Ubiquitin orchestrates proteasome dynamics between proliferation and quiescence in yeast

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ABSTRACT Proteasomes are essential for protein degradation in proliferating cells. Little is known about proteasome functions in quiescent cells. In nondividing yeast, a eukaryotic model of quiescence, proteasomes are depleted from the nucleus and accumulate in motile cytosolic granules termed proteasome storage granules (PSGs). PSGs enhance resistance to genotoxic stress and confer fitness during aging. Upon exit from quiescence PSGs dissolve, and proteasomes are rapidly delivered into the nucleus. To identify key players in PSG organization, we performed high-throughput imaging of green fluorescent protein (GFP)-labeled proteasomes in the yeast null-mutant collection. Mutants with reduced levels of ubiquitin are impaired in PSG formation. Colocalization studies of PSGs with proteins of the yeast GFP collection, mass spectrometry, and direct stochastic optical reconstitution microscopy of cross-linked PSGs revealed that PSGs are densely packed with proteasomes and contain ubiquitin but no polyubiquitin chains. Our results provide insight into proteasome dynamics between proliferating and quiescent yeast in response to cellular requirements for ubiquitin-dependent degradation.

INTRODUCTION

The proteasome is a 2.5-MDa, multisubunit protease complex responsible for the degradation of proteins conjugated to polyubiquitin chains. Failures in ubiquitin-proteasome-dependent protein degradation result in intracellular accumulations of irreversible and immotile ubiquitin-containing protein inclusions, which are indicative for age-related neurodegenerative diseases (Ciechanover and Brunold, 2003; Goldberg, 2003; Zheng et al., 2016). However, not all protein inclusions detected in centenarians’ brain tissues consist of irreversible and immotile proteins (Haller et al., 2013; Xekardaki et al., 2015). Protein inclusions that are reversible and motile, such as proteasome granules, may even protect from the early onset of neurodegenerative diseases (Amen and Kaganovich, 2015).

Proteasome granules have an enigmatic structure. The mechanism underlying their organization is unknown but seems to be conserved, as proteasome granules are found in nondividing quiescent yeast and human neuronal cells (Enenkel, 2014).

Quiescence is a reversible state in which cell proliferation is temporarily arrested during chronological aging (O’Farrell, 2011; Valcourt et al., 2012). Drastic metabolic changes accompany the transition from proliferation to quiescence. The genome undergoes significant topological reorganizations (Rutledge et al., 2015), and multiple molecules accumulate in storage compartments (Gray et al., 2004; De Virgilio, 2012). Global repression of transcription and translation ensures longevity of the quiescent state (Miles et al., 2013; McKnight et al., 2015).

Proteasome granules are the result of proteasome reorganizations during quiescence, as thousands of individual proteasomes gather within one to two membrane-less organelles. In yeast, proteasome granules were originally named proteasome storage...
granules (PSGs), presumably protecting proteasomes from degradation (Laporte et al., 2008). PSGs are primarily detected at a young replicative age. Their presence confers cell fitness during chronological aging (van Deventer et al., 2015) and resilience toward genotoxic and proteotoxic stress (Ratnakumar et al., 2011; Doherty et al., 2012; Weberbuss et al., 2013; van Deventer et al., 2015).

Proteasomes are the second most abundant protein complexes in eukaryotic cells (Marguerat et al., 2012). Composed of ~40 different subunits, proteasome holo-enzymes consist of the proteolytic core particle (CP) flanked by one or two regulatory particles (RPs) (Baumeister et al., 1998). The CP comprises two outer and two inner rings of seven distinct α and β subunits, respectively. The β-rings harbor the proteolytic sites. The α-rings control the access of protein substrates that are delivered by the RP. The RP is composed of approximately nine lid and approximately 10 base subunits, of which distinct Rpn (regulatory particle non-ATPase) subunits recognize the polyubiquitin chain. The ubiquitin moieties are cleaved before substrate degradation (Peth et al., 2010; Rosenzweig et al., 2012). The RP lid subunit Rpn11 catalyzes the en bloc removal of the polyubiquitin chain (Verma et al., 2002; Yao and Cohen, 2002), while the proteasome-associated protein Ubp6, the yeast homologue of USP14, catalyzes the removal of supernumerary polyubiquitin chains (Lee et al., 2016), thus allowing gradual deubiquitylation of the substate (Crosas et al., 2006; Hanna et al., 2006). The deubiquitylated substrate is translocated into the proteolytic chamber of the CP through Rpt (regulatory particle triple-A (AAA) ATPase) subunits adjacent to the CP α-ring (Rabl et al., 2008). During cell proliferation, when the intracellular ATP level is high (Laporte et al., 2011), the RP is attached to the CP, yielding proteasome holo-enzymes that are primarily located in the nucleus and are functionally active for degrading polyubiquitylated proteins (Tanaka, 2009; Enenkel, 2014).

