MAT α <i>nas2::CloNat hsm3::G418</i>	4g	(4)
sJR556	MAT α <i>rpt5::rpt5Δ3-(HYG)</i>	4g	(3)
sJR778	MAT α <i>rpt5::rpt5Δ3-(HYG) rpn1::RPN1-GFP (HIS3)</i>	4k S3	(6)
sJR780	MAT α <i>rpt5::rpt5Δ3-(HYG) rpn1::RPN1-GFP (HIS3) ecm29::G418</i>	4k	(6)
sJR798	MAT α <i>rpn1::RPN1-GFP (HIS3) ecm29::TRP</i>	3g,h 4g	(6)
sJR861	MAT α <i>rpn1::RPN1-GFP (HIS3)</i>	1a-c,g 2a-e 3a-c 4k 5a-e 6e,f S1-3,5	(2)
sJR862	MAT α <i>nas2::CloNat hsm3::G418 rpn1::RPN1-GFP (HIS3)</i>	4h,i S3	(6)
sJR863	MAT α <i>nas6::TRP rpn14::HYG rpn1::RPN1-GFP (HIS3)</i>	S3,4	(6)
sJR915	MAT α <i>rpn1::RPN1-GFP (HIS3) nat3::HYG</i>	5a-e S3	(6)
sJR921	MAT α <i>rpn1::Rpn1-GFP (HIS3) ecm29::ECM29-mCherry (HYG)</i>	3d-f	(6)
sJR924	MATA <i>ecm29::Ecm29-mCherry (G418)</i>	4d-g	(6)
sJR934	MAT α <i>rpt5::rpt5Δ3-(HYG) ecm29::Ecm29-mCherry (G418)</i>	4d-g	(6)
sJR967	MAT α <i>nas2::CloNat hsm3::G418 ecm29::Ecm29-mCherry (HYG)</i>	4d-g	(6)
sJR1084	MAT α <i>scl1::SCL1-GFP (HIS3)</i>	4h-j 6a-d S4	(5)
sJR1135	MAT α <i>rpn1::RPN1-GFP (HIS3) ecm29::pGPD-Ecm29 (CloNat)</i>	4a-c	(6)
sJR1136	MAT α <i>rpn1::RPN1-GFP (HIS3) ecm29::pADH-Ecm29 (G418)</i>	4a-c	(6)
sJR1143	MATA <i>rpn1::RPN1-mCherry (G418)</i>	1e,f	(6)
sJR1149	MATA <i>rpn1::RPN1-mCherry (G418) GFP-Atg8 (-ura, pJR683)</i>	1d	(5)
sJR1151	MAT α <i>rpn1::RPN1-GFP (HIS3) mpk1::G418</i>	6a-d S3	(5)
sJR1198	MAT α <i>rpn1::RPN1-GFP (HIS3) rpn4::(G418)</i>	4j	(6)
sJR1216	MAT α <i>scl1::SCL1-GFP (HIS3) mpk1::HYG</i>	6a-d	(5)
sJR1224	MAT α <i>rpn1::RPN1-GFP (HIS3) fus3::G418</i>	S5	(6)
sJR1232	MAT α <i>rpn1::RPN1-GFP (HIS3) hog1::G418</i>	S5	(6)
sJR1264	MAT α <i>rpn1::RPN1-GFP (HIS3) ggc1::G418</i>	S1	(6)
sJR1270	MAT α <i>rpn1::RPN1-GFP (HIS3) snf1::G418</i>	S1	(6)
sJR1308	MAT α <i>rpn1::RPN1-GFP (HIS3) adc17::G418</i>	S5	(6)
sJR1315	MAT α <i>nas6::TRP rpn14::HYG scl1::SCL1-GFP (HIS3)</i>	S4	(6)
sJR1316	MAT α <i>nas2::CloNat hsm3::G418 scl1::SCL1-GFP (HIS3)</i>	4h,i	(6)
sJR1317	MATA <i>ecm29::Ecm29-mCherry (G418) rpn1::RPN1-GFP (HIS3) atg1::HYG</i>	S1	(6)
sJR1326	MAT α <i>rpn4::(G418) scl1::SCL1-GFP (HIS3)</i>	4j	(6)
sJR1328	MAT α <i>rpn1::RPN1-GFP (HIS3) bck1::HYG</i>	6f	(5)
sJR1387	MAT α <i>rpn1::RPN1-GFP (HIS3) mkk2::HYG</i>	6e	(5)
sJR1390	MAT α <i>rpn1::RPN1-GFP (HIS3) mkk1::G418</i>	6e	(5)
sJR1392	MAT α <i>rpn1::RPN1-GFP (HIS3) mkk2::HYG mkk1::G418</i>	6e	(5)
sJR1419	MAT α <i>rpn1::RPN1-GFP (HIS3) ecm29::TRP nat3::HYG</i>	5a-e	(6)
a) All strains have the DF5 background genotype (<i>lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1</i>) 1. Finley, D., Ozkaynak, E., and Varshavsky, A. (1987) Cell 48, 1035-1046 2. Waite, K.A., De La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2015) JBC M115.699124 3. Lee, Stella Yu-Chien, Alina De la Mota-Peynado, and Jeroen Roelofs. (2011) JBC 286(42): 36641–51. 4. De La Mota-Peynado, Alina et al. (2013). JBC 288(41): 29467–81. 5. Chapter 3 6. This study			

Table 4-2. Primer List

Primer	Genotype	Template	Sequence (5' to 3')
rvrs/rpn1	<i>rpn1::rpn1-mCherry (G418)</i>	pBS34 ³	TTTGAATTTTCTATTCTGTTGATATTGCCAAAAGCTATTCAGTTTAATCGATGAATTCGAGCTCG
pRLs2-Rpt5	<i>rpt5::rptD3 (HYG)</i>	pYM24 ²	AATATGTAGATATGTGAATGGCGGCTTGATAAATCAAAATATTATTATTATCGATGAATTCGAGCTCG
pRL-s3Rpt5d3	<i>rpt5::rptD3 (HYG)</i>	pYM24 ²	TCGTTGAGGGTATAAGTGAAGTTCAAGCAAGAAAATCGAAATCGGTATCCTAGGGCGCGCCAGATCTGTT
pRL303	<i>hog1::G418</i>	pFA6a-kanMX6	CGGTAACCAGGCCATACAGTACGCTAATGAGTTCCAACAGCGTACGCTGCAGGTCGAC
pRL303	<i>hog1::G418</i>	pFA6a-kanMX6	ACATCAAAAAGAAGTAAGAATGAGTGGTTAGGGACATTAAATCGATGAATTCGAGCTCG
pRL327	<i>Ecm29::pAdh-Ecm29 (G418)</i>	pYM-N6 ²	CTGTTCTAACGGAGGCATG
pRL328	<i>Ecm29::pAdh-Ecm29 (G418)</i>	pYM-N6 ²	GTATGTGAATGCTGGTCGC
pRL344	<i>nat3::HYG</i>	pFA6a-hphNT1 ²	AAGAAAAAAATCCCTCGTCGATAATATAAATCGTACGCTGCAGGTCGAC
pRL345	<i>nat3::HYG</i>	pFA6a-hphNT1 ²	GGTTCATTATTATGTTCTGAGTATGAGGACGAGGTAATACATCGATGAATTCGAGCTCG
pRL385	<i>ecm29::ECM29-mCherry (HYG) (G418)</i>	pBS34 ³ , pBS35 ³	AACGAGCAGAATTGAGTTGGAATTGAAAAATATTGGTCTAGGTCGACGGATCCCCGGG
pRL386	<i>ecm29::ECM29-mCherry (HYG) (G418)</i>	pBS34 ³ , pBS35 ³	TTTATTAAGTGTCTATTATCAATATGAAAAAGTTTCGGATCGATGAATTCGAGCTCG
pRL401	<i>rpn1::rpn1-mCherry (G418)</i>	pBS34 ³	AGTAATTTTAAAGAAGAACCCCTGACTATCGTGAAGAGGAGGGTCGACGGATCCCCGGG
pRL564	<i>rpn4::G418</i>	pFA6a-kanMX6	TTGTATCTTTTCAAAAGTTTCTAGAATTTTCAAGCAATCCGTACGCTGCAGGTCGAC
pRL565	<i>rpn4::G418</i>	pFA6a-kanMX6	GGTTTTCTTCTTTATCTCCTATATAATTTGAACCTTAAATCGATGAATTCGAGCTCG
pRL652	<i>fus3::G418</i>	pFA6a-kanMX6	CTACAAGGAAATAAGGCAGAGAAAAAGAAAGGAAAAATAATCGTACGCTGCAGGTCGACG
pRL653	<i>fus3::G418</i>	pFA6a-kanMX6	ACATTGTTCTTCGGGTTGATATTTAATGATAATGATGGCATCGATGAATTCGAGCTCG
pRL698	<i>hog1::G418</i>	pFA6a-kanMX6	ATCAAAACAAAGCATCTTCAAAGTATTAGAAAAGGGAATCGTACGCTGCAGGTCGACG
pRL699	<i>hog1::G418</i>	pFA6a-kanMX6	AGCTGAATGCCAAGGAAAAATAGACAAGAAGTTCATGTACTATCGATGAATTCGAGCTCG
pRL702	<i>snf1::G418</i>	pFA6a-kanMX6	TTTGTAAACAGTTTGTCTACACTCCCTAATAAAGTCAACCGTACGCTGCAGGTCGACG
pRL703	<i>snf1::G418</i>	pFA6a-kanMX6	AAAAAAGGGAACCTCCATATCATTCTTTACGTTCCACCAATCGATGAATTCGAGCTCG
pRL718	<i>adc17::G418</i>	pFA6a-kanMX6	TAAAGCAAATCAAACATAATAACTACTACAAGTAACATACGTACGCTGCAGGTCGACG
pRL719	<i>adc17::G418</i>	pFA6a-kanMX6	TGACGTGAAAATGATGCGCAGTAAACTAAATCCCGTCTCATCGATGAATTCGAGCTCG
- Goldstein, A. L. & McCusker, J. H. Three new dominant drug resistance cassettes for gene disruption in <i>Saccharomyces cerevisiae</i>. <i>Yeast</i> 15, 1541–53 (1999). - Janke, C. et al. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. <i>Yeast</i> 21, 947–62 (2004). - Hailey DW, Davis TN, Muller EG. Fluorescence resonance energy transfer using color variants of green fluorescent protein. <i>Methods Enzymol.</i> 351:34-49 (2002).			

Results

Proteasome Granule Formation is Affected by Different Carbon Sources—Carbon starvation does not induce proteasome autophagy, otherwise known as proteophagy (7, 36, 37), which suggests that some conditions that induce general autophagy, do not cause proteophagy. However, until recently it was debated whether carbon starvation induces general autophagy or not. Some of this confusion appears to derive from subtle differences in washing cells before starvation, but primarily it is due to the carbon source cells are grown in prior to starvation. This has recently been shown to determine if general autophagy is induced or not (45). For example, when yeast cells are grown on a preferred carbon source such as dextrose (D-glucose), and subsequently starved for carbon, they do not induce general autophagy. On the other hand, when cells are grown on raffinose, another fermentable carbon source, or a non-fermentable (ex. glycerol) carbon source, general autophagy was induced upon carbon starvation. This was partly attributed to catabolite repression where genes associated with respiration and the utilization of carbon sources other than dextrose are repressed (45, 46). Thus, the metabolic state of the cell and transcriptional profile, appear to determine whether or not autophagy is induced. Our previous experiments looking at proteasome autophagy following carbon starvation were performed following growth in dextrose. Therefore, we tested whether proteasome autophagy would be induced if cells were grown in raffinose or glycerol prior to starvation. We utilized the Rpn1-GFP fusion protein previously described to monitor proteasome autophagy and localization (47). Like dextrose, growth in these carbon sources did not result in robust proteasome autophagy upon switching to carbon starvation, because the amount of “free” GFP produced upon carbon starvation, was minimal (Fig. 4-1A). Indeed, the amount of “free” GFP detected at 24 and 48 hrs of carbon starvation was less than or equivalent to “free” GFP detected at 6 hrs of nitrogen starvation where minimal proteasome autophagy is observed (7) (Chapter 3, submitted). However, we did observe a difference in the amount of proteasome granules induced depending on the carbon source. Pre-growth in dextrose resulted in a large number of cells with multiple granules (~ 43%), while pre-growth in raffinose resulted in less than 5% of cells with multiple granules (Fig. 4-1B). Proteasome granules are also induced when cells enter quiescence (36, 48) (Chapter 3, submitted).

We hypothesized that PSGs induced in quiescence were affected by carbon source. Prolonged growth in dextrose and raffinose media induced proteasome granules. While there appeared to be more granules at 72 hrs in cells pre-grown in dextrose (Fig. 4-1C), we did not observe a difference in the number of granules formed per cell. Here, both conditions showed the formation of a single dominant granule.

When yeast were switched from rich dextrose media to carbon starvation media, it was observed that multiple phagophore assembly sites (PAS) formed in yeast which typically have one, for example during nitrogen starvation. This likely affects autophagy induction and thus contributes to the lack of autophagy in this condition. On the other hand, pre-growth in other carbon sources resulted in a single PAS and general autophagy (45). The number of proteasome granules observed per cell when we switched from dextrose or raffinose to carbon starvation resembled these observations. We wondered if proteasomes may be co-localized with these structures during carbon starvation. Using a strain expressing GFP-Atg8 and Rpn1-mCherry, we monitored proteasome and PAS localization upon carbon starvation after pre-growth in dextrose, raffinose, or glycerol. We did not detect proteasome co-localization with PAS either early (1hr) or late (24hrs) in carbon starvation (Fig. 4-1D). Consistent with this observation, factors required for autophagy induction under carbon starvation (Atg1, Snf1 and Ggc1) were not required for proteasome granule formation (supplementary figure 1). Atg1, Snf1 and Ggc1 activate Mec1 in a process that tethers it to the mitochondrial outer membrane upon carbon starvation. This mitochondrial localization is required to maintain mitochondrial respiration, a prerequisite for autophagy induction (49). That these same factors were not required for proteasome granule formation shows that induction of autophagy under carbon starvation is not required for the formation of proteasome granules.

