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Nucleocytoplasmic Shuttling of Rpt6 in Saccharomyces cerevisiae

Des'ree Groover
desree.groover@bobcats.gcsu.edu

Nucleocytoplasmic Shuttling of Rpt6 in *Saccharomyces cerevisiae*

Des'ree D. Groover

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Dr. Y. Ellen France, Faculty Advisor

Department of Biological and Environmental Sciences
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Georgia College & State University

College of Arts and Sciences

Department of Biological and Environmental Sciences

We hereby approve the thesis of

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Des'ree D. Groover

Candidate for the degree of Master of Science

Ellen France — 6/25/2020

Dr. Ellen France, Major Professor — Date

Michael L. Gleason — 7/17/2020

Dr. Michael Gleason, Committee Member — Date

Ashok N. Hegde — 7/17/2020

Dr. Ashok Hegde, Committee Member — Date

Eric G. Tenbus — 7-21-20

Dr. Eric Tenbus, Dean of College of Arts and Sciences — Date

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Table of Contents

List of Figures

Abstract

The 26S proteasome is a highly conserved proteolytic complex responsible for regulation of targeted degradation of proteins and cellular processes. It consists of two subassemblies: the 20S core particle (CP) and 19S regulatory particle (RP), which can further be divided into base and lid complexes. The base contains a heterohexameric ring (Rpt1-6) that interacts with the lid and CP. In *Saccharomyces cerevisiae*, the proteasome primarily localizes in the nucleus of proliferating cells. Inducing a cellular stress response such as carbon exhaustion causes the proteasome to shuttle from the nucleus to cytoplasmic compartments called proteasome storage granules (PSGs). Our study specifically focuses on the nucleocytoplasmic shuttling behavior of base subunit Rpt6. In this study, we observed under carbon exhaustion, Rpt6 moves from the nucleus to the cytoplasm into PSGs and colocalizes with Hsp42, a chaperone protein previously reported to localize to PSGs. A canonical nuclear localization signal does not appear to be present on Rpt6; therefore, we decided to truncate Rpt6 and observe its localization in order to identify the region required for Rpt6 nuclear shuttling. First, we attempted serial C-terminal truncations via PCR. However, C-terminal deletion caused a decrease in overall Rpt6 expression level and growth defect in a dominant-negative manner. Currently, we are exploring N-terminus region deletions using CRISPR-Cas9 technology, to target the first 44 amino acids of Rpt6 to be truncated. Identifying the specific region required for Rpt6 nuclear translocation will shed light on the mechanism of Rpt6 function and its role within the 26S proteasome in *S. cerevisiae.*

Introduction

The composition of all living organisms is determined by its genes. While cells of the same organism contain the same genes, the variation between cells and cell types is caused by how their genes are expressed. Gene expression can vary over the life span of a cell based on signals received from environmental cues. Based on growth and environmental conditions, cells must activate or inactivate specific genes necessary for development. Regulating gene expression begins with the process of transcribing genes in a spatial and temporal manner.[1] Transcription is a highly regulated processes requiring a variety of transcriptional components such as transcription factors, activators, mediators, and others.[2] To ensure this process is highly regulated, cells acquire a variety of regulatory mechanisms for proper regulation of transcribing genes such as the ubiquitin-proteasome system (UPS), which is known to play an important role in maintaining proper level of various transcription regulators.

Regulation of General Biological Processes via Proteolysis

Availability of transcriptional factors to trigger gene expression is a major mechanism the cell relies on. Degrading proteins, also known as proteolysis, is responsible for some intracellular housekeeping and as a response to cellular stresses. It functions in removing damaged or non-essential proteins to supply amino acids required for generating new proteins that are needed.[3] Damaged or misfolded proteins can form aggregates and cause proteotoxicity; therefore, the removal of these proteins via regulated proteolysis is crucial for cell survival. Another interesting function of regulated proteolysis is how it serves as a controller of an internal cellular clock driven by fluctuations in various protein levels which ultimately leads to the unidirectional progression of cellular processes. For example, the cell cycle is driven by cyclin proteins which

have a short lifespan where they are degraded within minutes after their synthesis.[4] Various cyclins rise and fall based on the combined effect of transcription followed by proteolysis, which ultimately ensures cells move forward through different stages of the cell cycle. Cytoplasmic proteolysis is generally mediated by two major pathways—lysosomal degradation and the ubiquitin-proteasome system. Lysosome-based degradation mediates removal of proteins that are transported through secretory pathway by riding vesicles to endosome-lysosomal compartments. However, proteolysis of cytoplasmic proteins is mediated through a highly regulated mechanism known as ubiquitin-proteasome system (UPS). Cells have acquired the ability to successfully regulate transcription by utilizing proteolytic UPS.[1] In particular, the proteasome is recognized as the central component in handling proteolysis that is connected to regulation of gene expression.

Ubiquitin-proteasome system regulation

The ubiquitin-proteasome system is a substrate-specific machinery that requires the tagging of a substrate with polyubiquitin chain for rapid proteolysis (Figure 1). The targeted substrates for degradation are tagged with attachment of several ubiquitin molecules, which is required for recognition by the 26S proteasome. Ubiquitin is a highly conserved small protein consisting of 76 amino acids.[5] Ubiquitination is a post-translational modification that can significantly alter tagged substrates and their potential fate. Two well studied ubiquitin modifications are monoubiquitin and polyubiquitin. Monoubiquitylation often signals for non-proteolytic signals, such as endocytosis, whereas polyubiquitination often involves proteasome degradation.[6] Substrate-fate specificity is determined through specific polyubiquitin linkages. For example, the interglycine lysine-48 (K48) linkage to glycine 76 is the most prevalent chain linkage in

signaling proteasome-mediated to degrade the tagged substrate.[7] In contrast, lysine-63 (K63) linkage can regulate non-proteolytic activity that is independent of proteasomal functions.[6]

Tagging a protein requires a series of ATP driven enzymatic reactions mediated by three different enzymes: ubiquitin activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligases (E3).[8] The ubiquitination reaction begins with E1 catalyzing the activation of ubiquitin by interacting with the carboxyl group on the ubiquitin C-terminus. The activated ubiquitin gets transferred by E1 to the cysteinyl residue found on E2. The final step involves E2 forming a complex with E3 to transfer ubiquitin to tagged substrate for subsequent proteolysis. The ubiquitin is transferred by attaching to the ε-amino found on lysine residues of targeted substrate.[8] E3, the ubiquitin ligase, mediates the final step in this ubiquitination reaction by recognizing and covalently attaching the polyubiquitin chain to the substrate. After recognition of the polyubiquitin chain by the proteasome lid complex, the substrate becomes deubiquitinated, allowing the ubiquitin to be recycled. The protein substrate is unfolded by the enzymes found in the base complex, preparing the substrate for translocation to the proteolytic chamber. A conformational change of Rpt2, Rpt5, and Rpt4 of the α-ring opens the channel initiating the translocation of substrate.[9] Once the substrate reaches the proteolytic chamber, it gets cleaved by the β-subunits, and the cleaved substrates are released as small peptides.

Besides maintaining general protein homeostasis, the proteasome function is particularly critical for cellular processes such as the cell cycle and DNA replication and repair, which are both driven by fluctuations of specific protein levels in the cell. Consequently, a defective proteasome leading to non-selective degradation or decrease in processivity releasing partial degraded polypeptides can lead to severe consequences.[10] For example, the effects of impaired

proteasomal activity can be linked to different biological factors including aging, cancer, neurodegenerative diseases, and other late onset diseases.[11]

Overview of 26S Proteasome

The 26S proteasome is a highly sophisticated complex consisting of two subassemblies: the catalytic core particle (CP; 20S proteasome) and the regulatory particle (RP; 19S proteasome) (Figure 2). The 20S CP is formed by α- and β-subunits, seven each, on the outer and inner component of the structure, respectively.[10] The α- and β-subunits is stacked to form a barrel-shaped cylinder. The α-ring functions to regulate accessibility of substrate to the proteolytic chamber whereas β-ring is proteolytically active – specifically β_1, β_2, β_5.[10]

When the 20S CP is capped by 19S RP, the proteasome becomes a mature 26S holoenzyme. The 19S RP is a subcomplex divided into lid and base, consisting of a regulatory particle of triple-A ATPase (Rpt) subunits and regulatory particle of non-ATPase (Rpn) subunits, respectively. Functions of these individual complexes vary in regulating the proteasome by substrate entry or translocation of substrates to the proteolytic chamber. The 19S RP can be capped on one or both terminals of the 20S CP, making the proteasome enzymatically active.[14] The lid complex is the inferior component of the 19S RP and functions in deubiquitination of tagged substrates specifically for degradation.[14] The base component of 19S is comprised of six homologous AAA-ATPase subunits (Rpt1-Rpt6) and four non-ATPase subunits (Rpn1, Rpn2, Rpn10, and Rpn13). The ATPase domain of the Rpt subunits is highly conserved at the C-termini whereas the N-termini sequences are more divergent (Figure 3). The six ATPase subunits form a hexameric ring enclosing a channel through its center for substrate translocation. In addition, this hexameric ATPase ring functions by recruiting tagged substrates by recognizing

the polyubiquitin chain, inducing substrate unfolding, and opening of the α-rings for translocating unfolded substrates to the proteolytic chamber.[14]

Multiple studies have shown the significance of UPS function in regulating transcriptional activators through limiting activator abundance and assisting transcription.[7] Intricate mechanisms of the proteasome have evolved to ensure an approach that is efficient in maintaining accurate protein selection for degradation. After conducting a genome-wide analyses of 19S and 20S subunit mapped to chromatin association sites, there was an overlap of 19S and 20S at active transcription sites.[7] This indicates that the 26S proteasome is actively involved in the transcription process. Therefore, in this context, it is important to understand the mechanism of proteasomal subunits independently shuttling in and out of the nucleus to better understand their role in transcription regulation.