In yeast, the ATP level declines with the transition from proliferation to quiescence due to glucose depletion in stationary phase (Laporte et al., 2011). The intracellular pH drops, and the protoplasmic fluidity decrease impacts the dynamics of soluble proteins (Laporte et al., 2011; Parry et al., 2014; Munder et al., 2016). Proteasomes accumulate at the inner side of the nuclear envelope (Laporte et al., 2008), pass through the nuclear pore, and gather in an early intermediate of the PSG, which yields the mature PSGs separate from insoluble protein deposits (IPOD) (Kaganovich et al., 2008; Peters et al., 2016).

Although CP and RP colocalize within the PSGs, CP–RP assemblies are less stable in carbon- (Bajorek et al., 2016) or nitrogen-starved cells (Waite et al., 2016). The instantaneous gain of ATP that occurs upon glucose feeding of carbon-starved cells triggers the resumption of growth and the immediate clearance of PSGs. Within a few minutes, CP–RP assemblies are formed that relocate to the nucleus, illustrating the reversible and motile nature of the PSGs (Laporte et al., 2008; Weberbuss et al., 2013).

A genome-wide systematic screen in proliferating yeast revealed that lowering the intracellular pH induces premature PSG formation (Peters et al., 2013). It is conceivable that pH and metabolites influence proteasome assembly and intracellular movement, but the key proteins controlling proteasome dynamics and PSG organization remained unknown. Based on our previous studies, Blm10 is the only protein known to be involved in the sequestration of the CP into PSGs. Its absence affects the sequestration of the CP but not of the RP (Weberbuss et al., 2013).

PSGs, which share the feature of reversibility with juxtanuclear quality compartments (JUNQ) (Kaganovich et al., 2008), are not the only spherical structures of proteasome sequestrations. Cell cycle arrest by proteasome inhibition or nitrogen starvation also induces the sequestration of proteasomes, although in irreversible and immobile protein inclusions that resemble IPOD-like structures (Marshall et al., 2016). When either the proteasome is inactivated or essential amino acids are depleted, the proteasome becomes polyubiquitylated and is sacrificed for Atg8-mediated autophagic degradation (Marshall et al., 2015; 2016; Cohen-Kaplan et al., 2016). Dysfunctional and misassembled proteasomal subunits are also sequestered into IPOD-like compartments for final degradation (Peters et al., 2015). The lysosomal targeting of these proteasomal components appear to be specific for nitrogen starvation but is not observed under glucose starvation (Waite et al., 2016).

Yeast cells were grown in yeast peptone glucose (YPD) media at 30°C throughout this work to avoid mixed phenotypes with reversible and irreversible proteasome granules due to limitations of carbon and nitrogen sources.

Applying high-throughput microscopy to the yeast null-mutant collection, we screened for genes required for the orchestrated sequestration of the CP and RP into the PSGs. We also screened the yeast collection of green fluorescent protein (GFP)-labeled open reading frames (ORFs) for proteins that form reversible and motile granules in quiescence. Both screens identified genes that are involved in balancing the load of polyubiquitin chains on the proteasome versus the concentration of free ubiquitin. Reduced levels of free ubiquitin were parallel with impaired PSG formation. Using mass spectrometry and direct stochastic optical reconstruction microscopy (dSTORM) analysis, in vivo cross-linked PSGs were found to be densely packed and primarily composed of CP, RP, and free ubiquitin. Overexpression of lysine-less ubiquitin variants induced premature PSG formation during proliferation. On the basis of these findings, we propose a model for proteasome movements in proliferating and quiescent cells in response to cellular requirements for ubiquitin-dependent degradation.