Nevertheless, we did observe a difference in PSG dynamics that seems to correlate with mitochondrial respiration. Most cells formed granules at 24hrs when grown in dextrose (80%) or raffinose (60%), while growth in glycerol resulted in substantially less granule formation (40% at 24 hrs and 10% at 48 hrs) (Fig. 4-1D). There was a reverse correlation with the level of general autophagy induced (45). When yeast are deprived of dextrose (like growth in raffinose and particularly glycerol),

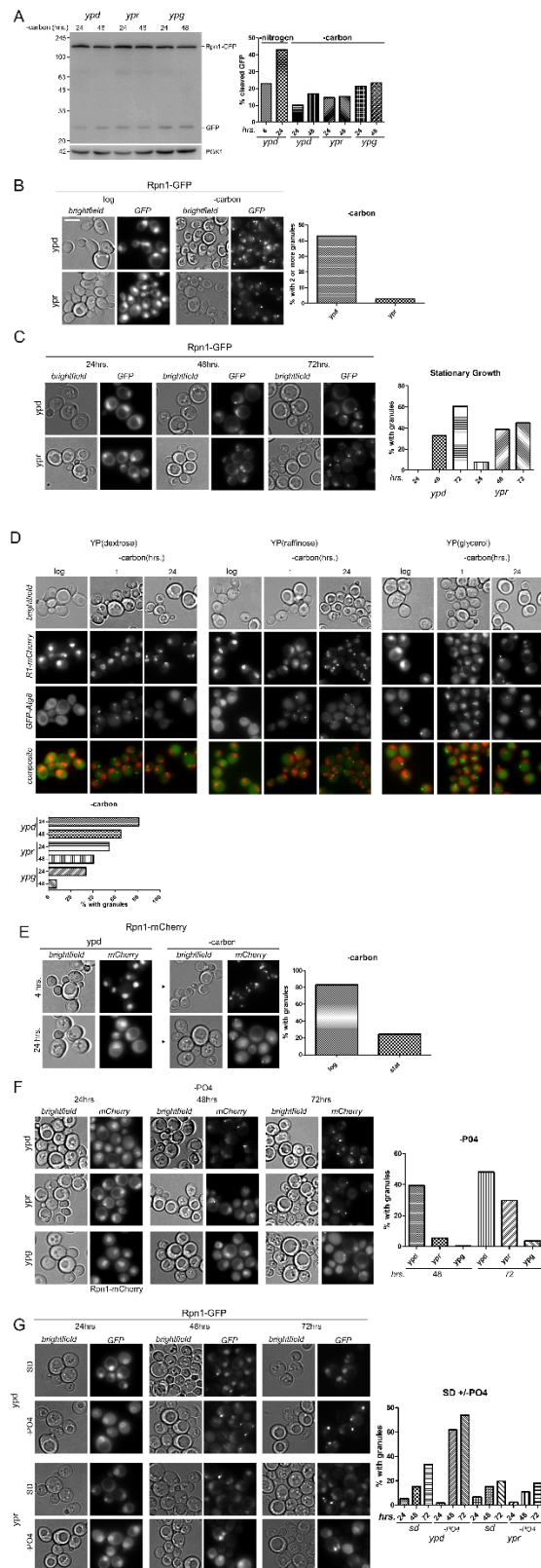
catabolite repression is released, and the yeast will utilize mitochondria instead of glycolysis/fermentation to produce the majority of their ATP (46). It also has been shown that the extent of autophagy induction upon carbon starvation is highly correlated with the oxygen consumption rate and cellular ATP levels (45). It appears that for the proteasome, conditions that maintain mitochondrial function correlate with nuclear localization (Fig. 4-1D). To further test this, we compared starvation response after growth for 4 or 24 hrs in rich media containing dextrose. At 24 hrs, all dextrose has been fermented and yeast switches to respiration for energy production. Indeed, starving yeast after 24 hrs of growth in dextrose showed a reduction in granule formation compared to 4 hrs (Fig. 4-1E). In all, the above observations led us to hypothesize that mitochondrial function and regulation of cellular ATP levels not only control autophagy induction under carbon starvation, but also proteasome localization.

To further test this, we starved cells for phosphate following growth in different carbon sources. This was based on the idea that cells lacking phosphate sources would be unable to maintain ATP levels as inorganic phosphate is required for ATP production (50). We indeed show a reduction in ATP compared to rich media when cells were phosphate starved (Fig. 4-2G, H). Phosphate starvation is also known to induce cell cycle arrest and a similar stress response as carbon starvation (51, 52). We have previously reported that phosphate starvation induced proteasome autophagy but to a lesser extent than nitrogen starvation at 24hrs (Chapter 3, submitted). Here, we show that prolonged phosphate starvation induced proteasome granules (Fig. 4-1F). Like PSGs induced by carbon starvation, these granules were also affected by the initial carbon source. Cells grown in raffinose that were starved for phosphate showed less PSGs compared to cells grown in dextrose. Even more striking, granules were almost entirely absent when cells were grown in glycerol (Fig. 4-1E). This is despite the fact that our phosphate starvation media always contained dextrose as a carbon source. Catabolite repression does not appear to play a role here as raffinose and glycerol pre-growth resulted in distinct phenotypes. Because granules induced by phosphate starvation are evident only at later timepoints (48 hrs), they could result from prolonged growth like granules formed during stationary phase. However, we found granules were induced to a much larger extent in SD media lacking phosphate compared to SD

complete media (Fig. 4-1G). When grown in raffinose, the number of cells with granules is largely reduced in complete media and phosphate starvation media (Fig. 4-1G). Here SD media and phosphate starvation media contained the indicated carbon source. These results are consistent with our observations made with carbon starvation. In all, proteasome granule formation appears to be in part dependent on the cellular energy status. Under conditions where mitochondrial respiration and ATP levels are perturbed, proteasomes appear to re-localize to PSGs more efficiently.

Mitochondrial Inhibition Induces Proteasome Granule Formation—The observed correlation between mitochondrial respiration and proteasome localization suggests cellular energy levels play a role in proteasome localization. This is interesting since ATP is an essential requirement for a fully functional 26S proteasome (14, 19). Furthermore, proteasome complexes are unstable *in vitro* in the absence of ATP (21), providing a potential mechanism between ATP maintenance, proteasome stability, and PSG formation. To test if other conditions that impact cellular ATP levels would also affect proteasome localization, we inhibited mitochondrial function using drugs targeting different components of the electron transport chain (ETC). Sodium azide, antimycin A and oligomycin A were used to target complex IV, complex III, and ATP synthase respectively. Consistent with our model, all three drugs induced proteasome granules in rich (dextrose) media (Fig. 4-2A). When we compared the formation of proteasome granules after sodium azide treatment following growth in dextrose vs raffinose, we observed that PSG formation was reduced when cells were grown in raffinose, as observed with phosphate starvation (Fig. 4-2B). Besides drugs, the absence of the final electron acceptor in the ETC, oxygen, would also inhibit the process of oxidative phosphorylation. Indeed, upon growth in anoxic conditions, proteasome granules were induced, and like granules described above, these were similarly affected by the initial carbon source (Fig. 4-2C).

Figure 4-1. Proteasome granule formation is affected by different carbon sources.



(A) Rpn1-GFP expressing cells were grown in rich media containing dextrose (ypd), raffinose (ypr) and glycerol (ypg) for four hrs then switched to SD media lacking carbon. 2 ODs of cells were harvested at indicated times and lysed as in Material and Methods. Immunoblotting for GFP and PGK1 were performed as in Materials and Methods. Quantifications of western blots were performed as previously described and compared to the amount of free GFP observed following nitrogen starvation at 6 and 24 hrs. **(B)** Cells grown as in A were imaged at log phase and following 20 hrs carbon starvation. Quantifications shows the percentage of cells with 2 or more granules. **(C)** Rpn1-GFP expressing cells were inoculated at OD=.38 and grown in rich media and microscopy performed at indicated times. Quantifications shows the percentage of cells with granules. **(D)** Rpn1-mCherry; GFP-Atg8 expressing cells were grown in rich media then switched to carbon starvation. Microscopy was performed at indicated times and quantifications show the percentage of cells with granules following 24 and 48 hrs of carbon starvation. **(E)** Rpn1-mCherry cells were grown logarithmically for 4 hrs or grown to stationary phase for 24 hrs and then switched to carbon starvation media. Quantifications show the percentage of cells with granules. **(F)** Rpn1-mCherry cells were grown in rich media for 4 hrs then switched to SD media lacking phosphate. Microscopy was performed at indicated times and quantifications show the percentage of cells with granules. **(G)** Rpn1-GFP expressing cells were grown in rich media for 4 hrs and then switched to SD complete media or SD lacking phosphate. Microscopy was performed at indicated times and quantifications show the percentage of cells with granules.

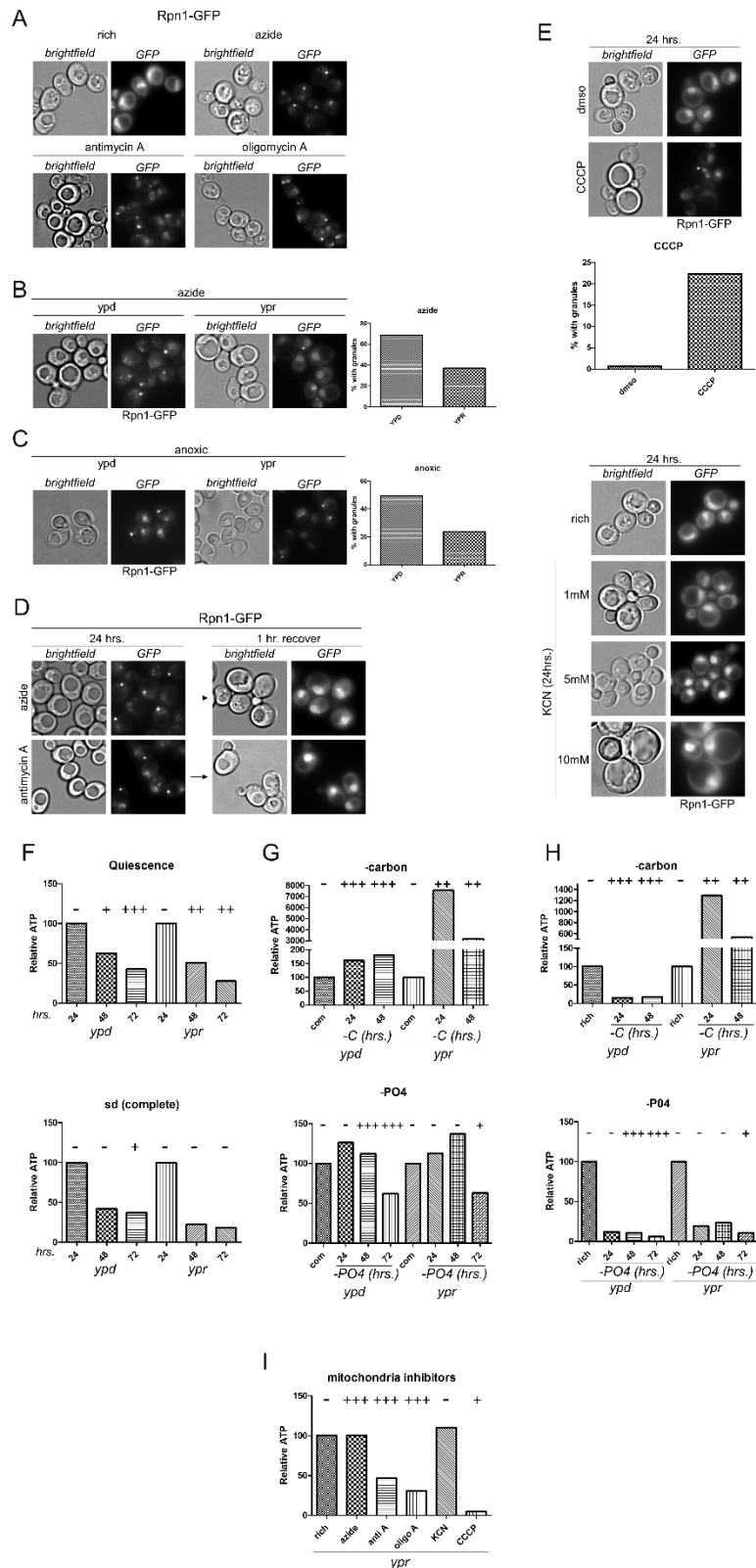
To test if granules induced through mitochondrial inhibition behave largely like proteasome granules induced by carbon starvation, we explored the reversibility of these granules. Carbon starvation-induced PSGs quickly disappear with proteasomes re-localizing to cell nuclei upon re-addition of a carbon source (36, 53). Similarly, granules induced by mitochondria inhibitors quickly returned to cell nuclei when we washed away the drugs (Fig. 4-2D). It appears some shared signal exist upon mitochondrial stress and carbon starvation that results in proteasome granule formation. Intriguingly, we did not detect induction of proteasome granules to a similar extent with all mitochondria inhibitors tested. For example, with the uncoupler CCCP, a more modest increase in proteasome granule formation was observed, while we did not detect any granules when we used potassium cyanide (KCN) at various concentrations (Fig. 4-2E). This is despite the fact that this drug targets the same complex as sodium azide (cytochrome c oxidase). KCN-treated cells grew slower than untreated cells and showed a similar growth rate as other mitochondria inhibitors (not shown). Furthermore, we observed an increase in cell size at high concentrations (Fig. 4-2E), indicating KCN is active in our hands but does not induce proteasome granules. Apparently mitochondrial inhibition or stress alone is not sufficient for this outcome.

We next wanted to determine the ATP levels upon the different treatments to see how that correlates with PSG induction. Prolonged growth in YPD or YPR resulted in a reduction in ATP over time which corresponded with PSG formation (Fig. 4-2F). Similarly, growth in SD-complete media also resulted in reduced ATP levels over time and an increase in PSGs (Fig. 4-2F, Fig. 1F). However, when we compared the amount of ATP detected following carbon or phosphate starvation, to ATP detected in SD-complete media, we found that ATP levels were lower in SD-complete (Fig. 4-2G). This was unexpected as this media contains dextrose, but it may be explained by the fact that these cells continue to grow and thus the carbon source is likely used up more quickly. We observed that with carbon starvation, ATP levels remained higher when cells were switched from raffinose instead of dextrose, consistent with previous reports (Fig. 4-2G, H) (45). Similarly, we observed that ATP remained higher when cells were pre grown in raffinose before phosphate starvation (Fig. 4-2G, H). These data are consistent with mitochondrial respiration playing a role in PSG formation as conditions

with more ATP correlated with less granule formation. The observation that ATP levels were lower in cells switched from dextrose to carbon starvation further supported this as these cells formed more granules (Fig. 4-2G). However, the opposite was seen when cells were switched from raffinose, where we detected higher ATP levels in carbon starvation than in rich media (Fig. 4-2H). This observation is again consistent with previous reports showing that cells pre-grown in raffinose are able to maintain respiration (45). Since we detected granules in this condition, albeit to a lesser extent, the fact that lower ATP was detected in rich media shows that ATP levels alone do not govern granule formation. Consistently, when we monitored ATP in phosphate starvation media, we observed that cells switched from raffinose maintained ATP levels longer than cells switched from dextrose. However, though phosphate starvation at all time points tested resulted in lower ATP than detected in rich media, proteasome granules are not detected at 24 hrs. (Figs. 4-1F, 4-2H).

When we monitored the effects of mitochondria inhibitors on the levels of ATP, we found that antimycin A, oligomycin A and CCCP led to a reduction in ATP levels (Fig. 4-2I). CCCP led to the largest reduction in ATP but displayed the most modest induction of granules (Fig. 4-2E, I). Sodium azide and KCN did not result in a reduction in ATP, despite the induction of granules in the case of sodium azide (Fig. 4-2I). The fact that both drugs inhibit cytochrome c oxidase and showed similar results in our ATP measurements serves as an internal control for our assays. In all, a clear correlation between ATP levels and PSG formation was not observed as we hypothesized. Though ATP may still play a signaling role in the process of PSG formation, low levels alone do not appear to explain this phenotype. One possible explanation is that in addition to ATP levels, other signaling molecules generated upon mitochondrial disruption promote granule formation.

Figure 4-2. Mitochondrial inhibition induces proteasome granule formation.



(A) Rpn1-GFP expressing cells were grown in rich media containing dextrose and then treated with mitochondria inhibitors as described in Materials and Methods.

Microscopy was performed 24 hrs following treatment. (B) Rpn1-GFP expressing cells were grown in media containing dextrose or

raffinose for 4 hrs and then treated with sodium azide as described in Materials and Methods. Quantifications show the percentage of cells with granules. (C) Rpn1-GFP

expressing cells were grown in media containing dextrose or raffinose for 4 hrs, then transferred to sealed culture tubes and incubated for 24 hrs.