Rpt6 Subunit

Rpt6 is a polypeptide of 45 kDa. in size and serves as an essential component of the AAA-ATPase hexameric ring. Recent studies have shown the importance of Rpt6 in assembly of the 26S proteasome.[15] In the hexameric ring, Rpt6 is located between subunits Rpt2 and Rpt3 where Rpt6 forms a dimer with Rpt3 to initiate proteasomal assembly.[15] The tail of Rpt6 plays an important role in assembling the hexameric ring by specificity of binding to its $\alpha 3$ pocket.[16] The C-termini of Rpt6 becomes an anchor allowing the rest of the hexameric ring to assemble in the proteasome. Not only has Rpt6 shown to be significant in the proper assembly of proteasome but there is evidence that it shuttles in and out of the nucleus possibly for a role in transcription.

Interestingly, proteasome subunits including Rpt6 appear to play a significant role in regulating transcription in mammalian neurons. Formation of long-term memory via changes in the synapses requires active transcription of genes that allows replacement of previously

degraded proteins while increasing the level of pre-existing proteins.[17] In our collaborator's laboratory, chemical stimulation to trigger synapses change, mimicking long-term memory formation, coincided with individual Rpt subunits including Rpt6 translocating to the nucleus from the cytoplasm, but independently from one another rather than as subcomplex (Bach S.V. and Hegde, A.N., unpublished). At present, specific regions of individual Rpt proteins that are recognized and regulated by nuclear pore complex to allow this nucleocytoplasmic shuttling behavior are not well known. Therefore, we deemed that the identification of specific regions within individual proteasome subunits necessary for the movement as an important first step in understanding the link between proteasome function and transcription regulation in the nucleus.

Our goal in this study was to identify the region critical for the nucleocytoplasmic shuttling of Rpt6 in *Saccharomyces cerevisiae.* As proteasome subunits are highly conserved through eukaryotes, *S. cerevisiae* serves as a convenient model system that allows various genetic modifications such as generating a fusion protein with a green fluorescent protein (GFP) tag and tracking protein movement by performing live microscopy. We observed carbon exhaustion, which will trigger systemic gene expression changes, can reproducibly cause nucleocytoplasmic shuttling of proteasome subunits.[18] Interestingly, when carbon source is depleted, proteasome subunits leave the nucleus and retreat to proteasome storage granules (PSGs) as cells enter into quiescence state (Figure 4). Theses PSGs present themselves as cytoplasmic puncta found in the cytosol, harboring intact forms of the 26S proteasome and protecting against degradation based on autophagy.[19] In an attempt to identify a potential region of the yeast Rpt6 subunit responsible for the shuttling behavior, we generated several versions of Rpt6 in which the C-terminal is serially truncated. Nucleocytoplasmic shuttling behavior of these

truncation mutants were then examined under the carbon exhaustion condition with or without. non-exhausted condition.

Materials and Methods

Yeast strains. All yeast strains used in this study have BY4742 background. Specific genotypes of strains used in this study are listed in Table 1. C-terminal deletions and fusion of GFP and td-Tomato on chromosomal copy of the respective genes were generated by PCR-mediated homologous recombination.[20]

Growth Conditions. Wild type cells were grown in synthetic complete medium (SC), which contains 2% glucose. Deletion constructs were grown in either selective media (SC-his-ura) or SC. All yeast cultures for experiments were grown at 30°C. For carbon exhaustion experiment, cells at OD_{600} of 0.1 in fresh media were allowed to grow continuously for 96 hrs, following the method in a published study to deplete carbon source in the media.[21] At the end of the 96-hrs incubation, cells were collected, quickly washed three times in 1X PBS, and resuspended in either fresh media or filtered carbon-depleted media that the cells originally grew in. After 15-mins incubation, cells were prepared for microscopy. For growth curve measurements, wild type cells and deletion construct strains at OD_{600} of 0.1 were set to grow in SC or SC-his-ura, respectively, and over a time course up to 72-144 hrs, an aliquot of each culture was taken for OD_{600} measurement to generate a growth curve. The experiment was repeated for three times.

Deletion Construct. Serial deletion and tagging of GFP to endogenous *RPT6* genes at their 3'-end was performed by generating PCR-based cassette containing *HIS3* marker according to the

published method.[18] PCR mixture included sterile ddH_2O, NEB Q5 Buffer, 10 mM dNTPs, forward and reverse primer, pFA6a-GFP-His3 template, and NEB Q5 DNA Polymerase. The PCR cycle settings were initial denaturation at 98°C for 2 mins, denaturation at 98°C for 10 seconds, annealing at 68°C for 30 seconds, extension at 72°C for 60 seconds, and final extension 72°C for 10 mins. Denaturation, annealing, and extension undergo 30 cycles. To verify deletion construct, DNA gel electrophoresis was performed. A wild type strain harboring a *CEN* plasmid with wild-type *RPT6* under endogenous promoter and terminator was transformed with the deletion construct cassettes to be integrated at the 3' end of the chromosomal *RPT6* gene via homologous recombination. The transformants were grown on SC-his-ura plate, and individual colonies were picked and grew in liquid SC-his-ura media at 30°C overnight and processed for live fluorescence microscopy. The wild-type strain, expressing Rpt6-GFP was used as a control to compare with localization of Rpt6 C-terminal deletion GFP strains.

Microscopy. Slides were prepared by adding 2 µl of diluted cells and 2 µl of EverBrite DAPI Mounting Medium for nuclear staining. Cells were observed using Zeiss Axio Scope.A1 with X-Cite Xenon light source using oil immersion 63X objective lens (1.4 NA Plan-APOCHROMAT) and 100X lens (1.3 NA Plan-Neoflaur) along with FITC, TRITC, and DAPI filters to visualize excitation of GFP, td-Tomato, and nucleus, respectively. Images were acquired using AxioCam MRm Rev3 camera controlled by AxioVision Special Edition (Zeiss International). Merged fluorescence images were generated using Image J (NIH). The fluorescence microscopy per each strain was performed three independent times, and n>200 cells were counted each time for quantification.

Western Blot Analysis. For protein gel samples, 2.5 OD_{600} unit of overnight culture were harvested. After suspending cell pellets in 100 µl of sterile ddH_2O, 100 µl of 0.2M NaOH was added, and the mixture was incubated at room temperature for five minutes, then centrifuged at top speed (21,100 x g). After supernatant was discarded, pelleted cells were resuspended in 50 µl of 2x Laemmli Buffer with DTT (BIO-RAD Cat. #161-0737) and boiled for three minutes.[21] The crude lysates were spun again at 21,1000 x g and a 15 µl of protein sample was loaded onto a 4 – 12% pre-cast gel (Mini-PROTEAN® TGX™; BIO-RAD Cat. #456-1095) for SDS-PAGE. The gel ran at 120V for 90 minutes in SDS running buffer (25mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). After completion of SDS-PAGE, proteins were transferred onto a 0.45 µm nitrocellulose membrane (BIO-RAD Cat. #162-0145) via wet transfer running at 120V for 90 minutes. For western blot analysis, the nitrocellulose membrane was first incubated in 1X PBS (pH 7.4) for 15 minutes followed by Odyssey® Blocking Buffer (PBS) (LI-COR® Lot #927-40000) to block for 1 hour at room temperature with gentle rocking. The anti-GFP primary antibody (SIGMA Lot #019M4760V) was diluted to 1:500 in Odyssey® Blocking Buffer and incubated for 1 hour at room temperature. Membrane was washed three times, alternating between 1X PBS and 1X PBS + 0.1% Tween20 for five minutes. The secondary antibody (IRDye® 680LT Goat anti-Rabbit 925-68021; LI-COR Lot #C81022-09) was diluted to 1:10,000, and the blot was incubated for 1 hour at room temperature, then washed as previously. Odyssey® Fc Dual-Mode Imaging System (LI-COR® Model 2800) was used to visualize the blot.