**RESULTS**

Systematic identification of yeast null mutants with deficiencies in PSG formation

To shed light into proteasome dynamics in yeast, we set out to explore genes that are involved in the sequestration of proteasomes into PSGs by exploiting high-throughput imaging screens based on automated image acquisition coupled with the quantification of specific morphological phenotypes (Boone et al., 2007; Tong et al., 2001). To systematically survey the yeast deletion collection for defects in PSG formation, we created a library of ~4300 null mutants expressing the GFP-labeled variant of the β5-subunit Pre2 (Pre2-GFP), which is a well-established reporter protein of the CP (Figure 1A) (Enenkel, 2012). In addition to Pre2-GFP, mCherry-labeled histone Hta2 (Hta2-mCherry) and the ribosomal RPL39 promoter region driving expression of tdTomato (RPL39pr-dTomato) were crossed into the arrayed collection of null mutants as markers of the nucleus and cytoplasm, respectively (Supplemental Figure S1).

Forty-five null mutants were identified with deficiencies in PSG formation (Supplemental Table S1). The mutants were grouped according to their biological functions: 1) nuclear transport; 2) ubiquitin modifying proteins; 3) proteins regulating energy levels; 4) kinases and phosphatases; 5) DNA repair and chromatin remodeling; and 6) proteins with miscellaneous functions. V-type ATPases involved in premature PSG formation in proliferating cells (Peters et al., 2013) were not among our hit genes, consistent with the finding that V-type ATPases disassemble with glucose deprivation in quiescence (Martinez-Munoz and Kane, 2008).
A high-throughput imaging screen of temperature-sensitive mutants of essential genes turned out to be hardly feasible, because each mutant requires a specific restrictive temperature and incubation time to display its phenotype in quiescence (e.g., {\textit{ubu1-1}} mutants of the ubiquitin-activating enzyme {\textit{Uba1}} [Shimada et al., 2002] formed PSGs upon growth for 3 d at 35°C).

Because CP and RP colocalize within PSGs, our main focus was directed toward proteins that orchestrate the sequestration of the CP and RP into the PSGs. The hit mutants that were identified using Pre2-GFP were assayed with GFP-labeled RP base subunit Rpn1 and lid subunit Rpn11 (Rpn1-GFP, Rpn11-GFP). Once again, Hta2-mCherry and cytosolic RPL39pr-tdTomato marked the nucleo- and cytosol, respectively. Twenty-one null mutants showed perturbed sequestration of the CP into PSGs. Because {\textit{BLM10}} deletion does not impair the sequestration of the RP into the PSGs (Webberrus et al., 2013), Blm10 does not fulfill the criterion of a protein orchestrating PSG formation (see the Supplemental Material for {\textit{blm10Δ}} strains with two different genetic backgrounds; Supplemental Figure S2).

As shown by our previous studies on {\textit{blm10Δ}} mutants, the deficiency in PSG formation is linked with a delay in the resumption of growth upon exit from quiescence, notably after DNA damage by phleomycin (Webberrus et al., 2013). To test the correlation between delayed outgrowth from quiescence and failures in PSG formation in our hit mutants, we plotted regrowth curves for lag-phase differences with and without phleomycin. Eight hit mutants showed delays in the resumption of growth upon exit from quiescence and perturbed PSG formation (Supplemental Figure S3A). Among them were null mutants of \textit{UBP6}, \textit{UBI4}, and \textit{DOA1} involved in the regulation of the cellular ubiquitin concentration (Kraut et al., 2007) (Supplemental Figure S3B). SDS–PAGE and Western blot analysis verified that each GFP-labeled proteasomal reporter subunit is expressed during proliferation and quiescence (Figure 1C, wild type, \textit{ubp6Δ}, and \textit{ubi4Δ}).