Microscopy was then performed and quantifications show the percentage of cells with granules. (D) Rpn1-GFP expressing cells were grown in media containing dextrose and treated with sodium azide and antimycin A as described in materials and methods.

Microscopy was performed at this timepoint and following transfer of cells back to rich media for 1 hr. (E) Rpn1-GFP

expressing cells were grown in media containing dextrose then treated with CCCP or KCN for 24 hrs. Quantifications show the percentage of cells with granules after CCCP treatment. (F, G, H, I) ATP measurements under indicated conditions was carried out as described in materials and

methods. Quantifications show relative ATP as compared to untreated controls. “+” and “-” indicate the percentage

of cells that formed granules (- indicates 0 to 20 % of cells formed granules, + indicates more than 20 % of cells formed granules, ++ indicates more than 40 % of cells formed granules, and +++ indicates more than 60 % of cells formed granules).

Proteasome Inhibitors and the Chaperone Ecm29 Restrict Proteasome Granule

Formation—RP and CP appear to not be associated in PSGs, suggesting that factors that lead to RP-CP dissociation might facilitate PSG formation (35, 36). *In vitro*, the removal of ATP and ADP from 26S proteasomes leads to their dissociation into CP and RP subcomplexes, (21) providing a potential link between cellular energy levels and proteasome stability. However, our ATP measurements do not perfectly correlate with granule formation. To explore the need for dissociation further, we tested conditions that are known to stabilize the RP-CP interaction *in vitro*, namely proteasome inhibitors and the proteasome associated protein Ecm29 (21). We have previously shown that proteasome inhibitors strongly reduced PSG formation under carbon starvation (Fig. 4-3A, Chapter 3, submitted). Upon phosphate starvation the percentage of cells with granules remains very similar with a modest reduction at 48 hrs. However, we observed a reduction in granule intensity of about 50% (Fig. 4-3B, quantification). It should be noted that the phosphate starvation-induced granules appear larger in size than those induced by carbon starvation (Fig. 4-3B), suggesting they are different in nature and thus might respond differently to specific conditions. Next, we tested how proteasome inhibitor treatment changed the granules that form upon anoxic growth. Here, a reduction in granules was observed (supplementary figure 2). Overall, the inhibition of proteasomes seems to interfere with their re-localization to granules in the conditions tested.

To determine if proteasome inhibitors impacted granule formation or granule maintenance, we added proteasome inhibitors after granules had formed. Here, granules induced by carbon starvation persisted as long as 24 hrs following inhibitor addition (Fig. 4-3C top panel), showing the proteasome inhibitor does not somehow cause granule dissolution. The effect of inhibitors on proteasome re-localization from the nucleus led us to wonder if these drugs may impact re-localization back to the nucleus. To test this, we returned carbon starved cells to rich media with or without proteasome inhibitors. We did not detect any difference in the re-localization of

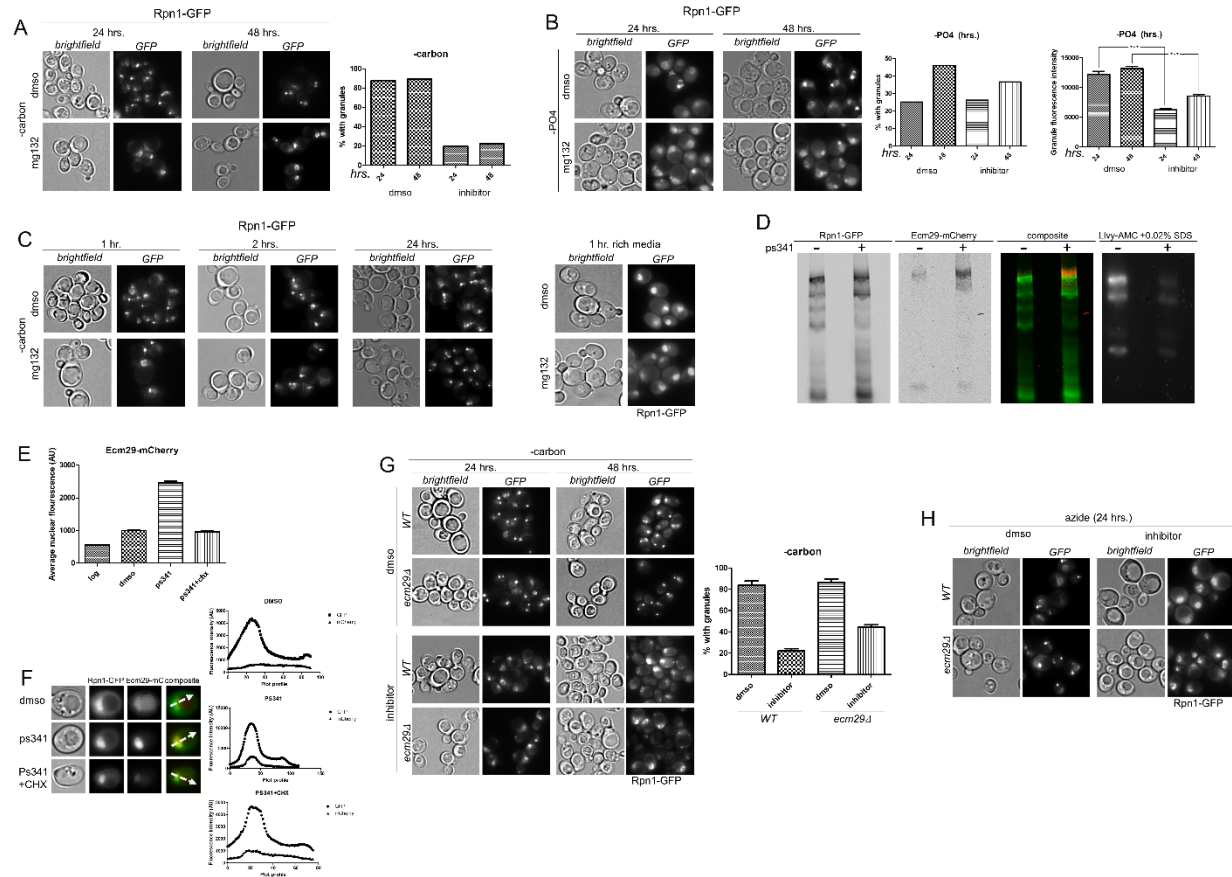
proteasome back to the nucleus which occurs upon carbon re-addition (Fig. 4-3C bottom panel). These data suggest that proteasome inhibitors affect proteasome re-localization in one direction, namely proteasome nuclear export.

We observed that proteasome inhibitor treatment caused an increase in the abundance of Ecm29, a proteasome associated protein. This was not surprising as the transcription factor Rpn4, a known proteasome substrate, regulates transcription of proteasome and proteasome-related genes amongst others (54–56). Upon proteasome inhibition, Rpn4 is stabilized, resulting in the upregulation of proteasome associated genes. Since, Ecm29 is an inhibitor of the 26S complex (56, 57) and we observed increased Ecm29 associated with 26S proteasomes upon inhibitor treatment (Fig. 4-3D), Ecm29 might augment the effect of proteasome inhibitors or proteasome inhibitors might act via Ecm29 upregulation. Interestingly, the increased level of Ecm29 in the presence of inhibitors was largely localized to the nuclei of cells where it co-localized with proteasomes (Fig. 4-3E, F). Plot profiles show increased Ecm29 and proteasome abundance, as well as colocalization (Fig. 4-3F). The apparent vacuolar mCherry localization in the DMSO control was shown to result from autofluorescence (not shown). Increased Ecm29 abundance in the presence of inhibitors was dependent on protein synthesis, as the translation inhibitor cycloheximide largely abolished this phenotype (Fig. 4-3E, F). Similar to proteasome inhibitors, Ecm29 alone is able to stabilize proteasome complexes *in vitro* in the absence of ATP (21). Therefore, we wanted to test if proteasome inhibitors regulate proteasome localization (in part) through Ecm29. Ecm29 was not required for efficient induction of PSG as both WT and *ecm29Δ* cells starved for carbon showed similar levels of granules (Fig. 4-3G). When we tested if proteasomes inhibitor treatment was still able to efficiently block granule formation in an *ecm29Δ* strain, we observed that without Ecm29 present, more granules formed after inhibitor treatment (Fig. 4-3G). Thus, proteasome inhibitors in part act through the upregulation and recruitment of Ecm29 to proteasomes. In all, it appears that upon proteasome inhibition, the drug and Ecm29 work together to restrict proteasome granule formation. We next treated cells with sodium azide in the presence of inhibitors and found a similar reduction in PSG formation as seen with carbon starvation. Surprisingly, when we tested if Ecm29 was involved in the formation of these granules, we did not

observe a difference between *WT* and *ecm29Δ* cells (Fig. 4-3H) This shows that proteasome inhibitors alone can restrict granule formation. Further, it appears Ecm29 is involved in regulating PSGs only under certain conditions. We sought to determine further the role of this chaperone in regulating these granules.

Ecm29 Restricts Proteasome Granule Formation—To further elucidate the role of Ecm29 in regulating proteasome localization, independent of the Rpn4 feedback loop, we generated mutants expressing Ecm29 from different promoters. We replaced the endogenous Ecm29 promoter with an ADH (alcohol dehydrogenase) or a GPD (glyceraldehyde 3-phosphate dehydrogenase) promoter. This resulted in a high level of overexpression from the pGPD-*ecm29* mutant, whereas the pADH-*ecm29* mutant appeared to express close to WT levels or less of Ecm29 under carbon starvation (Fig. 4-4A). In the presence of proteasome inhibitors, we observed that the WT had more Ecm29 bound proteasome than the ADH mutant, while the GPD mutant retained the highest expression level. This suggested that expression of Ecm29 in these mutants is no longer under Rpn4 regulation. Ecm29 binding to proteasomes caused a slower migration of 26S proteasomes on native gel (Fig. 4-4A, middle three lanes) as we have reported previously and also confirmed by immunoblotting for Ecm29 (56, 57). The levels of Ecm29 did not appear to impact granule formation upon carbon starvation as both the overexpression as well as a deletion of *ECM29* displayed similar percentages of cells with granules upon microscopic analyses (Fig. 4-3G). However, upon inhibition of proteasomes during carbon starvation, Ecm29 levels appeared to influence granule formation. Microscopically, we observed that cells expressing pGPD-*ecm29* formed similar amounts of granules as WT, which is consistent with the ability of WT to upregulate Ecm29 levels upon proteasome inhibitor treatment. However, pADH-*ecm29* showed more granules under this condition, as only a subset of proteasomes bound Ecm29 and feedback mechanism to upregulate Ecm29 was lost (Fig. 4-4B). When we tested different inducers of proteasome granules, we observed large reductions in granule formation when *ECM29* was over-expressed. When cells were grown to stationary phase in raffinose (but not dextrose), we did not detect proteasome granules

Figure 4-3. Proteasome inhibitors and the chaperone Ecm29 restrict proteasome granule formation



(A) Rpn1-GFP expressing cells were grown in media containing dextrose for 4 hrs. then switched to carbon starvation media with either DMSO as a control or MG132 as described in materials and methods. Microscopy was performed at indicated times. Quantifications show the percentage of cells with granules following treatment. (B) Rpn1-GFP expressing cells were grown as in A and switched to SD media lacking phosphate with or without MG132. Microscopy was performed at indicated times and quantifications show the percentage of cells with granules and granule fluorescence intensity. Error bars indicate SEM and p-values were determined using an unpaired, two-tailed Students t-test (* <0.01 , ** <0.001 , *** <0.0001) (C) Carbon starved cells expressing Rpn1-GFP were treated with DMSO or MG132 for indicated timepoints (top) or switched to rich media containing DMSO or MG132. (D) Rpn1-GFP; Ecm29-mCherry expressing cells were grown to log phase (~4 hrs.) then treated with DMSO or PS341 for 24 hrs. Native lysis was performed as described in materials and methods and protein samples were run on native gel. GFP and mCherry images were collected using a typhoon imager. Suc-LLVY-AMC peptidase assay was performed as described in materials and methods. (E) Quantifications showing the average nuclear fluorescence intensity following PS341 treatment for mCherry tagged Ecm29. Cells were grown to log phase the treated with DMSO, PS341, or PS341+cycloheximide. Error bars indicate SEM. (F) Cells from E were analyzed using FIJI plot profile to monitor fluorescence intensity and colocalization of Ecm29-mCherry and Rpn1-GFP. (G) WT and *ecm29Δ* yeast expressing Rpn1-GFP were grown to log phase and switched to carbon starvation media with DMSO or PS341. Microscopy was performed at indicated times and quantifications show the percentage of cells with granules at 24 hrs. (H) WT and *ecm29Δ* yeasts were grown to log phase then treated with sodium azide as described in materials and methods. Microscopy was performed at 24 hrs.

in the pGPD-*ecm29* mutant while an increase was observed for the pADH-*ecm29* mutant (Fig. 4-4C). A similar increase for this mutant was observed when cells were treated with oligomycin A, while the pGPD-*ecm29* mutant again showed a decrease in granule formation (Fig. 4-4C). When we treated these strains with antimycin A, both mutants showed a decrease in granule formation, however the pGPD-*ecm29* mutant showed a much larger decrease (Fig. 4-4C). In all, the pGPD-*ecm29* mutant consistently has less PSGs than the pADH-*ecm29* mutant or the WT in these conditions. Thus, it appears increased levels of Ecm29, in part dependent on Rpn4, restrict proteasomes to the nucleus and prevent granule formation.

We next tested mutant yeast strains that have increased levels of Ecm29 bound to proteasomes. Namely, mutants with deletion of the last 3 amino acids of the proteasome subunit Rpt5, or deletion of the molecular chaperones Nas2 and Hsm3 (57, 58). We also included a *NAS6* and *RPN14* mutant in some of our analyses, as proteasomes in these mutants are enriched with Ecm29 as well. Upon tagging of Ecm29 with mCherry in *WT*, *Rpt5Δ3*, and *nas2Δhsm3Δ* strains, we detected increased levels of Ecm29 in the nuclei of mutants compared to WT. In particular this was seen when we subjected the *nas2Δhsm3Δ* mutant to temperature stress, as previously reported (Fig. 4-4D,E) (59, 60). Consistently, we detected increased levels of Ecm29 by western blot in these mutants (Fig. 4-4F, supplementary Fig. 4-3). We also confirmed that this increased the amount of Ecm29-mCherry that migrated with 26S proteasomes on native gel with and without heat stress (Fig. 4-4G). At 37° C, we observed a faster migrating mCherry positive band (asterisk) that may represent free Ecm29-mCherry, Ecm29 bound to a subcomplex, or a break down product. When we treated *nas2Δhsm3Δ* and *nas6Δrpn14Δ* mutants with antimycin A, we observed that RP and CP behaved similarly where granule formation was restricted in both the *nas2Δhsm3Δ* mutant and the *nas6Δrpn14Δ* mutant (Fig. 4-4I, supplementary Fig. 4-4). However, when we starved the *nas2Δhsm3Δ* double mutant for carbon or treated cells with sodium azide, we found that the RP reporter Rpn1-GFP showed more than 50% reduction in granule formation compared to WT. The CP reporter α 1-GFP was affected substantially less (Fig. 4-4H). RP also remained largely nuclear while CP formed

granules in a *nas6Δrpn14Δ* mutant treated with sodium azide (supplementary Fig. 4). Similarly, a reduction in granule formation upon carbon starvation was observed for both the CP and RP reporter in the *nas6Δrpn14Δ* mutant, albeit to a lesser extent than in the *nas2Δhsm3Δ* mutant (supplementary Fig.4). However, we observed a larger reduction in CP granules than RP, suggesting that the *nas6Δrpn14Δ* mutations have some distinct effects of granule formation (supplementary Fig. 4). This was only evident in carbon starvation as Oligomycin A did not induce RP granules in *nas2Δhsm3Δ* or *nas6Δrpn14Δ* strains, whereas CP formed granules like WT (Fig. 4-4I, supplementary Fig. 4). In all, these data strongly suggest that CP and RP can form granules independently of each other.