Results

Rpt6 translocates from nucleus to the cytoplasm upon carbon depletion

It is well established that 26S proteasome primarily localizes to the nucleus of actively proliferating yeast cells[18,20,22-2]. In order to track nucleocytoplasmic shuttling of the Rpt6 subunit, we obtained a strain where the endogenous *RPT6* is tagged with GFP as a sole copy of *RPT6*. We confirmed that GFP tagged Rpt6 localizes to nucleus as expected while displaying the growth rate comparable to that of untagged wild-type cells (Figure 5). In order to induce Rpt6 shuttling out of the nucleus, however, we needed a condition that would induce systemic gene expression change. Based on a published study by Laporte *et al.* (2008), we identified carbon depletion as an inducible condition to trigger the movement of Rpt6 out of the nucleus and into the cytoplasm. Interestingly, as cells enter into stationary phase upon carbon depletion, proteasomal subunits in yeast are known to shuttle from the nucleus into cytoplasmic puncta, presumably proteasome storage granules (PSG) that have been reported by others.[23-25] We verified the published observation by monitoring Rpt6-GFP under carbon depletion using direct fluorescent microscopy. Indeed, Rpt6-GFP was primarily detected in the nucleus of active proliferating cells in fresh media (Figure 6A) while Rpt6-GFP signal starts to shift more and more to cytoplasmic puncta as cells were continuously grown over 24 and 48 hours (Figure 6A and B).

Rpt6 relocalizes from the nucleus in a reversible manner and colocalizes with Hsp42, proteasome storage granule marker

Next, we tested to see if the localization of Rpt6 in the proteasome storage granule (PSG) upon reaching quiescence could be reverted to the nucleus upon cells being released into fresh

media as previously reported. First, we grew a culture of Rpt6-GFP cells continuously over four days to deplete carbon source, inducing the ejection of Rpt6-GFP from the nucleus, then released an aliquot of cells from aged culture into fresh SC media for 15 minutes, then subjected cells from both conditions to fluorescent microscopy. In aged culture, Rpt6-GFP was primarily found in PSGs as expected, whereas Rpt6-GFP in cells that were shifted from aged media to fresh media localized back to the nucleus only after 15 minutes (Figure 7). This result clearly showed that exiting quiescence and re-entrance into the proliferative phase, which is reflected by Rpt6-GFP relocalization, can be consistently triggered by restoring the carbon source to quiescent cells.

To confirm the identity of the cytoplasmic puncta seen by Rpt6-GFP localization upon carbon depletion, we examined the localization of Heat shock protein 42(Hsp42p) – an essential protein in formulating protein aggregates under cellular stress response.[26] Hsp42p is also an established marker for PSG. We tagged endogenous copy of *HSP42* with tdTomato in our Rpt6-GFP strain and examined the colocalization of both Rpt6-GFP and Hsp42-tdTomato over a time course of 48 hours using direct fluorescent microscopy (Figure 8). Our results showed that Hsp42-tdTomato localizes to cytoplasmic puncta distinct from nuclear Rpt6-GFP seen during proliferative cycle, showing 0% co-localization (Figure 8b). As the culture continues to grow, at the 24-hour time point, 60% of cells showed colocalization of Rpt6-GFP with Hsp42-tdTomato in PSGs (Figure 8b). By the 48-hour time point, nearly 100% of cells showed clear overlap between Rpt6-GFP and Hsp42-tdTomato in PSGs (Figure 8b), confirming that the cytoplasmic puncta of Rpt6-GFP seen under carbon depletion are indeed, PSGs.

C-terminal deletion mutants appear to cause severe growth defect and behave in "dominant negative" manner

The next logical step was to identify the region of Rpt6 required for nucleocytoplasmic shuttling. Analysis of Rpt6 amino acid sequence revealed that it does not contain a canonical nuclear localization signal (NLS).[27] To identify the region responsible for nuclear localization,, we introduced partial deletions on the endogenous *RPT6* gene to generate serial truncations of Rpt6 protein at the C-terminus (Figure 10). We reasoned that if we delete the region of Rpt6 protein required for exiting from the nucleus and localizing into the PSGs, such mutant Rpt6 would fail to localize to PSG upon carbon depletion. Due to the fact that *RPT6* is an essential gene and that the C-terminal deletions may disrupt the essential function of Rpt6, potentially causing lethality upon deletion, prior to performing deletion, we transformed the strain with wild-type *RPT6* on a low copy plasmid. The schematic diagrams of five deletion mutants with C-terminal GFP tag constructed (*rpt6-Δ1, rpt6-Δ2, rpt6-Δ3, rpt6-Δ4,* and *rpt6-Δ5*) are shown in Figure 9.

Next, we grew all five deletion mutants independently to check their growth in comparison to wild type Rpt6-GFP to make sure that the mutants were healthy enough for us to proceed with fluorescence microscopy for localization studies. Surprisingly, all deletion mutants poorly grew when compared to full length wild type Rpt6-GFP cells even though they harbor wild type copy of RPT6 gene on plasmids (Figure 10). Although, mutants *rpt6-Δ1* and *rpt6-Δ2* exhibited similar growth rate in respect to wild type cells. After 72 hours, *rpt6-Δ3, rpt6-Δ5* exhibited decline in growth rate rather than maintaining stationary phase compared to wild type cells.

Upon microscopic examination, the deletion mutants did not appear to express detectable Rpt6 with GFP signal when compared to Rpt6-GFP in wild type cells (Figure 11). In early logarithmic phase, Rpt6-GFP wild type cells were enriched in the nucleus as expected (100%;

n>150; Figure 11b). In *rpt6-Δ1* through *rpt6-Δ4* cells, less than 7% of cells showed GFP signal whereas deletion mutant *rpt6-Δ5* cells show no signal at all (0%; Figure 11b). Also, under differential contrast microscopy (DIC), cell morphology appeared to be somewhat abnormal with a disorganized cytoplasm upon attempted carbon depletion (data not shown).

C-terminally truncated Rpt6 mutant proteins cannot be detected at the protein level

Due to the fact that the GFP signal of all C-terminally truncated Rpt6 mutant proteins tagged with GFP were barely detectable, we performed an SDS-PAGE and western blot to check the expression of these truncated proteins (Figure 12). The three control strains with Rpt6-GFP consistently produced bands of 72 kDa in size, which is the expected size of a full-length Rpt6 with tagged GFP. However, even after multiple attempts of repeating western blots with modified protocols, we were unable to ascertain the expression level of truncated Rpt6-GFP constructs because of nonspecific background bands appearing on our blots.

Discussion

In this study, our goal was to identify the specific region of Rpt6 required for the nucleocytoplasmic shuttling behavior upon carbon depletion and subsequent entrance to quiescence. Toward this aim, we generated to generate serial truncations on the C-terminus of Rpt6 and we a view to observe which mutant *Rpt6* mutant may lose shuttling behavior upon carbon depletion in comparison to wild-type. To do so, we established the standard condition that can be reproducible for inducing Rpt6 exit from the nucleus to the cytoplasm based on established reports.[22] Indeed, we were able to confirm that carbon depletion by growing cells to stationary phase induces Rpt6 to exit from the nucleus to cytoplasmic puncta, which we

confirmed to be proteasome storage granules (PSGs). We also established that relocalization from the PSGs to the nucleus is readily reversible upon restoring carbon source[18,20].

In previously established studies from Laporte *et al*. (2008) and Peters *et al.* (2016), core particle and regulatory particle proteasomal subunits revealed shuttling behavior from the nucleus to cytoplasm when monitoring protein movement. The study conducted by Laporte *et al.* (2008) focused on establishing the significance of PSGs and its function to harbor functional proteasomes under unfavorable cellular conditions by evaluating Pre6p, a core particle subunit, protein movement.[18] However, recently, Peters *et al.* (2016) published a study evaluating the association between PSGs and insoluble protein deposits (IPODs) by following a regulatory particle subunit, Rpn5, protein movement using established techniques from Laporte *et al.* (2008).[21] Continuous growth of yeast culture in limited media forces cells into a state of starvation caused by the lack of available nutrients, forcing the cells to enter into replicative quiescence, which is associated with systemic gene expression change. [28] As a result of nutrient stress response, it is imperative that yeast cells must cease active dividing to conserve energy required for expression of only essential genes. In addition, PSGs harbor proteasomes in an effort to protect intact proteasomes from autophagic degradation and ensure cells have available mature proteasome for cell cycle progression when entering the proliferative phase.

Hsp42p is a chaperone protein that was previously identified in the context of the proteasome migrating to PSGs.[21] In our study, we further validated Rpt6-GFP translocation with respect to Hsp42-tdTomato movement. Hsp42p plays a vital role in the assortment of easily aggregated proteins to deposits and protein sequestration in quiescent yeast cells.[29-30] We provided evidence that supports the notion that Rpt6 colocalizes with Hsp42p under carbon exhaustion. As cells respond to stresses, a variety of cytoplasmic puncta are seen harboring

different cellular proteins. The use of Hsp42p in our study provided a PSG marker to confirm Rpt6-GFP translocation to PSG.