In particular, the deletion of \textit{UBP6} and \textit{UBI4} resulted in PSG frequencies below 10% (Figure 2A, wild type and \textit{ubp6Δ} cells; Figure 2C, \textit{ubi4Δ} cell; Figure 2, B and D, quantification). The null mutant of \textit{DOA1} displayed ~30% PSG formation and was omitted from follow-up investigations. In the hit mutants, proteasomes were equally abundant, showing that disturbed PSG formation is not caused by proteasome depletion (Supplemental Figures S7 and S8).

\textit{UBP6} codes for a proteasome-associated ubiquitin-specific protease that contributes to the replenishment of ubiquitin (Crosas et al., 2006; Sakata et al., 2011). \textit{UBI4} encodes a penta-repeat of ubiquitin molecules and is required to resist starvation and stress in stationary phase (Finley et al., 1987). Both \textit{ubp6Δ} and \textit{ubi4Δ} mutants are characterized by low levels of free ubiquitin (Figure 2E) (Hanna et al., 2006, 2007).

To test whether the perturbed PSG phenotype of \textit{ubp6Δ} mutants is caused by the lack of the ubiquitin hydrolase activity of Ubp6, we analyzed the active-site mutant Ubp6-C118A (ubp6Δ-C118A) for PSG formation. The \textit{ubp6Δ} cells were transformed with centromere-based plasmids expressing either wild type or Ubp6-C118A. In addition, Pre2-GFP, Rpn1-GFP, and Rpn11-GFP were coexpressed to monitor the localization of the CP, RP base, and RP lid, respectively. Fluorescence microscopy revealed that PSGs are formed in reconstituted wild type but not in the \textit{ubp6Δ-C118A} active-site mutant (Figure 3A). Again, the ubiquitin level in the \textit{ubp6Δ-C118A} active-site mutant was confirmed to be lower than in wild type (Figure 2E).
PSGs are unique cytosolic granules densely packed with proteasomes

To extend our search for possible PSG scaffold proteins, we performed a systematic survey for proteins forming PSG-like structures by high-throughput imaging of the collection of strains expressing ~4100 GFP-labeled ORFs (Huh et al., 2003). Hta2-mCherry was crossed into the arrayed strain collection as marker of the nucleus. The screen was performed by automated imaging analysis using the Evotec Opera microscope and analyzed manually by eye. Only when three individuals independently found matching results was a strain counted as a hit.

In follow-up experiments, each hit strain was manually analyzed for PSG-like structures. Specifically, their motility during quiescence and their reversibility upon exit from quiescence were tested. Most GFP-labeled foci formed during quiescence were irreversible, confirming previous work by other groups (Narayanaswamy et al., 2009; O’Connell et al., 2014). As expected, all proteasomal components accumulated in reversible cytosolic foci. GFP-labeled versions of proteins encoded by ADE4, DNM1, DOA1, NEM1, PAK1, SET3, SNAF1, SNAF5, SNAF6, SRO9, UBI4, UBPE6, and VPS24, whose deletions resulted in perturbed PSG formation, were also found to form cytosolic foci in quiescence (Table 1). To examine whether these cytosolic foci behave like PSGs, we tested their clearance upon exit from quiescence. With the exception of Ade4, Ubp6, and Ubi4, the cytosolic foci of all other gene products did not clear with the resumption of growth, showing that they did not behave like PSGs.

Only a small number of proteins, namely Ade16, Nam7, Kap95, Cm1, and Kap123, the latter three of which are members of the β-karyopherin family (Wozniak et al., 1998), joined the list of proteins forming motile and reversible cytosolic foci in quiescence (Supplemental Table S2). None of them colocalized with PSGs (Supplemental Figure S4). Proteins known to be associated with the proteasomes, such as Ubp6 and Blm10, colocalized with PSGs (Figure 4, A and B). Colocalization studies of GFP-labeled Ubi4-GG, which was chromosomally deleted for the diglycine motif to avoid C-terminal cleavage of the GFP moiety from ubiquitin, and mCherry-labeled Pre2 suggested the presence of GFP-tagged ubiquitin within PSGs (Figure 4C). Western blot analysis confirmed the presence of GFP-tagged ubiquitin, which results from proteolytic processing of the polyubiquitin precursor Ubi4 during stationary phase (Figure 4C, panel labeled Ub-GG-GFP). Attempts to replenish (tagged versions of) ubiquitin in quiescent ubp6Δ and ubi4Δ cells by using GAL1, ADH1, ADH2, and UBI4 promoter-driven plasmids failed, suggesting that the ubiquitin level is hardly manipulated in quiescence.