One possible explanation for the observation that RP does not form granules in these mutant strains defective in RP assembly, is that there is not enough properly assembled RP to seed granule formation. However, when we deleted the transcription factor RPN4, resulting in a reduction of all proteasome subunits, we still observed that RP remained nuclear while CP formed granules. With sodium azide and antimycin A, Rpn1-GFP granules were either not detected or very low in abundance (Fig. 4-4J). While there was a reduction in α 1-GFP granules in the Rpn4 mutant compared to WT, this was much less than for Rpn1-GFP (Fig. 4-4J). If proteasome subcomplex abundance regulated granule formation, we would not expect such a difference here, where all proteasome complexes are reduced. Thus, at least in the Rpn4 mutant, the difference between the RP and CP does not appear to be explained by the reduction in proteasome complexes. However, it is known that mutating RP chaperones results in an accumulation of free CP in cells and a lack of free RP (Fig. 4-4G) (21, 59, 60). This excess CP does not associate with Ecm29. Thus, we hypothesize that the increase in Ecm29 traps the majority of 26S into a granule-incompetent state. While the free CP can still go to granules, no free RP is present. If true, a deletion of *ECM29* should restore to some extent the ability of RP to enter in granules. Indeed, the reduction in proteasome granule formation in a *Rpt5Δ3* mutant for carbon starvation, sodium azide, antimycin A and oligomycin A treatment was in part dependent on Ecm29 (Fig. 4-4K).

Loss of PSG Formation and Growth Phenotypes in a Nat3 Mutant are Dependent on Ecm29—Seeing a role for Ecm29 in regulating proteasome localization, we wondered if

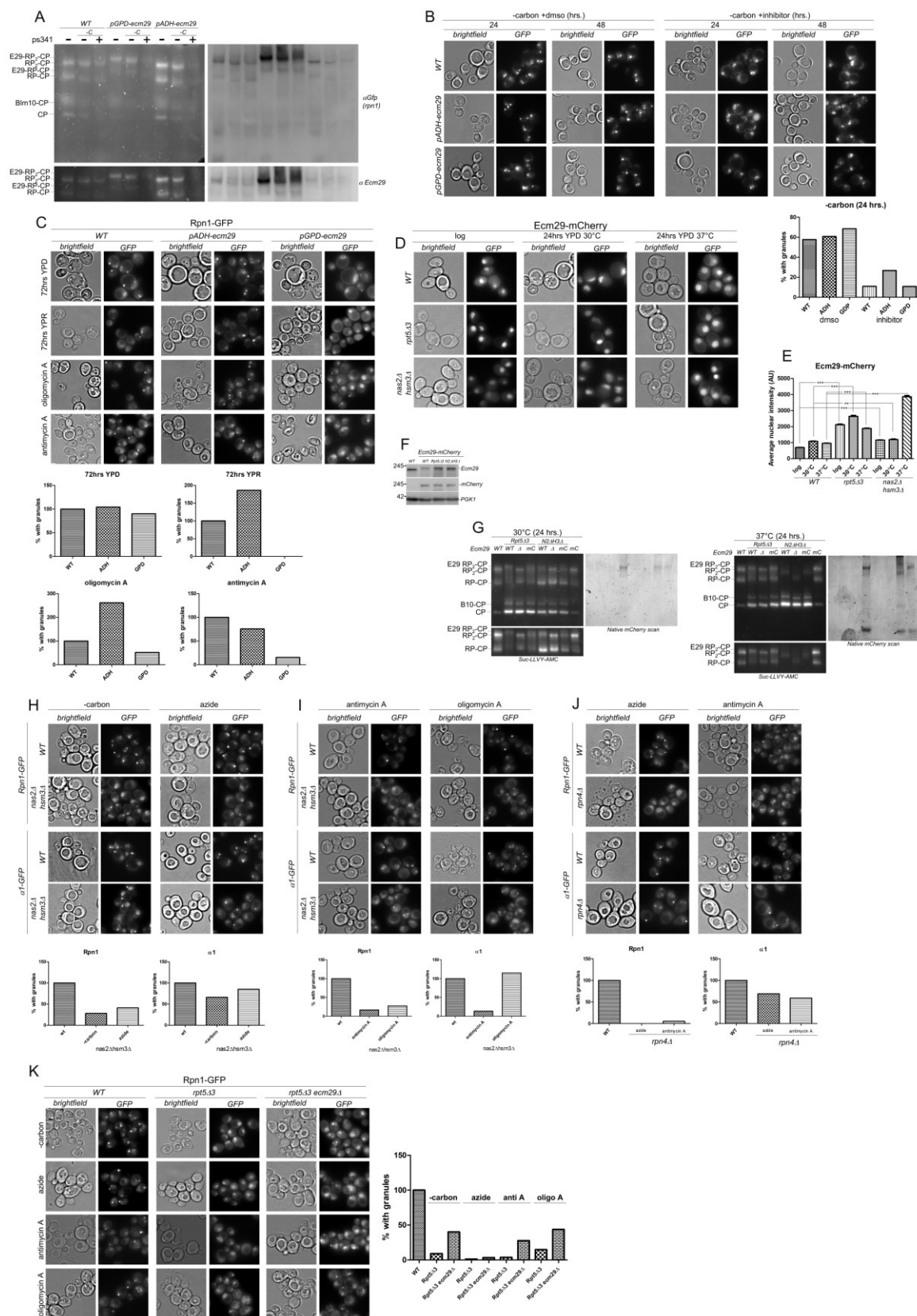
other mutants that fail to form granules are affected by this chaperone. We found that a previously characterized mutant, *NAT3*, that fails to form PSGs, also had proteasome assembly defects as native gel showed less assembled 26S proteasomes and more free CP. Additionally, we observed increased levels of Ecm29 (Fig. 4-5A, supplementary Fig. 3). *NAT3* encodes for an N-terminal acetylase and was found in a genetic screen to prevent proteasome granule formation (48). It was suggested that either proteasome subunits or another cellular substrate required N-acetylation for proteasomes to be re-localized. However, the increased levels of Ecm29 and the proteasome assembly defects we observed here might indicate Ecm29 is (partly) responsible for the observed phenotypes. As reported previously, *nat3Δ* mutants had a large reduction in granule formation in quiescence (Fig. 4-5B) (48). Here, we show that this mutant does not form granules upon carbon starvation or treatment with sodium azide (Fig. 4-5B). When we deleted *ECM29* in this mutant background, we found that PSG formation was indeed rescued (Fig. 4-5B). Further, phenotypes associated with the *nat3Δ* mutation were largely rescued by subsequent deletion of *ECM29*. *Nat3Δ* cells at stationary phase, following carbon starvation, or azide treatment, appear larger in size than *WT* and very “sick” (Fig. 4-5B). We observed a rescue in this appearance upon subsequent deletion of *ECM29*. Fig 5C shows that the growth phenotype of the *Nat3* mutant at 37° C was also rescued when *ECM29* was deleted.

We next performed phenotype screens and found growth defects in the *NAT3* deletion mutant with increased temperature and canavanine, reminiscent of phenotypes associated with proteasome assembly chaperone or sub-unit mutations. Like previously characterized proteasome mutants, deletion of *ECM29* rescued these phenotypes (Fig. 4-5D) (57, 59). As *nat3Δ* cells appeared to die following carbon starvation or growth at elevated temperature, we tested cell viability under these conditions. We observed that deletion of *NAT3* indeed resulted in less viable colonies. This was again partly rescued when *ECM29* was also deleted (Fig. 4-5E). Thus, it appears that *Nat3* limits proteasome granule formation by causing proteasome assembly defects. These proteasomes are then recognized by Ecm29 and restricted to cell nuclei. In all these data support a model whereby proteasome re-localization is restricted when proteasomes are inhibited, either with drugs or with their endogenous inhibitor Ecm29.

MAP Kinase Signaling is Required for Proteasome Granule Formation—Our data show that not all mitochondria inhibitors induce proteasome granules (Fig. 4-2E). This suggests that there is some regulator of the process that is only active under specific conditions. We sought to determine the signaling events involved in PSG formation. We have previously reported a requirement for the kinases Mpk1(Stt2), Mkk1 and Mkk2 in proteasome autophagy (Chapter 3, submitted). We hypothesized that the reduction observed was due to a reduction in the nuclear export of proteasomes. Further, Bck1, an upstream kinase in the cascade, and Mpk1 were shown to be required for induction of autophagy when antimycin A and potassium cyanide were added to cells. Interestingly, induction of general autophagy with these drugs was dependent on factors required for mitophagy including Atg32 and Atg11. However, mitochondria themselves were not targeted for autophagy (12). We have previously shown that like mitochondria, proteasomes are not degraded when autophagy is induced using KCN or antimycin A (Chapter 3, submitted). Indeed, we report here that antimycin A induces proteasome granules (Fig. 4-2A). This was not observed with varying concentrations of KCN (Fig. 4-2E). When we tested if deletion of *ATG32* or *ATG11* affected the formation of PSGs when mitochondria were inhibited, we observed no reduction (not shown). This suggest that the requirements for autophagy induction and PSG formation are not necessarily coupled. The role of map kinase signaling however suggest at least some overlap.

(A) WT, pGpd-Ecm29, and pAdh-Ecm29 yeast with Rpn1-GFP were grown to log phase. Cultures were divided into non-starved controls, or carbon starvation with DMSO or PS341. Native gel analysis and suc-LLVY-amc peptidase activity (+sds on top) were carried out as described in materials and methods. Immuno-blotting following native gel transfer for GFP and Ecm29 was carried out as described in materials and methods. (B) Strains as in A were starved for carbon as described above with DMSO (top) or PS341 (bottom). Microscopy was performed at indicated times and quantifications show the percentage of cells with granules at 24 hrs. (C) Strains as in A were grown to log phase and either treated with oligomycin A and antimycin A, or left to grow for 72 hrs. in rich media. Quantifications show the percentage of cells with granules relative to WT for each treatment. (D) WT, *rpt5Δ3*, and *nas2Δ hsm3Δ* yeast expressing Ecm29-mCherry were imaged following growth to log phase, and 24hrs at 30 °C or 37 °C. (E) Quantification of average nuclear mCherry fluorescence intensity of cells in D. Error bars indicate SEM and p-values were determined using an unpaired, two-tailed Students t-test (*<0.01, **<0.001, ***<0.0001) (F) Ecm29-mCherry yeast with *rpt5Δ3*, and *nas2Δ hsm3Δ* mutations, as well as WT yeast without mCherry, were grown to for 24 hrs. in rich media. 2 ODs of cells were collected and lysed as described in materials and methods. Immuno-blotting for Ecm29, mCherry, and PGK1 were performed as described in materials and methods. (G) Yeast strains as in D and F were grown for 24 hrs. in rich media at 30 °C and 37 °C. Native gel analysis including suc-LLVY-amc peptidase activity, and mCherry typhoon scan for Ecm29-mCherry, was performed as described in materials and methods. (H,I) WT and *nas2Δ hsm3Δ* yeast expressing Rpn1-GFP or α1-GFP were starved for carbon, or treated with sodium azide, antimycin A or oligomycin A for 24 hrs. following logarithmic growth. Quantifications show the percent of cells with granules relative to WT. (J) WT and *rpn4Δ* yeast expressing Rpn1-GFP or α1-GFP were treated with sodium azide or antimycin A for 24 hrs. following logarithmic growth. Quantifications show the percentage of cells relative to WT that form granules. (K) WT, *rpt5Δ3* and *rpt5Δ3 ecm29Δ* yeast expressing Rpn1-GFP were grown to log phase. Cells were then starved for carbon or treated with sodium azide, antimycin A or oligomycin A for 24 hrs. Quantifications show the percentage of cells with granules relative to the WT for each condition.

Figure 4-4. Ecm29 restricts proteasome granule formation

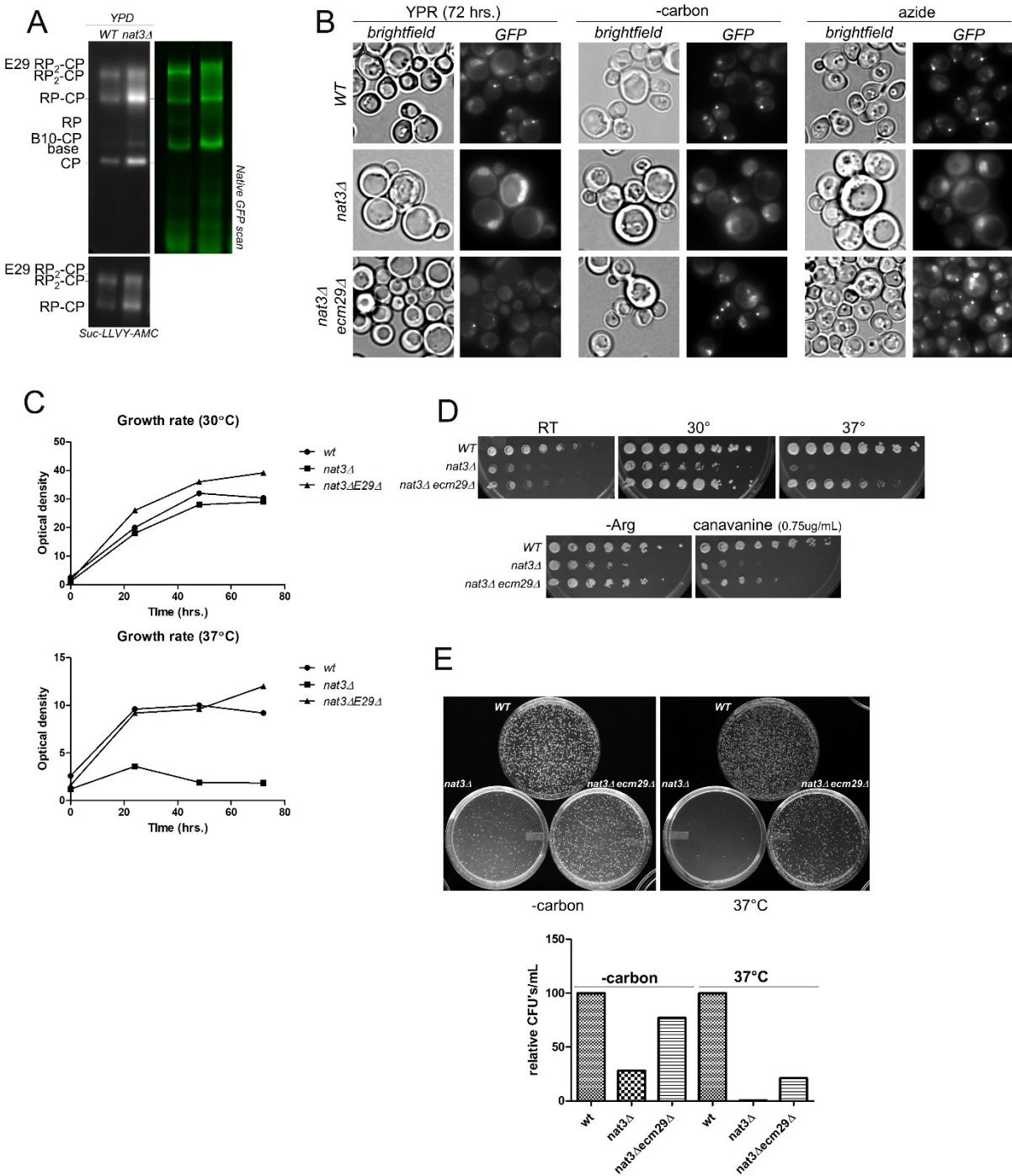


Bck1 and Mpk1 are involved in autophagy induction under mitochondria-inhibiting conditions, and Mpk1 is required for efficient proteasome autophagy induced by nitrogen starvation. When we tested if disruption of this cascade affected PSG formation, we observed a striking result. When *MPK1* was deleted, we found that PSG formation was unaffected when cells were starved for carbon following initial growth in dextrose (Fig. 4-6A). However, when we grew cells in raffinose, Mpk1 was required for both the RP and CP reporters to form granules (Fig. 4-6A). This is an intriguing correlation between the requirements for autophagy induction under carbon starvation, and the requirement for Mpk1 in PSG formation, as Mpk1 was only required for proteasome granule formation when mitochondrial respiration was increased. When we monitored granule formation following antimycin A or oligomycin A treatment, we found a similar requirement for Mpk1 when cells are grown in raffinose (Fig. 4-6B, C). However, RP granules were prevented when *mpk1Δ* cells were grown in dextrose or raffinose, then treated with sodium azide (Fig. 4-6D). CP granules on the other hand behaved very differently. When grown in dextrose, we observed a reduction in granule formation in the *mpk1Δ* mutant, whereas raffinose grown cells formed granules similar to WT (Fig. 4-6D). This observation further supports the idea of differential regulation of CP and RP in granule formation.