C-terminus is essential for growth and function of Rpt6

In order to identify the region that allows Rpt6 to shuttle between nucleus and PSGs, we proceeded to "cut off" the C-terminal region of Rpt6 and track its movement, reasoning that the loss of the required region for shuttling would result in failed shuttling behavior, hence identifying the required region. The reason we decided to proceed with C-terminal truncations rather than N-terminus was based on the availability of the yeast technique that would readily let us delete the 3'-end of the endogenous gene, while incorporating GFP tag at the end via PCR-based cassette and homologous recombination. Also, deleting the 5'-end of the endogenous gene via the conventional methods used in yeast is much more difficult due to the potential disruption of promoter sequence and regulatory sequences. Based on all examinations — growth rate monitoring and fluorescence microscopy seem to indicate that all five truncated Rpt6 mutant proteins appear to cause a "dominant negative" effect, causing severe growth defect even in the presence of wild-type Rpt6 proteins produced from a plasmid inside the cell. Perhaps the disruptive effects of C-terminal truncation have to do with the fact that the C-terminus of Rpt6 is important for Rpt6 function within the proteasome. Indeed, the C-terminus region of Rpt6 contains a highly conserved AAA-ATPase domain and is known to be responsible for stabilizing the interaction between CP and RP and mediating substrate translocation to cleavage sites in CP.[9, 31] Therefore, truncating C-terminus may disrupt these essential functions of Rpt6, which is critical for cell viability. To confirm the suspected lack of GFP signal under fluorescent microscopy, we performed western blot analysis using anti-GFP antibody. However, we were

unsuccessful at measuring mutant Rpt6 gene expression level as the results were inconclusive due to high background.

Having attempted C-terminal truncation to identify the region required for Rpt6 shuttling unsuccessfully, an alternative strategy would be to target the N-terminus of Rpt6 for deletion. As previously mentioned, the conventional methodology in yeast does not easily allow us to perform N-terminal deletion without causing disruption to the endogenous promoter region of the gene. However, recently, CRISPR-Cas9 technology in yeast became available for experimental use, and CRISPR-Cas9 with designed guide RNA can target specific regions of interests for deletion, which is otherwise difficult to target with other technologies. Previous studies showed the success of CRISPR-Cas9 in targeting the N-terminus of a variety of targeted genes (Kan^R, GFP, and mCherry) found in plasmids of *S. cerevisiae.*[32] Our experimental rationale in targeting the N-terminus region is based on the fact that Rpt subunits seem to display much more the divergent sequence variations on the N-terminus, which may dictate their individual behaviors when compared to the C-terminus containing highly conserved ATPase domain. Based on this rationale, we hypothesize that the N-terminal region may potentially contain a specific amino acid sequence responsible for Rpt6 shuttling behavior. Successful targeting of the N-terminal region for deletion can potentially provide us a specific region to perform site-directed mutagenesis to further dissect specific amino acids critical for Rpt6 shuttling behavior.

In conclusion, we determined that truncating at the C-terminus of Rpt6 leads to a decrease in gene expression and growth defects, which suggests that the C-terminus of Rpt6 harbors many essential factors required for proper functioning of the protein. However, our observations led us to confirm that under carbon depletion, Rpt6 translocates to cytoplasm entering a state of quiescence that was confirmed in previous studies. Also, it is unclear in *S.*

cerevisiae whether Rpt6 shuttles from the nucleus to cytoplasm independently of the 19S RP which should be further explored. Our future direction in elucidating the region required for Rpt6 shuttling mechanism is focusing on the N-terminus. With hope of finding our region of interest, we are optimistic about shifting our focus to this region on Rpt6 through the use of CRISPR-Cas9 technology. The initial step for identifying this potential region at the N-terminus is successfully constructing a plasmid containing Cas9 with guide RNA (gRNA). Groundwork for constructing the plasmid has begun and will be followed by truncating the first 18 amino acids at the N-terminus of Rpt6.

Appendix 1: Tables

Table 1. Yeast strains.

Strain	Genotype	Mating Type	Additional Information
EFY233	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	α	BY4742 background
EFY237	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; RPT6-GFP-HIS3*	α	BY4742 background
DGY4	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; RPT6-GFP-HIS3; HSP42-tdTomato-KANMX*	α	BY4742 background
DGY5	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; RPT6::GFP-pFA6a-HIS3[Cen pRS416-RPT6-URA3]*	α	BY4742 background
DGY6	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; *RPT6::[RPT6 aa 381-405Δ)-GFP-pFA6a-HIS3*	α	BY4742 background; Deletion construct 1
DGY7	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; *RPT6::[RPT6 aa 356-405Δ)-GFP-pFA6a-HIS3*	α	BY4742 background; Deletion construct 2
DGY8	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; *RPT6::[RPT6 aa 306-405Δ)-GFP-pFA6a-HIS3*	α	BY4742 background; Deletion construct 3
DGY9	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; *RPT6::[RPT6 aa 256-405Δ)-GFP-pFA6a-HIS3*	α	BY4742 background; Deletion construct 4
DGY10	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; *RPT6::[RPT6 aa 206-405Δ)-GFP-pFA6a-HIS3*	α	BY4742 background; Deletion construct 5

Table 2. Deletion construct primers.

Name	Primer Sequence	Notes
Rpt6-GFP Deletion 1 (F)	5' – AAGCTGGTATGTATGCTTTAAGAGAAAGAATACACGTTACTCAAGAACGGATCCCCGGGTTAATTAA – 3'	Amino acids 381 – 405 deletion; protein size ~ 69 kDa
Rpt6-GFP Deletion 2 (F)	5' – GTGGTATCAACTTGAGAAAGGTTGCTGAAAAGATGAACGGTTGTTCTGGTCGGATCCCCGGGTTAATTAA – 3'	Amino acids 356 – 405 deletion; protein size ~ 67 kDa
Rpt6-GFP Deletion 3 (F)	5' – AGATCATAATGGCCACGAATAGACTAGATATTCTAGATCCAGCACTTTTGCGGATCCCCGGGTTAATTAA – 3'	Amino acids 306 – 405 deletion; protein size ~ 61 kDa
Rpt6-GFP Deletion 4 (F)	5' – AACATGCTCCCTCAATTATCTTTATGGATGAAATCGATTCCATTGGCTCTCGGATCCCCGGGTTAATTAA – 3'	Amino acids 256 – 405 deletion; protein size ~ 55 kDa
Rpt6-GFP Deletion 5 (F)	5' – GCCCCCCTGGTACAGGGAAAACCTTATTGGCAAGAGCTGTCGCACATCACCGGATCCCCGGGTTAATTAA – 3'	Amino acids 206 – 405 deletion; protein size ~ 50 kDa
Rpt6 C-terminal Deletion (R)	5' – ATACACATACACTAAGTAACATATACAATGAGCCAAGTGAAACGAATACAGAATTCGAGCTCGTTTAAAC – 3'	Integrated with GFP tag

Appendix 2: Figures

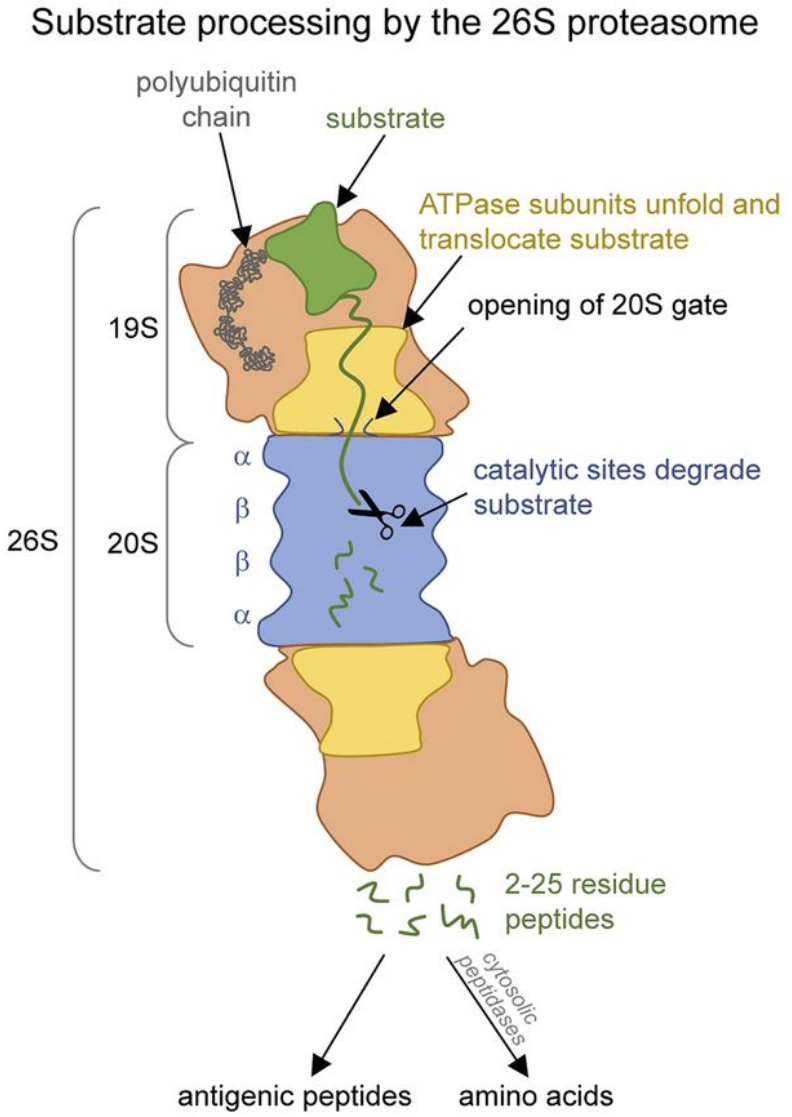


Figure 1. The process of substrate degradation by 26S proteasome. Substrates tagged with poly-ubiquitin chain are recognized by the 19S lid component where the hexameric ATPase ring will begin to unfold the substrate and translocate into the 20S core particle. In the 20S core particle, the unfolded substrate becomes degrade by the α and β rings. The degraded substrate will exit the 26S proteasome as 2-25 residue peptides and recycled for future use. (Modified from Thibaudeau, T.A. and Smith, D.M., *Pharma Rev,* (2019) 71(2):170-197.)