To investigate the structural organization of PSGs, we imaged cross-linked PSGs from wild-type cells expressing Rpn11-GFP via dSTORM (for enrichment of cross-linked PSGs, see Figure 5). The PSG signals appear as discrete isolated structures when imaged via wide-field total internal reflection fluorescence (TIRF) microscopy (Jain et al., 2016) (Figure 4D, left panel). The nanoscale resolution achieved via dSTORM revealed a finer organization not readily observable by conventional microscopy (Figure 4D, right and bottom panels). Three-dimensional surface projections of a representative dSTORM image (Figure 4E) revealed a highly heterogeneous structure with a high number of overlapping single-molecule localizations at its center (dark blue). This area likely represents the core of the PSG with a diameter of ~200 nm, similar to that reported by immunoelectron microscopy (Laporte et al., 2008), while single localizations at the periphery may arise from free proteasomes and/or

| Gene product | % CP | % RP base | % RP lid | Granule |
|--------------|------|----------|----------|---------|
| Ubiquitin-modifying proteins | | | | |
| Doa1 | – | – | – | + |
| Ubi4 | – | – | – | + + + |
| Ubp6 | – | – | – | + + + |
| Regulators of energy levels | | | | |
| Dnm1 | – | – | – | + |
| Hap4 | – | – | – | – |
| Hap5 | – | – | – | – |
| Kinases and phosphatases | | | | |
| Nem1 | – | – | – | + |
| Npr3 | – | – | – | n.d. |
| Pak1 | + | – | – | + |
| Snf1 | + | – | – | + |
| DNA repair, chromatin remodeling | | | | |
| Rad4 | + | – | – | – |
| Set3 | – | – | – | – |
| Snf5 | + | – | – | + |
| Snf6 | + | – | – | + |
| Sro9 | – | – | – | + |
| Proteins with miscellaneous functions | | | | |
| Ade4 | – | – | – | + |
| Cpr7 | + | – | – | – |
| Icl1 | – | – | – | n.d. |
| Sec66 | – | – | – | + |
| Tps2 | – | – | – | – |
| Vps24 | + | – | – | + |

Null mutants of selected hit genes expressing either Pre2-, Rpn1-, or Rpn11-GFP were manually analyzed for PSG formation in quiescence. Two hundred cells were monitored by direct fluorescence microscopy. The percentage of cells that sequester CP, RP lid, and RP base into the PSG was counted. –, less than 30%; +, between 30% and 50% of the respective proteasomal complex is sequestered into the PSGs. The sequestration of the hit gene products into cytosolic granules in quiescence was analyzed by monitoring wild-type cells expressing either Pre2-, Rpn1-, or Rpn11-GFP. Proteins with miscellaneous functions are designated in red (+), irreversible and immotile in black (+). Proteins colocalizing with PSGs were are designated (+ +), n.d. the localization could not be detected.

TABLE 1: Proteins required for PSG formation identified in imaging screen with deletion collection.