In order to determine if other yeast kinase pathways were required for PSG formation, we deleted *FUS3* and *HOG1*. Deletion of *FUS3* or *HOG1* did not prevent the formation of proteasome granules (supplementary Fig. 5). A more direct effector of Mpk1 with respect to proteasomes is the chaperone Adc17. This proteasome chaperone is known to be activated under stress, and is required for regulating proteasome assembly (61). Adc17 is also known to be activated by Mpk1 (62). We did not detect a role for this Mpk1 target in PSG formation (supplementary Fig. 5).

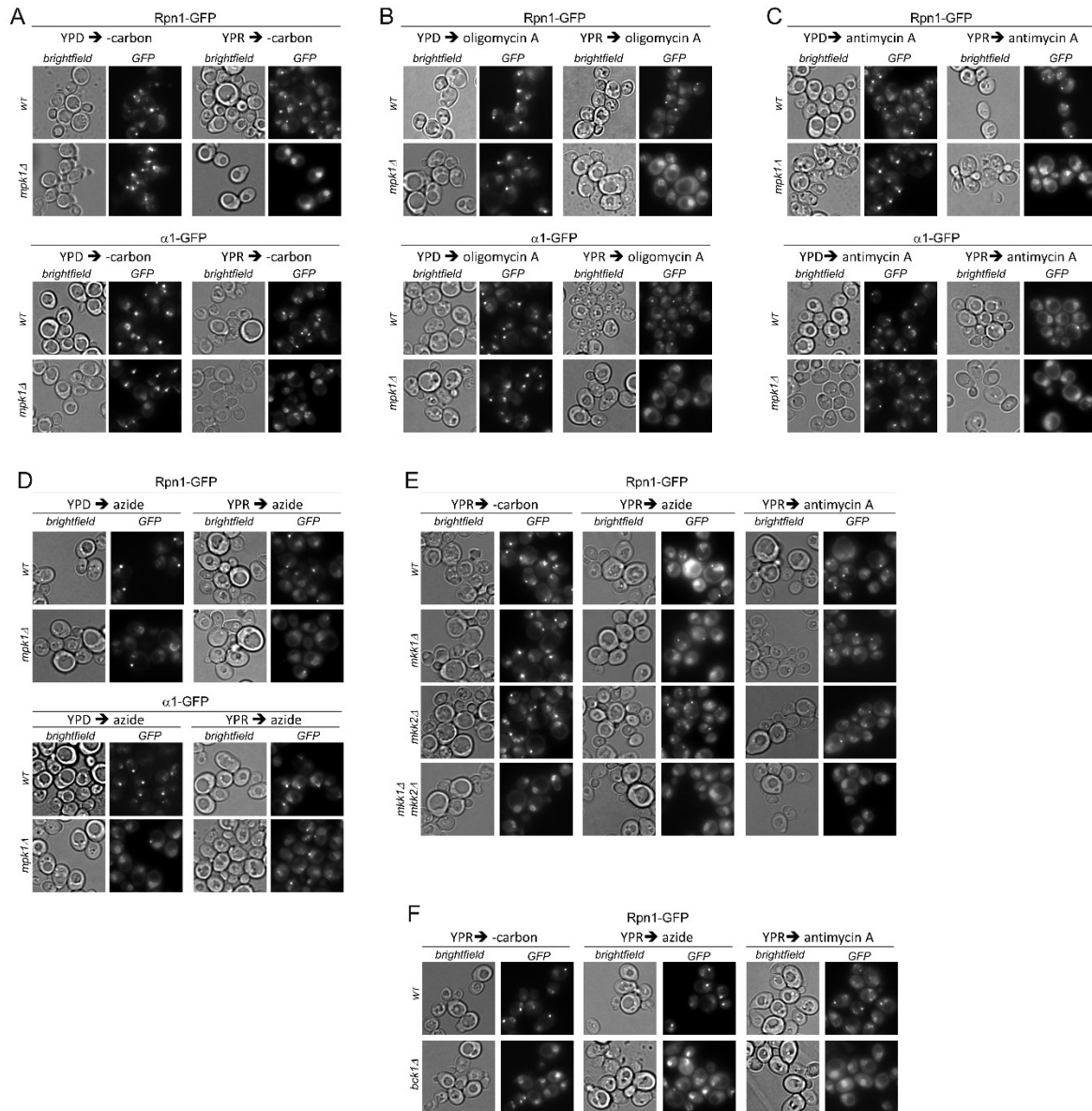
(A) WT and *nat3Δ* yeast expressing Rpn1-GFP were grown for 24 hrs. in rich media. Native gel analysis and suc-LLVY-amc peptidase activity were carried out as described in materials and methods. (B) WT, *nat3Δ* and *nat3Δ ecm29Δ* yeast were grown for 72 hrs. in rich media, starved for carbon, or treated with sodium azide. (C) Growth rates of yeast from B at 30 °C and 37 °C were monitored over time by optical density. (D) Yeast strains as in B were grown to log phase (~4 hrs.) and 1 OD collected by centrifugation. 4-fold serial dilutions were performed and spotted on YPD plates for temperature treatments or plates with or without canavanine. (E) Following carbon starvation or 37 °C growth for 24 hrs. of yeast strains as in B, 1 OD of cells was collected. Serial dilutions were performed, spread on YPD plates, and allowed to incubate at 30 °C for 24 hrs. 10⁻⁴ (-carbon) and 10⁻⁵ (37 °C) plates are shown. Quantifications show CFUs/mL relative to WT.

Figure 4-5. Loss of PSG formation and growth phenotypes in a Nat3 mutant are dependent on Ecm29



We next looked upstream of Mpk1 to Mkk1, Mkk2 and Bck1. We found that a double deletion of *MKK1* and *MKK2* largely prevented proteasome granules under carbon starvation, azide treatment, and antimycin A treatment for the RP (Fig. 4-6E). These proteins are redundant in function, thus PSGs were largely unaffected in the single deletion mutants. The upstream kinase Bck1 was also required for PSG formation under the same conditions (Fig. 4-6F). These data show that an intact map kinase pathway is required for proteasome granule formation. We have previously reported that Mpk1, Mkk1 and Mkk2 are required for efficient proteasome autophagy (Chapter 3, submitted). Whether Mpk1 directly phosphorylates proteasome subunits remains to be determined.

Figure 4-6. MAP kinase signaling is required for proteasome granule formation



(A, B, C, D) WT and *mpk1Δ* yeast expressing Rpn1-GFP or α1-GFP were grown to log phase in rich media containing dextrose or raffinose. Cells were then starved for carbon, or treated with oligomycin A, antimycin A, or sodium azide. (E) WT, *mkk1Δ*, *mkk2Δ* and *mkk1Δ mkk2Δ* yeast expressing Rpn1-GFP were grown to log phase in rich media containing raffinose then starved for carbon, treated with sodium azide, or treated with antimycin A. Microscopy was performed at 24 hrs. following treatments. (F) WT and *mpk1Δ* yeast expressing Rpn1-GFP were grown to log phase in rich media containing raffinose then starved for carbon, treated with sodium azide, or treated with antimycin A as in E. Microscopy was performed at 24 hrs. following treatments as in E.

Discussion

Organisms employ numerous mechanisms to adapt to environmental conditions. Proteasomal degradation of proteins plays crucial roles in this process (24, 63). As most proteasomes reside in cell nuclei, it is important to not only understand how cells control their abundance (synthesis and degradation), but their localization and quality as well. In this work, we have uncovered some of the molecular mechanisms that regulate proteasome localization. We show that yeast grown under conditions of catabolite repression (dextrose), regulate proteasome granules differently than when grown in other carbon sources (Fig. 4-1B, C, D). Importantly we show that proteasomes largely form granules in conditions of carbon starvation and mitochondrial inhibition, even though general autophagy is induced. We found that factors required for autophagy induction under carbon starvation were not required for PSG formation (49) (supplementary Fig. 1). Nor were proteasomes localized with phagophore assembly sites (PAS) at the timepoints tested, though (PAS) foci and proteasome granules behave similarly following carbon starvation from different carbon sources (45) (Fig. 4-3D). This suggests that proteasome granule formation is not linked to autophagy in carbon starvation. However, we did observe a correlation between proteasome granules, mitochondrial respiration, and autophagy. As mitochondrial respiration and autophagy increased (i.e. growth in glycerol vs raffinose or dextrose), decreasing numbers of cells formed proteasome granules (Fig. 4-1D, E, F, G). The reduction in proteasome granules observed when there is increased proteasome autophagy supports a recent model where PSGs were hypothesized to protect proteasomes from autophagic degradation (37). While we did observe a slight increase in proteasome autophagy when cells were grown in glycerol compared to raffinose or dextrose, this was minimal compared to proteasome autophagy induced by nitrogen starvation (Fig. 4-1A). Indeed, most proteasomes appeared nuclear rather than vacuolar when proteasome granules did not form. Consistent with this, we did not observe proteasome autophagy in a *NAT3* mutant as was previously reported (37). In our hands we found that *nat3Δ* mutants largely restricted proteasome granule formation through Ecm29. Here, it appears that Ecm29 retains proteasomes in the nucleus upon carbon starvation (Fig. 4-5B). We did not observe autophagy when *ECM29* was deleted, instead granule

formation was rescued. Similarly, in all conditions tested where proteasome granules failed to form, we observed predominantly nuclear localization of proteasomes (Fig. 4-1G, 4-2B,C, 4-3A,B,G,H, 4-4B,C,H,I,J,K, 4-6A,B,C,D,E,F, supplementary Fig. 2,4,). It is possible that nuclear retention of proteasomes and PSG formation are functionally equivalent in preventing proteasome autophagy. Another possibility is that PSGs serve as another layer of control for proteasome activity. The fact that proteasomes largely remain nuclear when PSG formation is disrupted shows that cells actively move proteasomes out of the nucleus and into PSGs under these conditions. Further, phenotypes associated with the inability to re-localize proteasomes (ex. *nat3Δ*), are rescued when PSG formation is restored. We propose that PSGs may function to limit nuclear proteasome activity under particular stress conditions.

It has previously been shown that proteasome mutations can lead to mitochondrial dysfunction (64, 65). Here, we show that inhibiting oxidative phosphorylation, like carbon starvation and quiescence, induced proteasome granules but not proteasome autophagy (Fig. 4-2A). In particular, antimycin A and KCN are known to induce general autophagy, but importantly not mitophagy in yeast (12). While antimycin A induced proteasome granules, KCN did not (Fig. 4-2A, E). Neither drug induced proteasome autophagy (Chapter 3, submitted). In all, these data further strengthen the link between proteasomes, autophagy, and mitochondria. This is intriguing as with other autophagy inducers, proteasomes are either degraded (nitrogen starvation, rapamycin treatment, caffeine treatment) or re-localized to granules (phosphate starvation, carbon starvation, antimycin A). Nuclear retention with KCN suggest that PSG signals may be absent depending on the mode of mitochondrial inhibition. In support of this, we surprisingly did not detect a reduction in ATP when KCN was used (Fig. 4-2I). When cells were grown in the presence of sodium azide or in anoxic conditions, proteasome granule formation was again affected by the initial carbon source (Fig. 4-2B). These conditions lead to a larger reduction in ATP than when cells are grown in another carbon source. We hypothesized that the ATP-dependent nature of the proteasome would sensitize the complex to perturbations in ATP. As respiration is maintained when cells are grown in non-fermentable carbon sources, this may explain why less PSGs are present in these conditions. While we did detect

reductions in ATP levels under some conditions where PSGs are induced, in others with lower ATP (ex. SD-Com, CCCP), PSG's were not induced to a large extent (Fig. 4-2F, G, H, I). This suggests that low ATP levels alone do not result in proteasome granule formation. Another possibility is that a sudden change in ATP levels, as occurs when cells are switched from dextrose, leads to a more drastic effect on proteasomes than a gradual change (45). Nevertheless, ATP levels alone do not control PSG formation. Some signaling events likely occur upon mitochondrial disruption that triggers PSG formation. Consistent with the hypothesis presented above, this would prevent the consumption of limited ATP pools by 26S proteasomes. In support of a signaling pathway regulating proteasome granule formation, we found that the MAP kinase cascade, which is involved in autophagy induction upon mitochondrial inhibition, is necessary for proteasome granule formation (Fig. 4-6). This was dependent on mitochondrial function as Mpk1 was dispensable for granule formation when cells were grown in dextrose. Our data clearly point to a role for mitochondria in regulating proteasome localization.