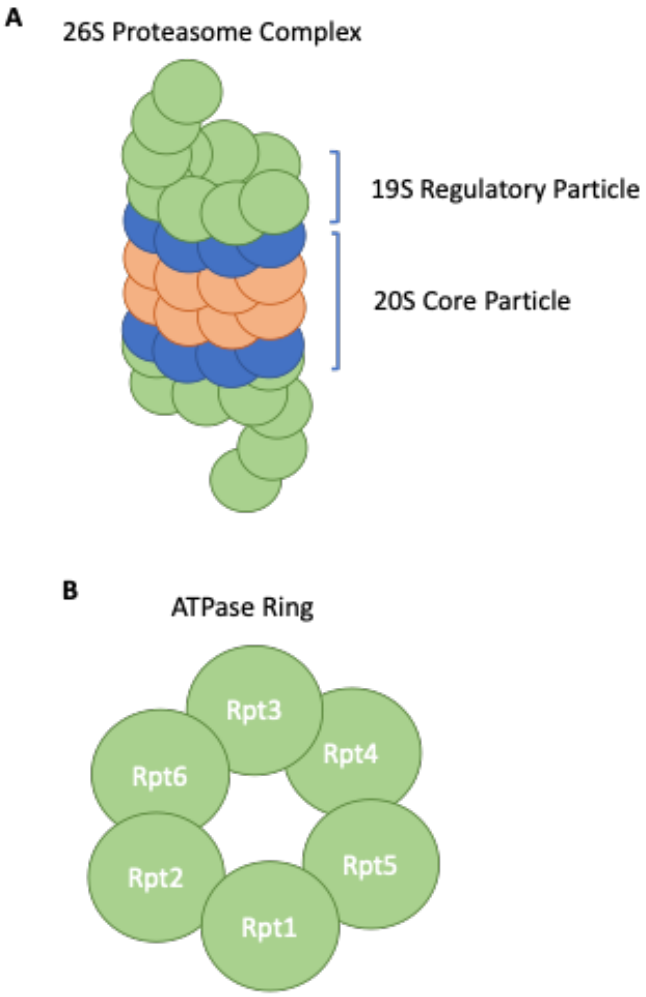


Figure 2. Structural overview of proteasome complex. (A) Simplified structural composition of 26S proteasome consisting of 20S core particle subassemblies (CP) and 19S regulatory particle (RP). The RP forms lid and base. (B) The base diagram of a hexameric ATPase ring composed by Rpt1-Rpt6.

Figure 3. Schematic overview of Rpt subunit structural domains. A visual representation of Rpt protein structural domains found in all six isoforms in *Saccharomyces cerevisiae*. N-terminal regions display much variability in amino acid sequence from protein to protein while the C-termini of Rpt proteins, which contain triple ATPase domain, is highly conserved. (Modified from Inobe, T. and Genmei, R., *PLoS One*. (2015) 10(7):e0134056.)

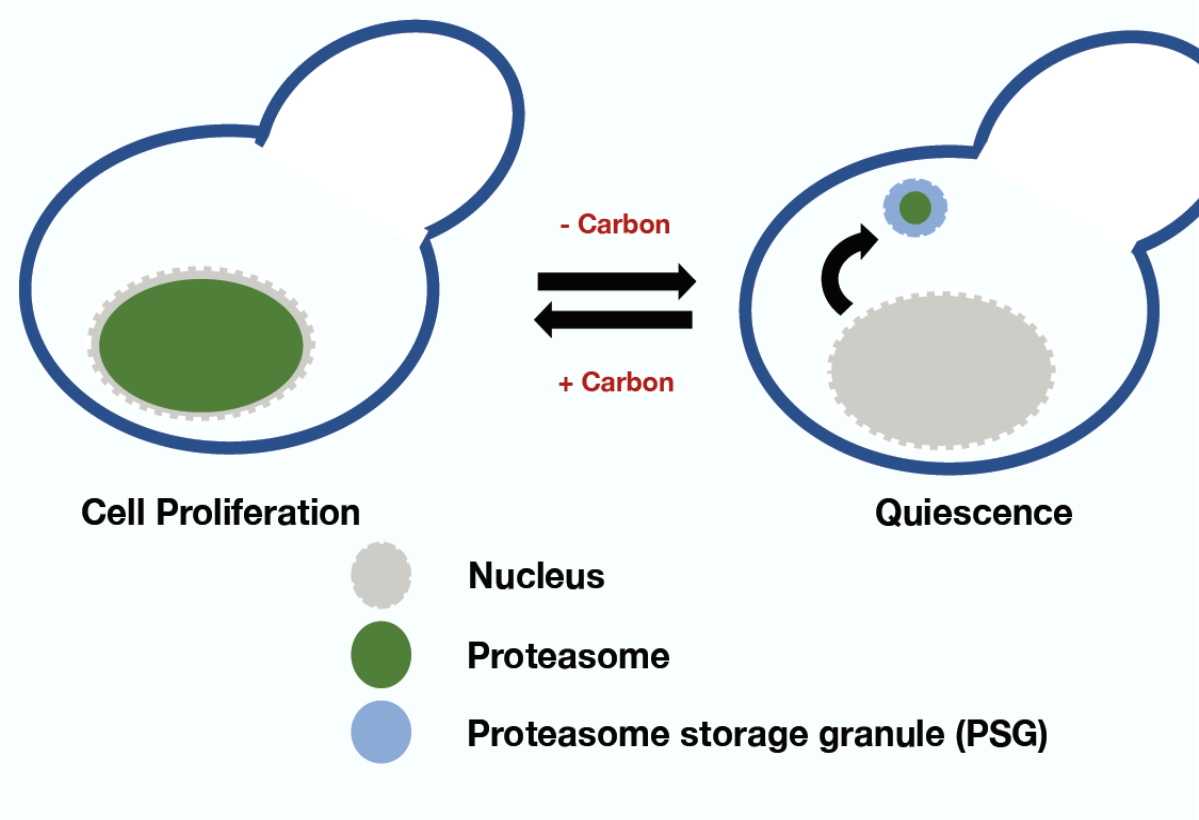


Figure 4. Proteasome Storage Granules (PSGs) formation under carbon depletion. Depleting carbon source in media results in formation of PSGs in the cytoplasm, which is reported to contain the intact subassemblies of the proteasome. Replenishing cells with a fresh carbon source induce the proteasome exiting from PSGs and return to the nucleus. (Modified from Peters L. *et al., JCS*. (2016) 129, 1190-1197.)