We next analyzed whether proteasome relocalization into the nucleus is delayed in ubp6Δ and ubp6-C118A mutants upon exit from quiescence, when PSGs resolve and mature proteasomes reappear within a few minutes in the nucleus of wild-type cells. In ubp6Δ and ubp6-C118A mutants lacking PSGs, the replenishment of nuclear proteasomes took more than one regular generation time (Figure 3, B and D, top graph); ubi4Δ cells displayed similar phenotypes (Figure 3, C and D, bottom graph). Thus perturbed PSG formation is connected with the inability to rapidly reposition proteasomes into the nucleus upon growth resumption. Perturbed PSG formation may also contribute to the hypersensitivity of ubp6Δ and ubi4Δ mutants toward a variety of agents inducing proteo- and genotoxic stress (Finley et al., 1987; Chernova et al., 2003).
nonspecific antibody reactions. Around 700 localizations were detected per sample (20 samples) versus ~10,000 existing proteasomes in a yeast cell (Kulak et al., 2014), suggesting a densely packed core in which the majority of GFP epitopes are sterically occluded and therefore not readily accessible for immunofluorescence. Alternatively, the finding of one-tenth of the proteasomes within the PSG sample could be explained by the fact that the PSGs were not isolated as intact organelles, collapsed during fixation, or do not contain all proteasomes of the cell.

**Mass spectrometry–based approaches determine the protein composition of PSGs**

To identify PSG-resident proteins in quiescent yeast cells, we used an integrated proteomic approach based on in vivo cross-linking of cells followed by lysis under denaturing conditions (Guerrero et al., 2006; Kaake et al., 2010). This cross-linking strategy was chosen because the PSG is a membrane-less droplet of soluble proteins (Laporte et al., 2008). For retention of transiently interacting proteins within PSGs, quiescent cells were cross-linked by formaldehyde. The cross-linked cells were disintegrated in the presence of urea, and proteasomes were harvested by tandem-affinity purification using a dodeca-histidine tag combined with the in vivo biotinylation signal peptide sequence (HBH) on Pre2. Wild-type and \textit{blm10}Δ cells with WCGα background were analyzed, because PSG formation occurs in nearly all wild-type cells and is abrogated in \textit{blm10}Δ cells with regard to the CP (Weberruss et al., 2013). Cross-linked quiescent cells from both strains were processed for tandem affinity purification. The eluates were analyzed by SDS–PAGE and Western blot before liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis (Supplemental Figure S5). Possible PSG scaffold proteins should be identifiable through the comparison of the proteomic profiles of quiescent wild-type and \textit{blm10}Δ cells. Two sets of duplicates were analyzed, and only peptides resolved in all four experiments were counted. As a control for nonspecific binding to the beads, lysates of cross-linked quiescent cells without HBH-tagged protein were subjected to tandem-affinity purification and LC-MS/MS analysis. In this control, no proteasomal peptides were isolated, but low numbers of peptides from metabolic enzymes and ribosomal subunits were identified (unpublished data), which usually arise from cross-linked and aggregation-prone proteins in quiescent cells (Narayanaswamy et al., 2009; O’Connell et al., 2014). The peptides of the control were subtracted from the peptides yielded from wild-type and \textit{blm10}Δ cells expressing Pre2-HBH. High peptide counts resulted for the complete set of proteasomal subunits in

![FIGURE 2: Proteasome localizations in \textit{ubp6}Δ and \textit{ubi4}Δ cells.](image)
Ubiquitin is a key component of proteasome storage granules

For analysis of the protein composition of PSGs in detail, cross-linked PSGs were enriched from quiescent BY4741 wild-type cells expressing GFP-linked PSGs were enriched from quiescent BY4741 wild-type cells for analysis of the protein composition of PSGs in detail, cross-linking of HBH-tagged proteasomal subunits (Guerrero et al., 2006).

Interestingly, polyubiquitinated proteins were hardly detected within cross-linked PSGs that were heated in sample buffer overnight to release high molecular mass proteins (Figure 5E, lanes 3 and 4). Instead of polyubiquitin, free ubiquitin was enriched within PSGs. The presence of ubiquitin within purified PSGs was also verified by mass spectrometry by excising the typical banding pattern of CP subunits, which is identical to the banding pattern of purified CP (Figure 5D, top panel, compare lanes 1 and 2). High molecular mass subunits of the RP and Blm10 were detected by Western blot analysis after overnight heating at 65°C (Figure 5D, bottom panel, lane 3). Finally, bands in the range of 60–240 kDa were excited and analyzed by mass spectrometry. Pre2-GFP, Rpn1, and Blm10 were identified but did not migrate at the expected size due to the overnight treatment (unpublished data). Because PSGs do not form under conditions of ubiquitin depletion, we tested whether ubiquitin is present within PSGs. As a control, proteasome holo-enzymes were affinity purified as GFP-tagged CP complexes from proliferating cells and probed for ubiquitin. Consistent with the literature (Besche et al., 2014), polyubiquitin chains were detected on proteasome holo-enzymes trapped during protein degradation (Figure 5E, lanes 1 and 2).