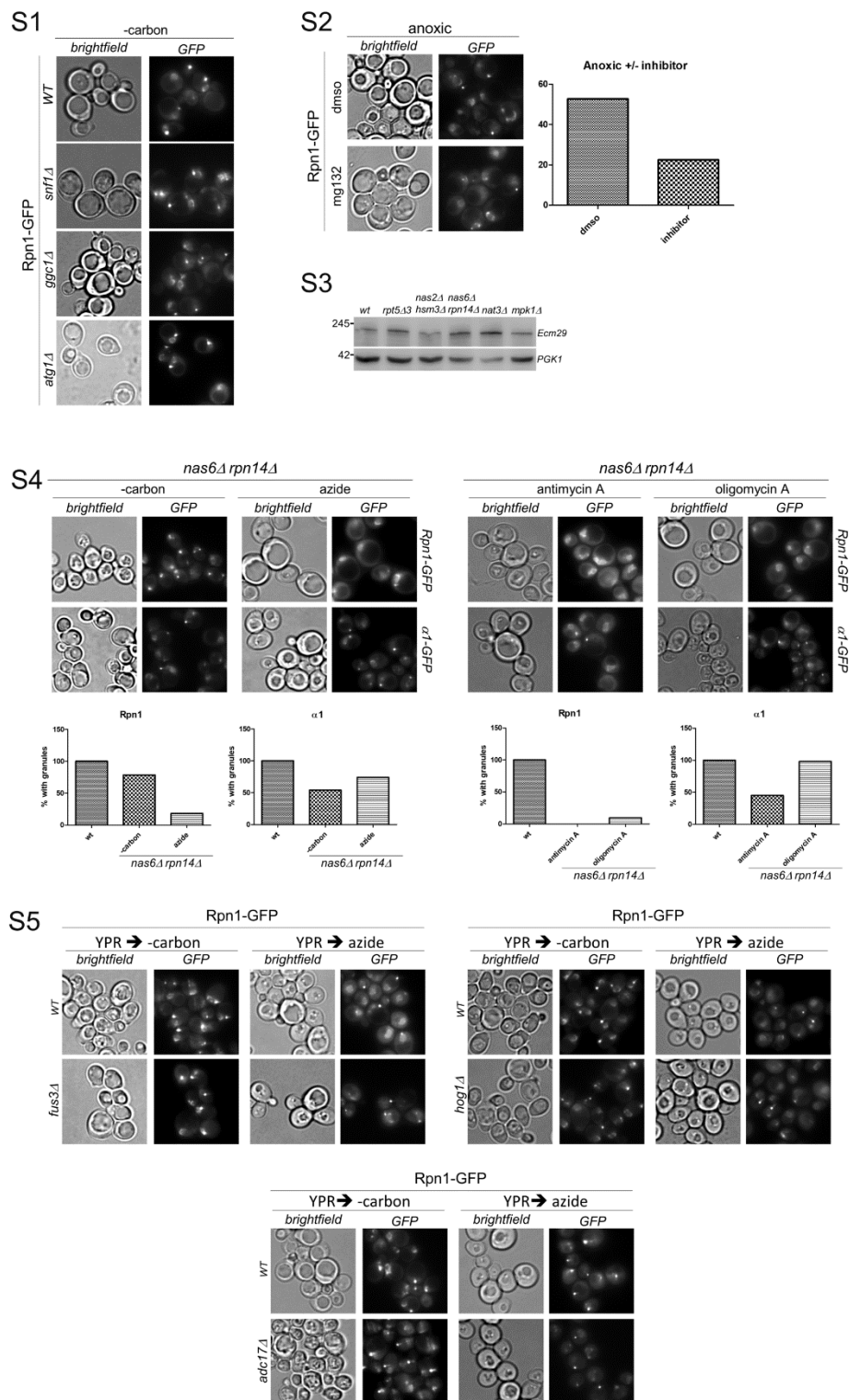
It is known that proteasomes are unstable in the absence of ATP *in vitro*. Though a clear correlation between ATP levels and PSGs was not observed, we found that factors known to stabilize RP-CP interactions restricted PSG formation. We show that proteasome inhibitors result in more Ecm29 bound to proteasomes and that both factors partly restricted proteasome re-localization (Fig. 4-3). Here, the effect of Ecm29 was only evident when proteasome inhibitors were included in the starvation medium. Suggesting under this condition, Ecm29 affects inhibited proteasomes differently than un-inhibited complexes. When we overexpressed *ECM29*, we observed that PSG formation was largely restricted in most conditions including stationary growth in raffinose, oligomycin A treatment, and antimycin A treatment, without proteasome inhibitors (Fig. 4-3C). The exception here was carbon starvation where less of a reduction was observed unless proteasome inhibitors were present (Fig. 4-1B). These observations support the idea that proteasome granules are not all regulated the same.

We found that proteasome chaperone mutations, which result in increased levels of Ecm29, resulted in a loss of PSG formation with various stimuli. For the *Rpt5Δ3* mutant, we found that deletion of *ECM29* restored the ability of those proteasomes to

form granules (Fig. 4-4K). Similarly, we found that *ECM29* was largely responsible for the loss of PSGs and growth phenotypes associated with a *NAT3* mutant (Fig. 4-5). We report proteasome assembly defects in this mutant that resulted in increased Ecm29 bound proteasomes. Intriguingly, we found that RP chaperone mutations largely did not prevent CP from forming PSGs (Fig. 4-4H. I). This points to independent regulation of CP and RP in terms of granule formation. Previous work supports the idea of independent targeting of CP or RP in to granules and autophagy (7, 34, 37). Our data suggest that Ecm29 plays a role in this regulation. It appears that in conditions where more Ecm29 is bound to proteasomes, PSG formation is prevented. This is likely through stabilization and nuclear retention of proteasome complexes. The reduced amount of assembled RP produced in these chaperone mutants would bind any CP, as CP would be in excess. These 26S complexes would then be accessible to Ecm29. Whereas most RP would be associated with Ecm29, the free CP would be available for potential modifications, nuclear export and granule formation. Consistent with this, deletion of *ECM29* resulted in a partial rescue of RP granule formation. However, the differential reduction in PSG formation observed when *RPN4* was deleted does not support this model. Whether there is an excess of free CP in this mutant will be determined.

In all, our data support a model whereby cells re-localize proteasomes to granules under particular stresses. The signaling events that govern this process involve mitochondria and the Mpk1 kinase cascade. When such a signal occurs, proteasomes become unstable, and sub-complexes become more accessible to modifications. Modified sub-complexes are then exported from the nucleus and packaged into PSGs. Ecm29 and proteasome inhibitors prevent this by stabilizing 26S complexes and preventing nuclear export. Experiments are currently under way to test these possibilities.

Figure 4-7 Supplementary Figures



Supplementary Figure 1. WT, *snf1Δ*, *ggc1Δ* and *atg1Δ* yeast expressing Rpn1-GFP were grown to log phase in rich media containing raffinose then starved for carbon. Microscopy was performed at 24 hrs.

Supplementary Figure 2. Rpn1-GFP expressing yeast were grown as in Fig. 2C with DMSO or MG132 for 24 hrs. Quantifications represent the percentage of cells with granules.

Supplementary Figure 3. WT, *rpt5Δ3*, *nas2Δ hsm3Δ*, *nas6Δ rpn14Δ*, *nat3Δ* and *mpk1Δ* yeast were grown for 24 hrs. in rich media and 2 ODs collected. Immuno-blotting was performed as in materials and methods for Ecm29 and Pgk1.

Supplementary Figure 4. WT and *nas6Δ rpn14Δ* yeast expressing Rpn1-GFP or α 1-GFP were grown to log phase in rich media containing raffinose, then starved for carbon or treated with sodium azide, antimycin A or oligomycin A. Quantifications show the percentage of granules compared to WT.

Supplementary Figure 5. WT, *fus3Δ*, *hog1Δ* and *adc17Δ* yeast expressing Rpn1-GFP were grown to log phase in rich media containing raffinose, then starved for carbon or treated with sodium azide. Microscopy was performed at 24 hrs.

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References-Chapter 4

1. Dikic, I. (2017) Proteasomal and Autophagic Degradation Systems. *Annu. Rev. Biochem.* **86**, 193–224
2. Finley, D., Ulrich, H. D., Sommer, T., and Kaiser, P. (2012) The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. *Genetics*. **192**, 319–360
3. Feng, Y., He, D., Yao, Z., and Klionsky, D. J. (2014) The machinery of macroautophagy. *Cell Res.* **24**, 24–41
4. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19**, 5720–8
5. Kraft, C., Reggiori, F., and Peter, M. (2009) Selective types of autophagy in yeast. *Biochim. Biophys. Acta - Mol. Cell Res.* **1793**, 1404–1412
6. Mochida, K., Oikawa, Y., Kimura, Y., Kirisako, H., Hirano, H., Ohsumi, Y., and Nakatogawa, H. (2015) Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus. *Nature*. **522**, 359–362
7. Waite, K. A., De La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2015) Starvation Induces Proteasome Autophagy with Different Pathways for Core and Regulatory Particle. *J. Biol. Chem.* 10.1074/jbc.M115.699124
8. Marshall, R. S., Li, F., Gemperline, D. C., Book, A. J., Vierstra Correspondence, R. D., and Vierstra, R. D. (2015) Autophagic Degradation of the 26S Proteasome Is Mediated by the Dual ATG8/Ubiquitin Receptor RPN10 in Arabidopsis. *Mol. Cell.* **58**, 1053–1066
9. Kamada, Y., Yoshino, K. -i., Kondo, C., Kawamata, T., Oshiro, N., Yonezawa, K., and Ohsumi, Y. (2010) Tor Directly Controls the Atg1 Kinase Complex To Regulate Autophagy. *Mol. Cell. Biol.* **30**, 1049–1058
10. BAO, W., GU, Y., TA, L., WANG, K., and XU, Z. (2016) Induction of autophagy by the MG-132 proteasome inhibitor is associated with endoplasmic reticulum stress in MCF-7 cells. *Mol. Med. Rep.* **13**, 796–804
11. Ogata, M., Hino, S., Saito, A., Morikawa, K., Kondo, S., Kanemoto, S., Murakami, T., Taniguchi, M., Tanii, I., Yoshinaga, K., Shiosaka, S., Hammarback, J. A., Urano, F., and Imaizumi, K. (2006) Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol. Cell. Biol.* **26**, 9220–31
12. Deffieu, M., Bhatia-Kissova, I., Salin, B., Klionsky, D. J., Pinson, B., Manon, S., and Camougrand, N. (2013) Increased levels of reduced cytochrome b and mitophagy components are required to trigger nonspecific autophagy following induced

- mitochondrial dysfunction. *J. Cell Sci.* **126**, 415–426
13. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–79
 14. Schrader, E. K., Harstad, K. G., and Matouschek, A. (2009) Targeting proteins for degradation. *Nat. Chem. Biol.* **5**, 815–22
 15. Finley, D., Özkaynak, E., and Varshavsky, A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell*. **48**, 1035–1046
 16. Fujii, K., Kitabatake, M., Sakata, T., Miyata, A., and Ohno, M. (2009) A role for ubiquitin in the clearance of nonfunctional rRNAs. *Genes Dev.* **23**, 963–974
 17. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **19**, 94–102
 18. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature*. **349**, 132–138
 19. Finley, D. (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu. Rev. Biochem.* **78**, 477–513
 20. Tomko, R. J., and Hochstrasser, M. (2013) Molecular architecture and assembly of the eukaryotic proteasome. *Annu. Rev. Biochem.* **82**, 415–45
 21. Kleijnen, M. F., Roelofs, J., Park, S., Hathaway, N. A., Glickman, M., King, R. W., and Finley, D. (2007) Stability of the proteasome can be regulated allosterically through engagement of its proteolytic active sites. *Nat. Struct. Mol. Biol.* **14**, 1180–1188
 22. Xie, Y. (2010) Structure, assembly and homeostatic regulation of the 26S proteasome. *J. Mol. Cell Biol.* **2**, 308–317
 23. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature*. **349**, 132–138
 24. Wang, X., Xu, H., Ha, S.-W., Ju, D., and Xie, Y. (2010) Proteasomal degradation of Rpn4 in *Saccharomyces cerevisiae* is critical for cell viability under stressed conditions. *Genetics*. **184**, 335–42
 25. Wang, Z., Gerstein, M., and Snyder, M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* **10**, 57–63
 26. Bax, M., McKenna, J., Do-Ha, D., Stevens, C. H., Higginbottom, S., Balez, R., Cabral-da-Silva, M. e C., Farrawell, N. E., Engel, M., Poronnik, P., Yerbury, J. J., Saunders, D. N., and Ooi, L. (2019) The Ubiquitin Proteasome System Is a Key Regulator of Pluripotent

Stem Cell Survival and Motor Neuron Differentiation. *Cells*. **8**, 581

27. Ji, C. H., and Kwon, Y. T. (2017) Crosstalk and Interplay between the Ubiquitin-Proteasome System and Autophagy. *Mol. Cells*. **40**, 441–449
28. Nam, T., Han, J. H., Devkota, S., and Lee, H.-W. (2017) Emerging Paradigm of Crosstalk between Autophagy and the Ubiquitin-Proteasome System. *Mol. Cells*. **40**, 897–905
29. Kelly, S. P., and Bedwell, D. M. (2015) Both the autophagy and proteasomal pathways facilitate the Ubp3p-dependent depletion of a subset of translation and RNA turnover factors during nitrogen starvation in *Saccharomyces cerevisiae*. *RNA*. **21**, 898–910
30. Yamamoto, K., Makino, N., Nagai, M., Honma, Y., Araki, H., and Ushimaru, T. (2018) TORC1 signaling regulates DNA replication via DNA replication protein levels. *Biochem. Biophys. Res. Commun.* 10.1016/j.bbrc.2018.10.018
31. Kleijnen, M. F., Kirkpatrick, D. S., and Gygi, S. P. (2007) The ubiquitin-proteasome system regulates membrane fusion of yeast vacuoles. *EMBO J*. **26**, 275–87
32. Cohen-Kaplan, V., Livneh, I., Avni, N., Fabre, B., Ziv, T., Kwon, Y. T., and Ciechanover, A. (2016) p62- and ubiquitin-dependent stress-induced autophagy of the mammalian 26S proteasome. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E7490–E7499
33. Marshall, R. S., McLoughlin, F., and Vierstra, R. D. (2016) Autophagic Turnover of Inactive 26S Proteasomes in Yeast Is Directed by the Ubiquitin Receptor Cue5 and the Hsp42 Chaperone. *Cell Rep*. **16**, 1717–1732
34. Nemec, A. A., Howell, L. A., Peterson, A. K., Murray, M. A., and Tomko, R. J. (2017) Autophagic clearance of proteasomes in yeast requires the conserved sorting nexin Snx4. *J. Biol. Chem.* **292**, 21466–21480
35. Gu, Z. C., Wu, E., Sailer, C., Jando, J., Styles, E., Eisenkolb, I., Kuschel, M., Bitschar, K., Wang, X., Huang, L., Vissa, A., Yip, C. M., Yedidi, R. S., Friesen, H., and Enenkel, C. (2017) Ubiquitin orchestrates proteasome dynamics between proliferation and quiescence in yeast. *Mol. Biol. Cell*. **28**, 2479–2491
36. Enenkel, C. (2018) The paradox of proteasome granules. *Curr. Genet.* **64**, 137–140
37. Marshall, R. S., and Vierstra, R. D. (2018) Proteasome storage granules protect proteasomes from autophagic degradation upon carbon starvation. *Elife*. 10.7554/eLife.34532
38. Goldstein, A. L., and McCusker, J. H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast*. **15**, 1541–53
39. Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-

- Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and Knop, M. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast*. **21**, 947–962
40. Hailey, D. W., Davis, T. N., and Muller, E. G. D. (2002) Fluorescence resonance energy transfer using color variants of green fluorescent protein. *Methods Enzymol.* **351**, 34–49
 41. Kushnirov, V. V. (2000) Rapid and reliable protein extraction from yeast. *Yeast*. **16**, 857–860
 42. Roelofs, J., Suppahia, A., Waite, K. A., and Park, S. (2018) Native Gel Approaches in Studying Proteasome Assembly and Chaperones. in *Methods in molecular biology (Clifton, N.J.)*, pp. 237–260, **1844**, 237–260
 43. Li, D., Song, J.-Z., Shan, M.-H., Li, S.-P., Liu, W., Li, H., Zhu, J., Wang, Y., Lin, J., and Xie, Z. (2015) A fluorescent tool set for yeast Atg proteins. *Autophagy*. **11**, 954–960
 44. Sundin, B. A., Chiu, C.-H., Riffle, M., Davis, T. N., and Muller, E. G. D. (2004) Localization of proteins that are coordinately expressed with Cln2 during the cell cycle. *Yeast*. **21**, 793–800
 45. Adachi, A., Koizumi, M., and Ohsumi, Y. (2017) Autophagy induction under carbon starvation conditions is negatively regulated by carbon catabolite repression. *J. Biol. Chem.* **292**, 19905–19918
 46. Kayikci, Ö., and Nielsen, J. (2015) Glucose repression in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 10.1093/femsyr/fov068
 47. Waite, K. A., De-La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2016) Starvation Induces Proteasome Autophagy with Different Pathways for Core and Regulatory Particles. *J. Biol. Chem.* **291**, 3239–53
 48. van Deventer, S., Menendez-Benito, V., van Leeuwen, F., and Neefjes, J. (2014) N-terminal acetylation and replicative age affect proteasome localization and cell fitness during aging. *J. Cell Sci.* **128**, 109–117
 49. Yi, C., Tong, J., Lu, P., Wang, Y., Zhang, J., Sun, C., Yuan, K., Xue, R., Zou, B., Li, N., Xiao, S., Dai, C., Huang, Y., Xu, L., Li, L., Chen, S., Miao, D., Deng, H., Li, H., and Yu, L. (2017) Formation of a Snf1-Mec1-Atg1 Module on Mitochondria Governs Energy Deprivation-Induced Autophagy by Regulating Mitochondrial Respiration. *Dev. Cell.* **41**, 59-71.e4
 50. Ko, Y. H., Hong, S., and Pedersen, P. L. (1999) Chemical mechanism of ATP synthase. Magnesium plays a pivotal role in formation of the transition state where ATP is synthesized from ADP and inorganic phosphate. *J. Biol. Chem.* **274**, 28853–6

51. Secco, D., Wang, C., Shou, H., and Whelan, J. (2012) Phosphate homeostasis in the yeast *Saccharomyces cerevisiae*, the key role of the SPX domain-containing proteins. *FEBS Lett.* **586**, 289–295
52. Petti, A. A., Crutchfield, C. A., Rabinowitz, J. D., and Botstein, D. (2011) Survival of starving yeast is correlated with oxidative stress response and nonrespiratory mitochondrial function. *Proc. Natl. Acad. Sci.* **108**, E1089–E1098
53. Weberruss, M. H., Savulescu, A. F., Jando, J., Bissinger, T., Harel, A., Glickman, M. H., and Enenkel, C. (2013) Blm10 facilitates nuclear import of proteasome core particles. *EMBO J.* **32**, 2697–707
54. Xie, Y., and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3056–61
55. Ju, D., Wang, L., Mao, X., and Xie, Y. (2004) Homeostatic regulation of the proteasome via an Rpn4-dependent feedback circuit. 10.1016/j.bbrc.2004.06.105
56. Park, S., Kim, W., Tian, G., Gygi, S. P., and Finley, D. (2011) Structural Defects in the Regulatory Particle-Core Particle Interface of the Proteasome Induce a Novel Proteasome Stress Response. *J. Biol. Chem.* **286**, 36652–36666
57. De La Mota-Peynado, A., Lee, S. Y.-C., Pierce, B. M., Wani, P., Singh, C. R., and Roelofs, J. (2013) The proteasome-associated protein Ecm29 inhibits proteasomal ATPase activity and in vivo protein degradation by the proteasome. *J. Biol. Chem.* **288**, 29467–81
58. Roelofs, J., Park, S., Haas, W., Tian, G., McAllister, F. E., Huo, Y., Lee, B.-H., Zhang, F., Shi, Y., Gygi, S. P., and Finley, D. (2009) Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature.* **459**, 861–5
59. Lee, S. Y.-C., De la Mota-Peynado, A., and Roelofs, J. (2011) Loss of Rpt5 protein interactions with the core particle and Nas2 protein causes the formation of faulty proteasomes that are inhibited by Ecm29 protein. *J. Biol. Chem.* **286**, 36641–51
60. Nahar, A., Fu, X., Polovin, G., Orth, J. D., and Park, S. (2019) Two Alternative Mechanisms Regulate the Onset of Chaperone-mediated Assembly of the Proteasomal ATPases. *J. Biol. Chem.* 10.1074/jbc.RA118.006298
61. Hanssum, A., Zhong, Z., Rousseau, A., Krzyzosiak, A., Sigurdardottir, A., and Bertolotti, A. (2014) An Inducible Chaperone Adapts Proteasome Assembly to Stress. *Mol. Cell.* **55**, 566–577
62. Rousseau, A., and Bertolotti, A. (2016) An evolutionarily conserved pathway controls proteasome homeostasis. *Nature.* **536**, 184–189

63. Schmidt, M., and Finley, D. (2014) Regulation of proteasome activity in health and disease. *Biochim. Biophys. Acta*. **1843**, 13–25
64. Rinaldi, T., Hofmann, L., Gambadoro, A., Cossard, R., Livnat-Levanon, N., Glickman, M. H., Frontali, L., and Delahodde, A. (2008) Dissection of the Carboxyl-Terminal Domain of the Proteasomal Subunit Rpn11 in Maintenance of Mitochondrial Structure and Function. *Mol. Biol. Cell*. **19**, 1022
65. Saunier, R., Esposito, M., Dassa, E. P., and Delahodde, A. (2013) Integrity of the *Saccharomyces cerevisiae* Rpn11 Protein Is Critical for Formation of Proteasome Storage Granules (PSG) and Survival in Stationary Phase. *PLoS One*. **8**, e70357

Chapter 5 - Discussion

Proteasome Autophagy

How cells control proteasome abundance and localization has historically been under appreciated. This is somewhat surprising given the amount of work that has been dedicated to understanding the ubiquitin proteasome system and proteasome assembly. The work presented here is one of the first attempts aimed at filling this knowledge gap. We hypothesized and demonstrated that proteasomes are degraded in yeast through an autophagy-dependent pathway (1). This occurred following nitrogen starvation, a condition where cells likely benefit from amino acids recycled from these abundant complexes. However, proteasome activity could also provide amino acids by degrading proteins in this condition. Nitrogen-starved cells could then benefit from this pool of amino acids. In support of a role for proteasome activity early in autophagy, our data suggested that proteasome autophagy occurs slower than general autophagy. Further analysis revealed that proteasome autophagy was generally slow under all conditions that induced it (Chapter 3). This idea was further supported by the observation that inhibiting TORC1 using the drug rapamycin induced proteasome upregulation (2). Our data show that this is a transient proteasome response that is followed by proteasome autophagy at later timepoints (Chapter 3). This may point to a role for proteasome activity at early timepoints in autophagy. One possibility is that the UPS and autophagy work together to maintain amino acid homeostasis. By upregulating protein degradation through proteasomes, cells are anticipated to better maintain necessary pools of amino acids. Proteasomes are readily available whereas autophagy must be induced, thus they may also provide an earlier source of free amino acids than can be provided through autophagy. This may help to restrict autophagy until cells become committed following more prolonged starvation. Finally, the UPS might play a role in regulating autophagy. Proteins inhibitory to the pathway may be proteasome targets when autophagy is induced. In support of this, vacuolar membrane fusion was shown to be regulated by the proteasomal degradation of a vacuolar Rab GTPase (3). Here, vacuoles became fragmented, and autophagy was inhibited, when proteasomal degradation of Ypt7 was blocked (4). Further, proteasomes help to regulate autophagy

initiation by degrading the kinase Ulk1 during autophagy (5). Thus, proteasome activity appears to be required for efficient autophagy regulation. Delayed nuclear export of proteasomes is another possibility for their slow autophagy. is another possible explanation for slow autophagy. Here, the only contribution of proteasome activity would be to potentially degrade some factor that keeps them nuclear over time. This would however be inconsistent with the upregulation seen with rapamycin, as autophagy would occur sooner.

In all, these observations point to a functional role for proteasomes early in autophagy. We observed that when cells were starved for amino acids, proteasome autophagy was not induced. Proteasome activity under this condition appears to be needed rather than their vacuolar targeting. If proteasomes were simply being used for raw materials, autophagy would be expected under this condition. Indeed, we show that a number of conditions that induce general autophagy do not induce proteasome autophagy (chapter 3). This could reflect a requirement for proteasomes under these conditions. Whether proteasome activity is required for efficient autophagy or plays a role in cell survival under autophagy inducing stimuli is of continuing interest.

Signaling Proteasome Localization

It is clear from our work that proteasomes can be degraded selectively and non-selectively through autophagy. Other than factors required for selective autophagy, evidence for selectivity is also seen when proteasomes are not degraded. In these conditions when general autophagy occurs, the machinery for the process is present. However, proteasomes either stay nuclear or are targeted to PSGs. This suggests that there are specific signals that regulate proteasome autophagy induction, granule formation, or nuclear retention. In support of this, we show that carbon starvation signals override nitrogen starvation signals, resulting in PSGs (1). It appears that the induction of general autophagy, depending on the stimulus, results in different signals that govern proteasome responses. One such signal is phosphorylation through MAP kinase signaling, which we found to be involved in proteasome autophagy and granule formation. Whether this pathway directly modifies proteasomes remains to be determined. Considering MAP kinase signaling works to orchestrate a number of stress responses (2, 6–8), the various proteasome outcomes might be controlled indirectly via

different effectors downstream of Mpk1. While we did not detect a role for several downstream effectors of Mpk1 (Chapters 3 and 4), it does not rule out the possibility of some unknown factor playing a role. Thus, it remains an open question whether direct post-translational modification of proteasomes is needed, or an indirect role for MAP kinase signaling is responsible for the loss in proteasome regulation. We hypothesize that the signaling governing the choice in localization is made prior to nuclear export. This is supported by the observation that preventing proteasome autophagy, or PSGs, results in nuclear retention of proteasomes. If the choice was made following export, we would expect an accumulation of cytoplasmic proteasomes upon preventing either pathway. It appears that nuclear localization is the default for proteasomes unless otherwise signaled.

An Alternative Model for PSG Formation

PSG formation is a more dominant signal for the proteasome than autophagy. We have described many novel conditions that induce general autophagy and proteasome granules similar to carbon starvation. The commonality among these conditions is their effects on cellular energy metabolism. Under conditions where cellular energy is depleted, normal levels of proteasomes would result in a larger deficit of ATP. Thus, one hypothesis is that PSGs serve to limit proteasome activity and cellular ATP use. In these conditions where it would cost energy to degrade these complexes, cells degrade other constituents such as plasma membrane proteins, and instead package proteasomes into granules (9). The inactive complexes therein would not utilize ATP and would be available for re-mobilization following a return to optimal conditions. In support of this, it has been shown that quiescent cells that are unable to form PSGs are less viable once returned to rich media (10, 11). Our data further show that loss of viability upon deletion of *NAT3* was rescued when we restored the ability of cells to form PSGs (chapter 4). These observations support a role for proteasome granules in promoting cell survival. A recent model proposed an alternative role for PSGs where they function to protect proteasomes from autophagy. This was based on observations made under carbon starvation where disruption in PSG formation resulted in proteasomes undergoing autophagy (12). Though our observation that general autophagy and PSG formation usually occur together give some support to this idea,

other data presented are inconsistent with this model. Rather than autophagic degradation when PSG formation is restricted, we observed nuclear retention, despite general autophagy (chapters 3 and 4). This occurred upon proteasome inhibitor treatment, as well as when *NAT3* or *MPK1* were deleted. Further, proteasome chaperone mutations show a loss of PSGs with no detectable proteasome autophagy. This is consistent with previous studies showing nuclear localization when PSG formation was disrupted (10). Indeed, the only time we observed an increase in proteasome autophagy, correlating with decreased granule formation, was following carbon starvation where raffinose and glycerol were used (chapter 4). However, this was less than proteasome autophagy observed at earlier timepoints in nitrogen starvation, showing that induction of proteasome autophagy here was minimal.

These and other observations led us to propose a model whereby PSGs help in stress survival. Here, proteasome activity and ATP use would be tailored to cellular needs by sequestering these complexes into granules. By selectively excluding proteasomes, autophagy factors are free to engage other cargo. Previous studies of PSGs are consistent with this model, as they show that upon elimination of PSG inducing conditions, proteasomes quickly return to the nucleus. Thus, some sensor, potentially re-establishment of mitochondrial function, signals PSG dissolution and proteasome nuclear localization. As proteasomes are already protected from autophagy in the nucleus, PSG formation would not be necessary.

Proteasome Assembly and Regulation of Sub-Complexes in Granules

As mentioned above, proteasome granule formation was disrupted when proteasome chaperones or subunits were mutated (chapter 4). This observation implied two intriguing possibilities: either these factors were directly required for PSG formation or damaged proteasomes did not go to PSGs. The latter hypothesis seemed unlikely as these factors are dedicated proteasome chaperones and intrinsic subunits. Defects in proteasome assembly seemed a more likely explanation, as proteasomes undergo quality control allowing cells to recognize faulty proteasomes. However, there is enough proteasome activity in these cells to maintain cellular functions, suggesting there are assembled proteasomes that in principle could form PSGs. The quality control factor Ecm29 is enriched on mutant proteasomes as compared to WT, providing a putative

distinguishing factor (Chapter 4). Indeed, we found that proteasome inhibitors prevented PSG formation partly through Ecm29 binding. We determined that *Ecm29* was responsible for the loss of PSGs observed in mutant proteasome strains. We also found that *Ecm29* was responsible for the loss of PSGs, as well as decreased viability and growth rate, in a *NAT3* deletion mutant. This was one of the first mutants identified that prevents PSG formation, and until now, the molecular mechanism was not clear. We showed that similar to proteasome chaperone mutants, *NAT3* mutants have faulty proteasomes and excess Ecm29 which explains the loss of PSG formation. Consistent with our model, we did not observe autophagy in these proteasome defective mutants. Intriguingly, we did observe that the CP and RP behaved differently in these strains. Whether or not proteasome holoenzymes are exported for PSG formation has remained unclear. One report using an Rpn11-truncation mutant suggested that PSGs are formed from 26S proteasomes (10), while we and others have presented data suggesting differential regulation of subcomplexes (1, 12–14).

With respect to PSG formation, we observed that CP was able to form granules independently of RP. In these chaperone mutants, RP assembly is defective while CP assembly is unaffected. Indeed, there is an upregulation of all proteasome subunits dependent on the Rpn4 feedback loop. Thus, excess CP is produced while cells try to compensate for defective RP assembly. Nevertheless, lower amounts of 26S, and higher amounts of free CP, are phenotypes observed in these mutants. It appears from these data that CP unassociated with Ecm29 is free to form granules, while those in 26S complexes remain nuclear. Importantly, this shows that the CP and RP can be subject to different regulation, an observation consistent with our model of proteasome dissociation prior to re-localization. The Rpn11 truncation mutants that fail to form PSGs for both the CP and RP, are likely to be regulated by distinct mechanism despite the excess CP present.