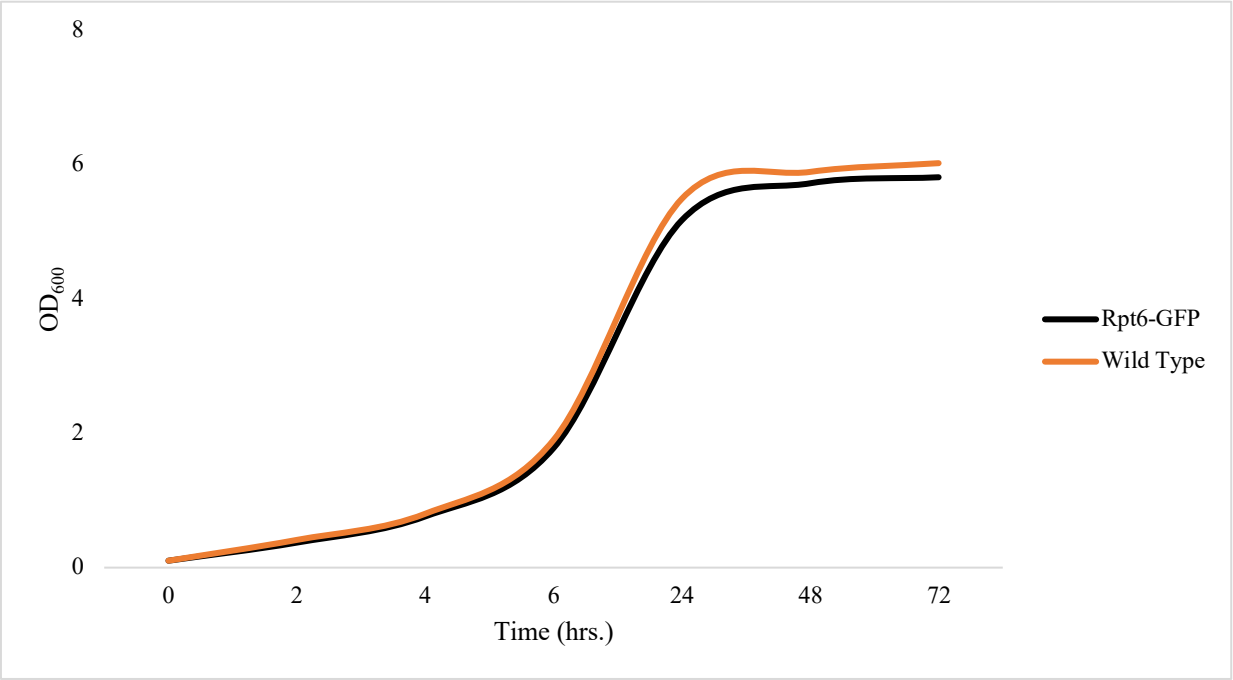
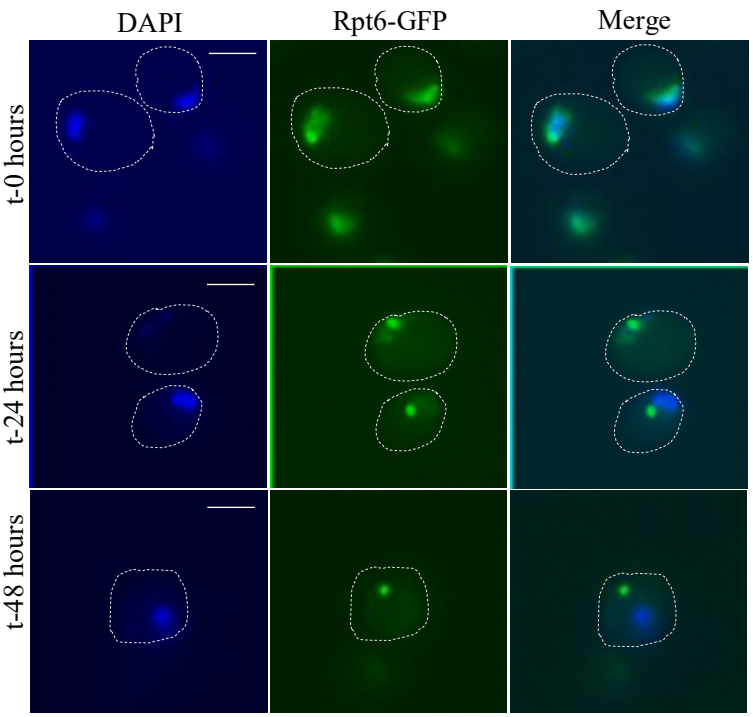


Figure 5. Growth curve of untagged wild-type and Rpt6-GFP. Both strains were grown at 30°C in SC overnight to logarithmic phase. The following morning, the cells were diluted to an OD600 of 0.1 and grown continuously for a time course of 96 hours.

A.



B.

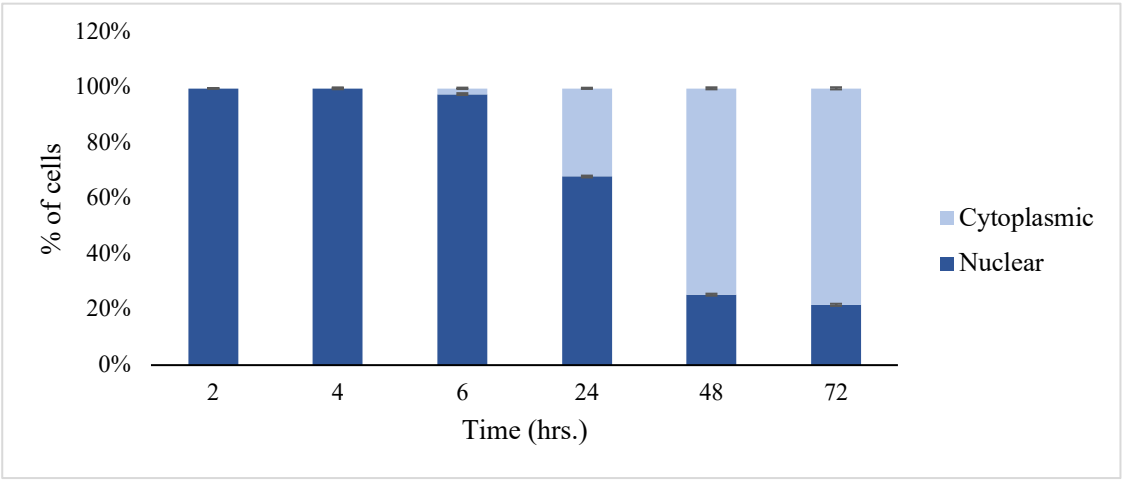


Figure 6. Rpt6-GFP translocate to Proteasome Storage Granules (PSGs) under carbon depletion. (A) Continuous growth of Rpt6-GFP wild-type grown at 30°C in SC for a period of 48 hours. Visualization of Rpt6-GFP nuclear shuttling is shown over a 48-hour period. Nuclei is visualized by the binding of DAPI to DNA. Scale bar represents approximately 2µm. (B) Cell culture grown continuously in SC for 96 hours to track the movement of Rpt6-GFP to the PSGs. At each time point, GFP fluorescence (Rpt6-GFP) and nucleus (via DAPI) were monitored using fluorescent microscopy. n ≥ 200 for each time frame were counted. Error bars show standard error between three independent experiments.

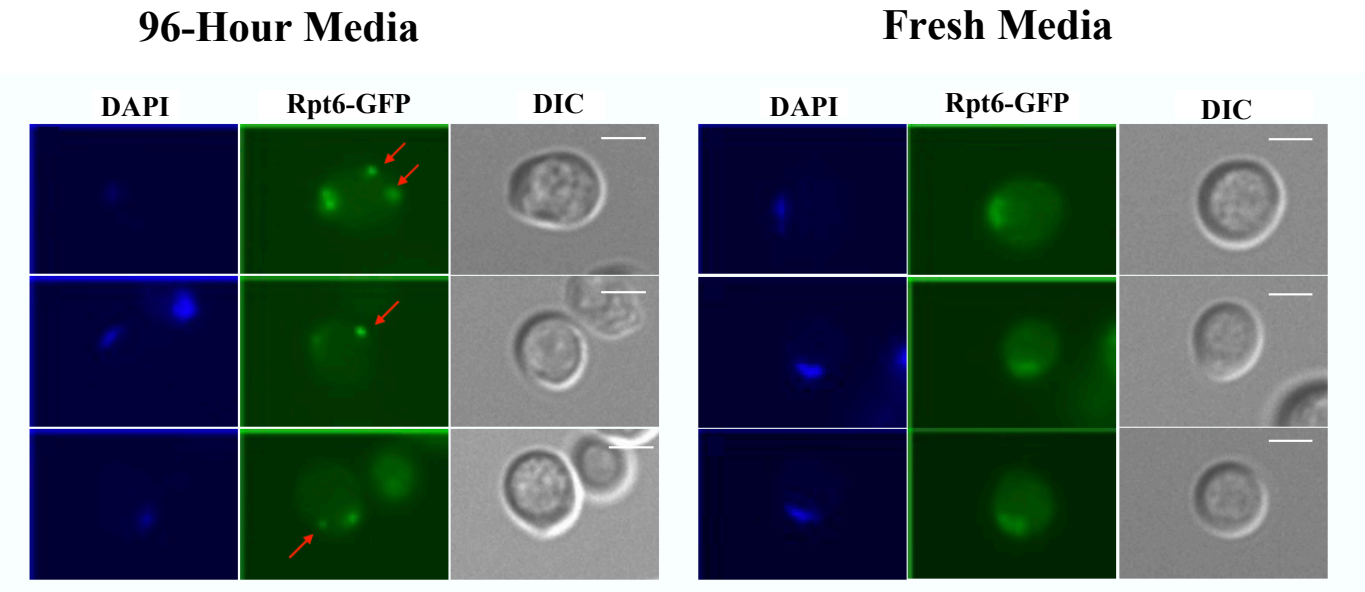
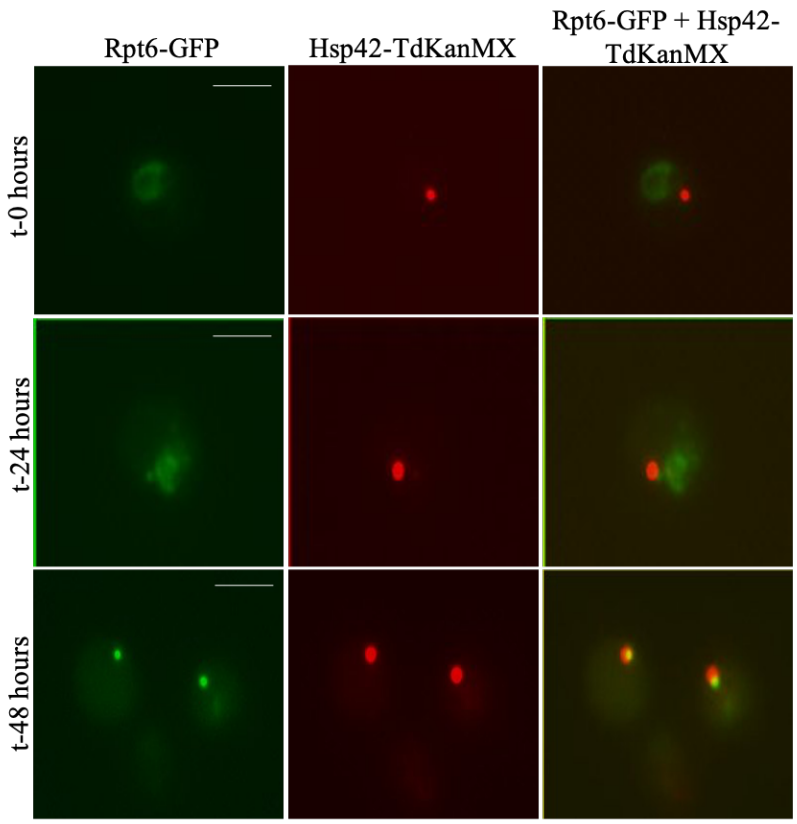