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Overexpression of lysine-less ubiquitin induces premature PSG formation and inhibits cell growth

For investigation of whether PSG formation is prematurely induced in proliferating cells by manipulating the level of free ubiquitin, the expression of His-tagged ubiquitin behind the CUP1-promoter was induced in wild-type cells expressing Pre2-GFP grown in YPD medium. As revealed by fluorescence microscopy, overnight overexpression of His-ubiquitin forced the sequestration of the CP into PSGs, while the CP remained nuclear in mock-treated cells with endogenous ubiquitin levels (Figure 6A, left panel). The expression of sucrose cushion ultracentrifugation yielded GFP-labeled PSGs, which cosedimented with fragments of 4′,6-diamidino-2-phenylindole (DAPI)-stained organelles as revealed by fluorescence microscopy (Figure 5A). Filters were used as specified in the Supplemental Materials to separate the PSGs from DAPI-stainable nuclear and mitochondrial remnants (Figure 5B), as confirmed by Western blot analysis using antibodies against histone H3 and Tim23, a protein of the mitochondrial translocase complex. Also, markers of ribosomes (Rps6) and autophagosomes (Atg8) did not react with PSGs enriched by sucrose cushion ultracentrifugation followed by filtration (Figure 5C). The enriched PSGs were briefly boiled to reverse formaldehyde cross-links. SDS-PAGE followed by Coomassie blue staining of the released proteins revealed the typical banding pattern of CP subunits, which is identical to the banding pattern of purified CP (Figure 5D, top panel, compare lanes 1 and 2). High molecular mass subunits of the RP and Blm10 were detected by Western blot analysis after overnight heating at 65°C (Figure 5D, bottom panel, lane 3). Finally, bands in the range of 60–240 kDa were excited and analyzed by mass spectrometry. Pre2-GFP, Rpn1, and Blm10 were identified but did not migrate at the expected size due to the overnight treatment (unpublished data). Because PSGs do not form under conditions of ubiquitin depletion, we tested whether ubiquitin is present within PSGs. As a control, proteasome holo-enzymes were affinity purified as GFP-labeled protein complexes from proliferating cells and probed for ubiquitin. Consistent with the literature (Besche et al., 2014), polyubiquitin chains were detected on proteasome holo-enzymes trapped during protein degradation (Figure 5E, lanes 1 and 2).

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Among pleiotropic effects, K0 ubiquitin inhibits proteasomal degradation (Bloom et al., 2003; Ziv et al., 2011). Again, K0 ubiquitin-GG was overexpressed behind the CUP1 promoter in wild-type cells expressing Pre2-GFP. After a few hours in YPD, PSGs were formed, and cell cycle progression was arrested, whereas mock-treated cells proliferated with continuous nuclear localization of proteasomes (Figure 6B). The cells forming PSGs upon K0 ubiquitin overexpression were serially diluted and spotted on YPD medium with and without copper. The cells did not resume growth on copper-containing medium, suggesting that K0 ubiquitin interferes with cell cycle progression. As a control, cells containing wild-type ubiquitin grew on copper-containing medium (Figure 6C).

Ideally, the overexpression of ubiquitin variants would have been performed in quiescent ubi4Δ cells. However, protein overexpression is only feasible in proliferating cells, where proteasomes are located in the nucleus independent of the genetic background. Galactose-induced overexpression of Ubi4 in ubi4Δ cells yielded unprocessed Ubi4, suggesting that the ubiquitin-processing hydrolase is not active during proliferation (unpublished data).