The Linkage Between Proteasomes, Mitochondria, and Autophagy

Regulation of mitochondrial dynamics and proteasome activity have been linked in mammalian cells. For example, removal of damaged mitochondria is mediated through stabilization of the serine threonine kinase Pink1 on the outer mitochondrial membrane (OMM). Pink1 then recruits the E3 ligase Parkin which ubiquitinates various

outer mitochondrial membrane proteins, resulting in mitochondrial fission and autophagic degradation. Proteasome activity is required for Pink1 degradation which prevents Parkin accumulation and promotes maintenance of healthy mitochondria (15, 16). Parkin, on the other hand, is an E3 ligase and mutations result in the accumulation of misfolded proteins. Mutations that result in loss of function of Pink1 or Parkin are associated with familial parkinsonism due in part to proteasome impairment, accumulation of damaged mitochondria, and accumulation of misfolded proteins (17–19).

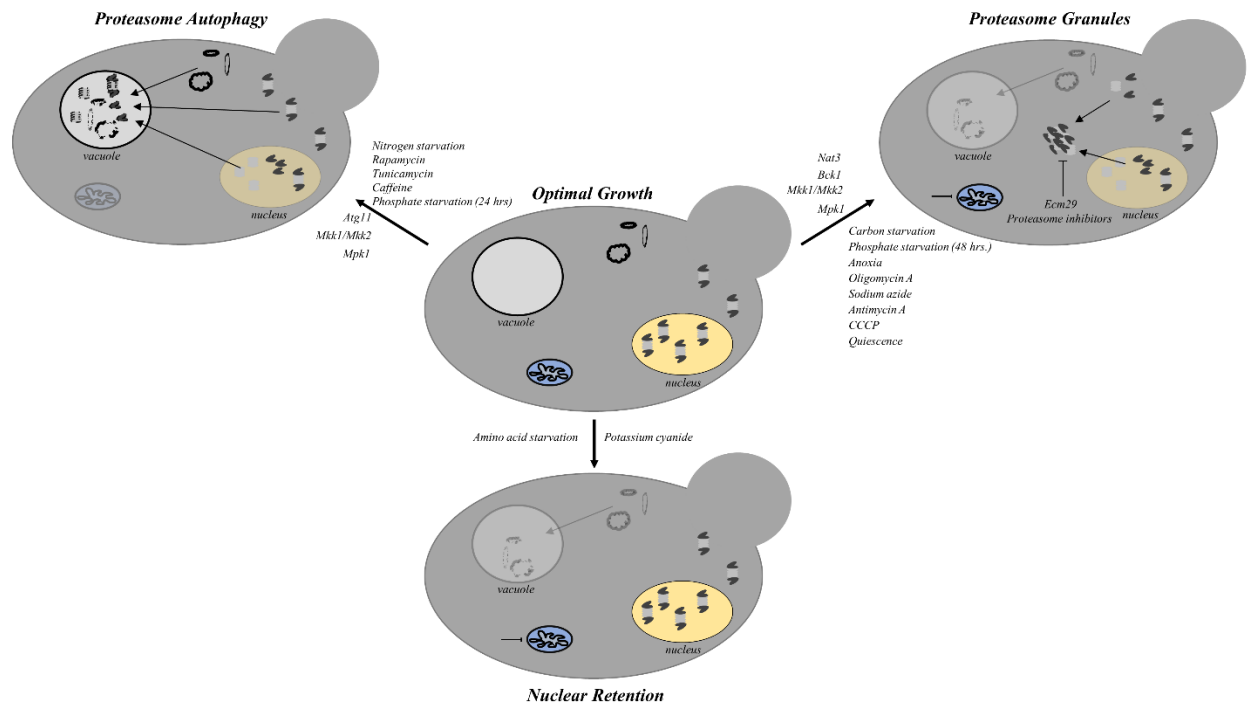
Mitochondrial function was further shown to be linked to proteasome activity in yeast. It was demonstrated that C-terminal truncations of the Rpn11 endogenous proteasome subunit prevented the formation of proteasome granules in quiescent cells (11, 20). Interestingly, this truncation was shown to affect mitochondria by leading to increased fragmentation resulting in decreased respiration. It was further shown that expression in trans of the C-terminal 45 amino acids restored mitochondrial morphology and PSG formation, demonstrating that these residues are required for normal mitochondrial dynamics. It appears under this condition that normal mitochondrial function was required for PSG formation. It is therefore intriguing that inhibiting mitochondrial function results in PSGs. We found that a number of mitochondria inhibitors induced the formation of PSGs in rich media. It is possible that disrupting mitochondrial function by these different means affects PSG formation differently. The Rpn11 truncation was shown to prevent granules in quiescence, and this was partly attributed to perturbations in mitochondrial function. However, the Rpn11 truncation mutant has proteasome assembly defects that could explain the lack of PSG formation, which would be consistent with what we observed in other proteasome assembly mutants. Though mitochondrial function is clearly linked to proteasome localization, the loss of PSG phenotype associated with the Rpn11 truncation might reflect an assembly defect rather than an effect of dysfunctional mitochondria. Indeed, another Rpn11 truncation mutant which showed mitochondrial defects, but had more assembled proteasomes, was able to induce PSGs (10). On the other hand, despite increased free CP in these mutants, no granules were detected for the CP or the RP. This may signify a differential role for Ecm29 in regulating granules in our mutant strains vs the

Rpn11 truncation strains. Nevertheless, a relevant question following these data is whether proteasomes behave similarly in mammalian cells. It will be of interest to determine how disruptions in mitochondrial function affect proteasome localization and activity.

Proteasomes have been shown to antagonize mitochondrial fission, maintain quality control of intermembrane proteins, and play a role in regulating respiration in mammalian cells (21–23). Mitochondrial respiration and autophagy induction are also linked. Carbon starvation does not lead to general autophagy in yeast unless cells are grown in respiring conditions (9, 24). Further, formation of a Snf1-Mec1-Atg1 module on mitochondrial membranes is required for carbon starvation induced autophagy (25). Our data support the notion of an intricate link between the UPS, mitochondrial respiration and autophagy. Our work has focused on how mitochondrial function and autophagy work to regulate proteasome activity. However, it appears proteasome activity can also regulate mitochondrial function and autophagy. The fact that cellular energy production would affect a AAA-ATPase is not necessarily surprising given that proteasome activity requires ATP. Preliminary data suggest that this might be a general response to ATP depletion, as mitochondrial inhibition also induced CDC48, another ATP utilizing machine, to form cytosolic granules (not shown). We envision that cells sequester AAA-ATPases into granules over time as ATP is gradually depleted to limit its use until levels can reach equilibrium; granules would help maintain this equilibrium until the stress is removed. This may indicate a cellular mechanism that works to sequester substrates to prevent their specific activity under particular conditions. In support of this, carbon starvation results in a larger decrease in ATP than nitrogen starvation, and consistent with our model, proteasomes are not packaged into granules in the latter (26).

Proteasome autophagy may serve two purposes in nitrogen starvation. The first would be to produce amino acids from proteasomes to facilitate cell survival. The second would be to prevent proteasomes from degrading proteins once cells have switched to a pro-survival state. This would be functionally equivalent to PSGs limiting proteasome activity. In all, our data support a model whereby proteasome localization and activity are tailored to the metabolic state. Future experiments will test this and other models of proteasome regulation.

Figure 5-1 Model for the regulation of proteasome localization



Approximately 80 % of proteasomes reside in the nucleus of yeast in optimal growth conditions (center). Whereas general autophagy induces the degradation of many cellular substrates, proteasomes are only degraded under certain general autophagy inducing conditions (left). Factors required for proteasome autophagy suggest that this is a specific autophagic pathway. Conditions that induce proteasome autophagy and proteasome granule formation appear to lead to nuclear RP-CP dissociation. This facilitates the nuclear export of proteasomes. Conditions that lead to granule formation instead of proteasome autophagy are shown on the right as well as factors required for this pathway. Mitochondrial function plays a role in this pathway and Ecm29 and proteasome inhibitors can prevent it. Other than autophagy or granule deposition, proteasomes can also remain in the nucleus following autophagy induction (bottom). Proteasomes appear to be dynamically regulated depending on the type of stress cells encounter.

References-Discussion

1. Waite, K. A., De La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2015) Starvation Induces Proteasome Autophagy with Different Pathways for Core and Regulatory Particle. *J. Biol. Chem.* 10.1074/jbc.M115.699124
2. Rousseau, A., and Bertolotti, A. (2016) An evolutionarily conserved pathway controls proteasome homeostasis. *Nature*. **536**, 184–189
3. Kleijnen, M. F., Kirkpatrick, D. S., and Gygi, S. P. (2007) The ubiquitin-proteasome system regulates membrane fusion of yeast vacuoles. *EMBO J.* **26**, 275–87
4. Tang, B., Cai, J., Sun, L., Li, Y., Qu, J., Snider, B. J., and Wu, S. (2014) Proteasome Inhibitors Activate Autophagy Involving Inhibition of PI3K-Akt-mTOR Pathway as an Anti-Oxidation Defense in Human RPE Cells. *PLoS One*. **9**, e103364
5. Nazio, F., Carinci, M., Valacca, C., Bielli, P., Strappazzon, F., Antonioli, M., Ciccocanti, F., Rodolfo, C., Campello, S., Fimia, G. M., Sette, C., Bonaldo, P., and Cecconi, F. (2016) Fine-tuning of ULK1 mRNA and protein levels is required for autophagy oscillation. *J. Cell Biol.* **215**, 841–856
6. Kuranda, K., Leberre, V., Sokol, S., Palamarczyk, G., and Francois, J. (2006) Investigating the caffeine effects in the yeast *Saccharomyces cerevisiae* brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. *Mol. Microbiol.* **61**, 1147–1166
7. Manjithaya, R., Jain, S., Farré, J.-C., and Subramani, S. (2010) A yeast MAPK cascade regulates pexophagy but not other autophagy pathways. *J. Cell Biol.* **189**, 303–10
8. Deffieu, M., Bhatia-Kissova, I., Salin, B., Klionsky, D. J., Pinson, B., Manon, S., and Camougrand, N. (2013) Increased levels of reduced cytochrome b and mitophagy components are required to trigger nonspecific autophagy following induced mitochondrial dysfunction. *J. Cell Sci.* **126**, 415–426
9. Lang, M. J., Martinez-Marquez, J. Y., Prosser, D. C., Ganster, L. R., Buelto, D., Wendland, B., and Duncan, M. C. (2014) Glucose starvation inhibits autophagy via vacuolar hydrolysis and induces plasma membrane internalization by down-regulating recycling. *J. Biol. Chem.* **289**, 16736–16747
10. Saunier, R., Esposito, M., Dassa, E. P., and Delahodde, A. (2013) Integrity of the *Saccharomyces cerevisiae* Rpn11 Protein Is Critical for Formation of Proteasome Storage Granules (PSG) and Survival in Stationary Phase. *PLoS One*. **8**, e70357
11. Rinaldi, T., Hofmann, L., Gambadoro, A., Cossard, R., Livnat-Levanon, N., Glickman, M. H., Frontali, L., and Delahodde, A. (2008) Dissection of the Carboxyl-Terminal Domain of the Proteasomal Subunit Rpn11 in Maintenance of Mitochondrial Structure and Function. *Mol. Biol. Cell.* **19**, 1022
12. Marshall, R. S., and Vierstra, R. D. (2018) Proteasome storage granules protect proteasomes from autophagic degradation upon carbon starvation. *Elife*. 10.7554/eLife.34532

13. Gu, Z. C., Wu, E., Sailer, C., Jando, J., Styles, E., Eisenkolb, I., Kuschel, M., Bitschar, K., Wang, X., Huang, L., Vissa, A., Yip, C. M., Yedidi, R. S., Friesen, H., and Enenkel, C. (2017) Ubiquitin orchestrates proteasome dynamics between proliferation and quiescence in yeast. *Mol. Biol. Cell.* **28**, 2479–2491
14. Nemec, A. A., Howell, L. A., Peterson, A. K., Murray, M. A., and Tomko, R. J. (2017) Autophagic clearance of proteasomes in yeast requires the conserved sorting nexin Snx4. *J. Biol. Chem.* **292**, 21466–21480
15. Lin, W., and Kang, U. J. (2008) Characterization of PINK1 processing, stability, and subcellular localization. *J. Neurochem.* **106**, 464–474
16. Narendra, D. P., Jin, S. M., Tanaka, A., Suen, D.-F., Gautier, C. A., Shen, J., Cookson, M. R., and Youle, R. J. (2010) PINK1 Is Selectively Stabilized on Impaired Mitochondria to Activate Parkin. *PLoS Biol.* **8**, e1000298
17. Liu, W., Vives-Bauza, C., Acín-Peréz, R., Yamamoto, A., Tan, Y., Li, Y., Magrané, J., Stavarahe, M. A., Shaffer, S., Chang, S., Kaplitt, M. G., Huang, X.-Y., Beal, M. F., Manfredi, G., and Li, C. (2009) PINK1 Defect Causes Mitochondrial Dysfunction, Proteasomal Deficit and α -Synuclein Aggregation in Cell Culture Models of Parkinson's Disease. *PLoS One.* **4**, e4597
18. Webb, J. L., Ravikumar, B., Atkins, J., Skepper, J. N., and Rubinsztein, D. C. (2003) α -Synuclein Is Degraded by Both Autophagy and the Proteasome. *J. Biol. Chem.* **278**, 25009–25013
19. Junn, E., Lee, S. S., Suhr, U. T., and Mouradian, M. M. (2002) Parkin accumulation in aggresomes due to proteasome impairment. *J. Biol. Chem.* **277**, 47870–7
20. RINALDI, T., PICK, E., GAMBADORO, A., ZILLI, S., MAYTAL-KIVITY, V., FRONTALI, L., and GLICKMAN, M. H. (2004) Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain. *Biochem. J.* **381**, 275–285
21. Tar, K., Dange, T., Yang, C., Yao, Y., Bulteau, A.-L., Salcedo, E. F., Braigen, S., Bouillaud, F., Finley, D., and Schmidt, M. (2014) Proteasomes associated with the Bln10 activator protein antagonize mitochondrial fission through degradation of the fission protein Dnm1. *J. Biol. Chem.* **289**, 12145–56
22. Lavie, J., De Belvalet, H., Sonon, S., Dupuy, J.-W., Lalou, C., and Bé Nard Correspondence, G. (2018) Ubiquitin-Dependent Degradation of Mitochondrial Proteins Regulates Energy Metabolism In Brief. *CellReports.* **23**, 2852–2863
23. Bragoszewski, P., Gornicka, A., Sztolsztener, M. E., and Chacinska, A. (2013) The Ubiquitin-Proteasome System Regulates Mitochondrial Intermembrane Space Proteins. *Mol. Cell. Biol.* **33**, 2136–2148
24. Adachi, A., Koizumi, M., and Ohsumi, Y. (2017) Autophagy induction under carbon starvation conditions is negatively regulated by carbon catabolite repression. *J. Biol. Chem.* **292**, 19905–19918

25. Yi, C., Tong, J., Lu, P., Wang, Y., Zhang, J., Sun, C., Yuan, K., Xue, R., Zou, B., Li, N., Xiao, S., Dai, C., Huang, Y., Xu, L., Li, L., Chen, S., Miao, D., Deng, H., Li, H., and Yu, L. (2017) Formation of a Snf1-Mec1-Atg1 Module on Mitochondria Governs Energy Deprivation-Induced Autophagy by Regulating Mitochondrial Respiration. *Dev. Cell.* **41**, 59-71.e4
26. Thomsson, E., Larsson, C., Albers, E., Nilsson, A., Franzén, C. J., and Gustafsson, L. (2003) Carbon starvation can induce energy deprivation and loss of fermentative capacity in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **69**, 3251–7