Figure 7. Rpt6-GFP re-enters the proliferative cycle when nutrients are replenished. Rpt6-GFP is primarily localized to the nucleus when cells are actively dividing. Cells grown in synthetic complete (SC) media for 4 days enters into quiescence where Rpt6-GFP localizes to the PSGs (A, B, and C). Replenishing the cells with fresh media triggers cells to re-enter the proliferative cycle, which coincide with the Rpt6-GFP re-localizing to the nucleus within 15 minutes (D, E, and F). Red arrows indicate PSGs. Scale bar represents 2 µm.

8a.



8b.

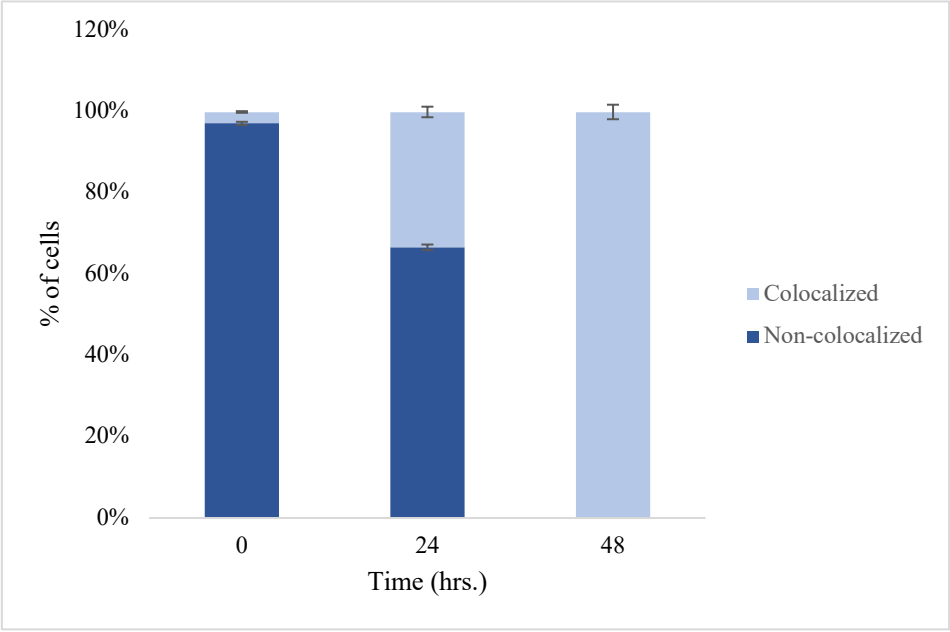


Figure 8. Rpt6-GFP colocalizes with heat shock protein (Hsp42) in PSGs. Cells expressing Rpt6-GFP and Hsp42-TdKanMX were continuously grown at 30°C in SC media for 48 hours. n ≥ 150 for each time point. Scale bar represents approximately 2µm.

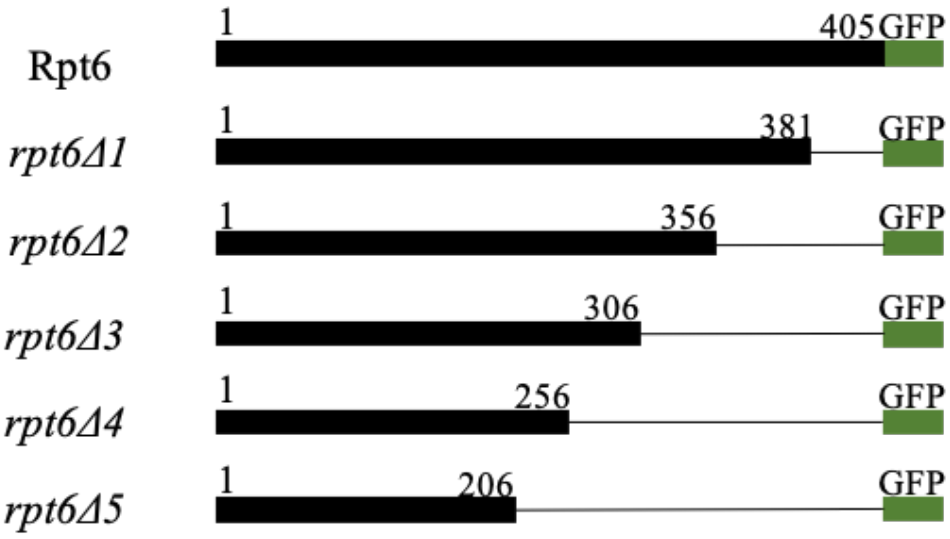


Figure 9. Rpt6 C-terminal truncation constructs. Schematic overview of constructs for serial C-terminal truncation of Rpt6 protein with GFP-tag at the C-terminus.

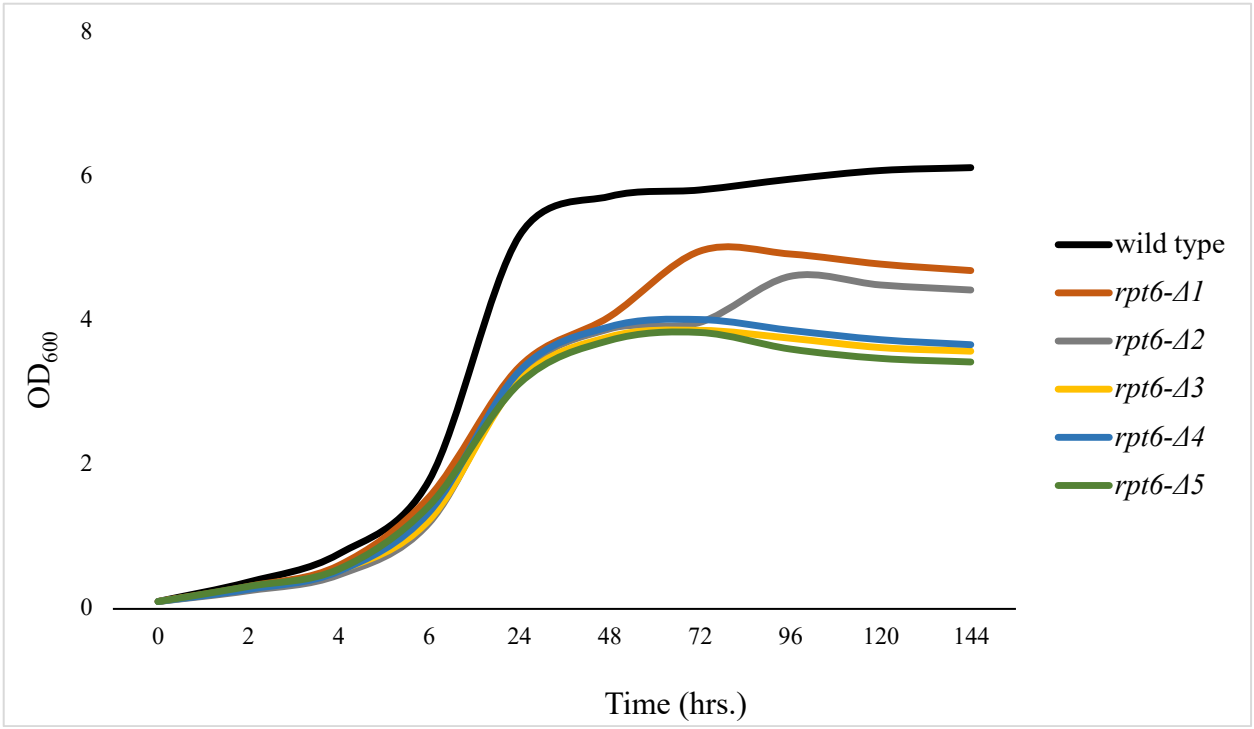
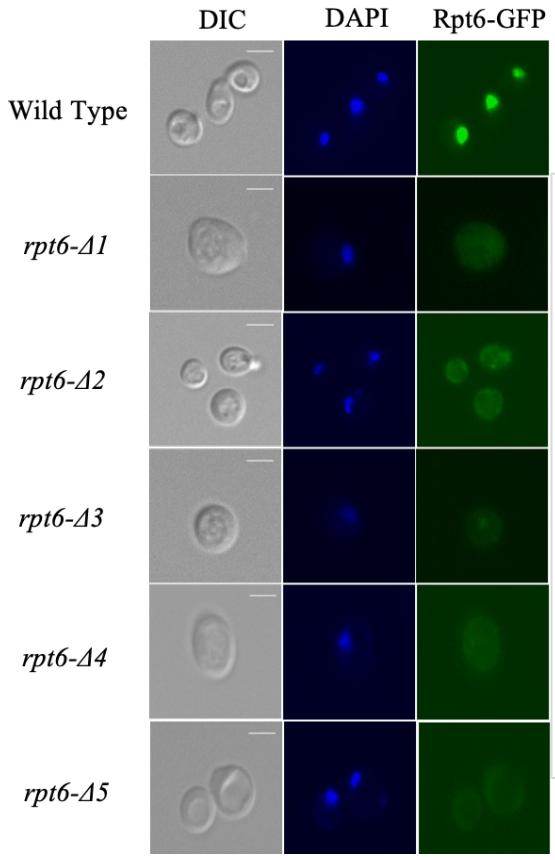


Figure 10. Growth curve containing deletion mutants and wild-type strain. Cells were grown continuously for six days at 30°C. Deletion mutants were grown in SC-ura-his and wild type strain was grown in SC.

A.



B.

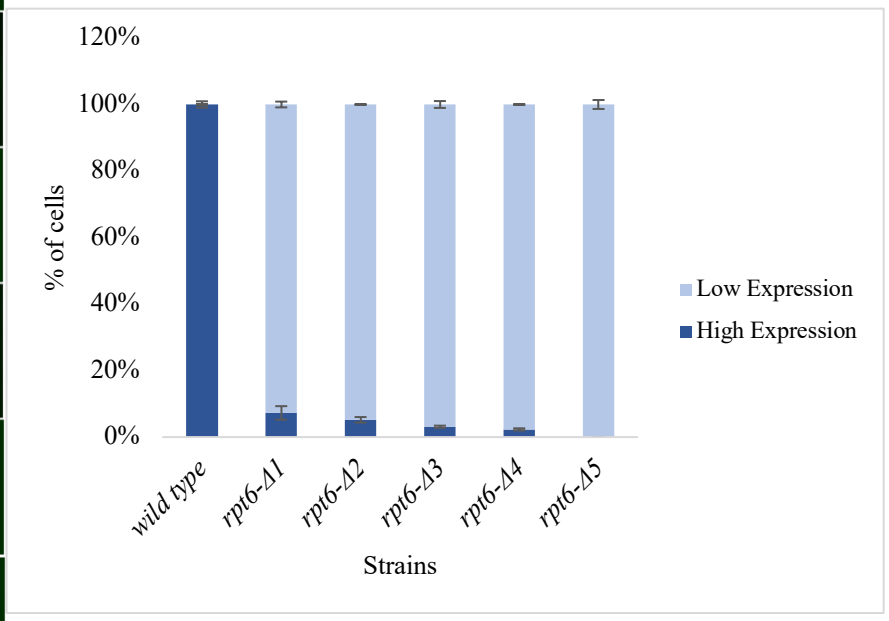


Figure 11. Deletion of the C-terminal decreases Rpt6 expression. (A) Strains containing Rpt6 C'-terminal deletion 1-5 were grown in rich media (SC) at 30°C to early logarithmic phase. (B) Cells were observed based on level of expression of Rpt6 deletion using fluorescent microscopy. n > 150 for each strain. Scale bar represents approximately 2µm.

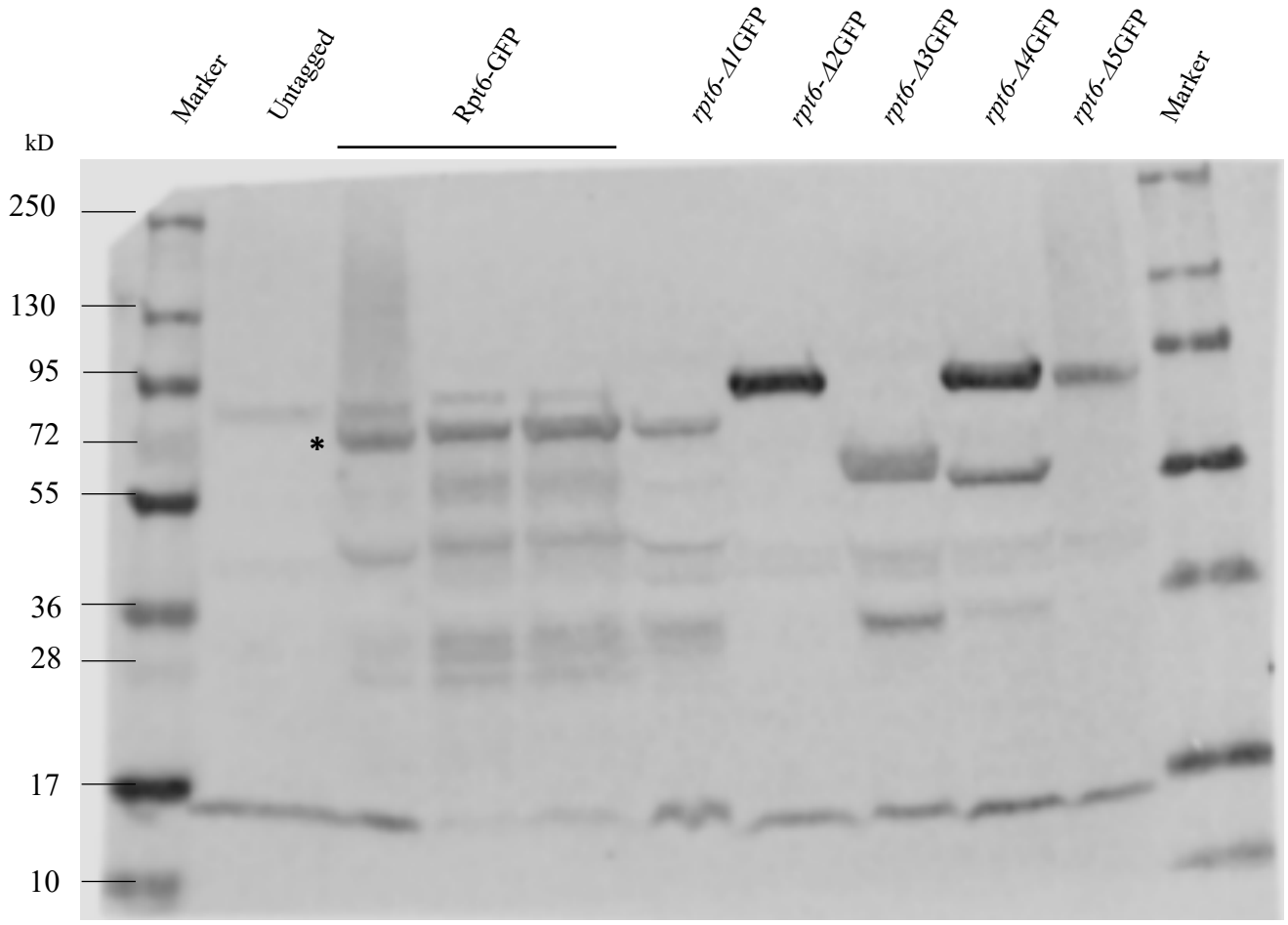


Figure 12. Western blot analysis of Rpt6-GFP deletion GFP proteins. Samples was run on 4-12% gradient gel and transferred to 0.45 µm nitrocellulose membrane. The membrane was blotted with anti0GFP antibody at 1:500 dilution. Lane 1: wt strain with no GFP; Lane 2-4: strains that contain Rpt6-GFP; Lane 5: *rpt6-1* GFP (69 kDa); Lane 6: *rpt6-2* GFP (67 kDa); Lane 7: *rpt6-3* GFP (61 kDa); Lane 8: *rpt6-4* (55 kDa); Lane 9: *rpt6-5* GFP (50 kDa). * indicates a band predicted to be full length Rpt6-GFP based on the calculated molecular size of 72 kDa.

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