Proteasome holo-enzymes are unstable in quiescence

As is well known from the literature, proteasome holo-enzymes remain stable in the presence of ATP and during the degradation of polyubiquitinylated substrates (Kleijnen et al., 2007; Kriegenburg et al., 2008). With the decline of ATP, proteasome holo-enzymes tend to dissociate. Thus proteasome holo-enzymes are unstable in quiescent cells (Bajorek et al., 2003). For testing this in our set of mutants, lysates of wild-type and mutant cells were subjected to native PAGE and analyzed by fluoroimaging of the GFP moieties attached to either CP, RP base, or RP lid subunit. Proteasome configurations were assigned according to our previous analysis using specific antibodies and mass spectrometry (Supplemental Figure S6) (Enenkel, 2012). Because ubp6Δ cells displayed one of the most striking phenotypes with regard to orchestrating PSG or organization, we depicted proteasome configurations from proliferating and quiescent ubp6Δ cells. Proteasome holo-enzymes with either RP–CP–RP or RP–CP configuration were present in proliferating cells (Figure 7A) and dissociated into RP and CP in quiescent cells (Figure 7B). In wild type, a considerable fraction of CP was bound to Blm10. In quiescent ubp6Δ cells, the overall proteasome configurations were similar to wild type, but less Blm10 was bound to the CP (Figure 7, 7A) and dissociated into RP and CP in quiescent cells (Figure 7B).

His-ubiquitin was confirmed by Western blot (Figure 6A, right panel). Additionally, we tested the effect of lysine-less (K0) ubiquitin in which all lysines are mutated to arginines and the C-terminal diglycine motif is deleted (–GG), so that it is not linkable to any substrate or proceeding ubiquitin molecule. Among pleiotropic effects, K0 ubiquitin inhibits proteasomal degradation (Bloom et al., 2003; Ziv et al., 2011). Again, K0 ubiquitin-GG was overexpressed behind the CUP1 promoter in wild-type cells expressing Pre2-GFP. After a few hours in YPD, PSGs were formed, and cell cycle progression was arrested, whereas mock-treated cells proliferated with continuous nuclear localization of proteasomes (Figure 6B). The cells forming PSGs upon K0 ubiquitin overexpression were serially diluted and spotted on YPD medium with and without copper. The cells did not resume growth on copper-containing medium, suggesting that K0 ubiquitin interferes with cell cycle progression. As a control, cells containing wild-type ubiquitin grew on copper-containing medium (Figure 6C).
Because the sequestration of the CP into PSGs is facilitated by Blm10 (Weberruss et al., 2013), diminished fractions of Blm10-bound CP may contribute to perturbed PSG organization in addition to low ubiquitin levels in ubp6Δ mutants. Consistent with our finding in ubp6Δ cells, our hit mutants with disturbed PSG formation showed RP and CP dissociated as well, confirming that the decline of ATP has a critical influence on proteasome stability during quiescence (selection of hit mutants, Supplemental Figures S7 and S8).

**DISCUSSION**

Here we explored different approaches to understand PSG organization and to identify key proteins apart from proteasomal components that are required to assemble these reversible and motile granules in the cytoplasm of quiescent yeast cells.

Our initial high-throughput screen for yeast null mutants with perturbed PSGs identified Blm10, a protein required for CP but not RP sequestration into PSGs (Weberruss et al., 2013) (Supplemental Table S1). To identify proteins that orchestrate the sequestration of both the CP and RP into PSGs, we extended our array to mutants with defective RP sequestration into PSGs (Table 1). The proteins affected in these mutants have diverse functions, being either involved in phosphorylation, the regulation of metabolic energy, genotoxic stress response, or ubiquitin regulation. Npr3 kinase, which acts on TORC1 kinase, Pak1, the upstream kinase of AMP-dependent kinase Snf1, and Snf1 kinase emerged in our extended screen for mutants with reduced PSG formation. TORC1 and Pak1 govern the sensing of glucose depletion in quiescence (Zhang et al., 2011) and may modify proteasomal components by phosphorylation, thus influencing proteasome dynamics and their propensity to assemble into PSGs. Eighteen kinases have been reported to form reversible cytosolic foci in quiescence, although Npr3, Pak1, and Snf1 were not among them (Shah et al., 2014). None of these kinases including Npr3, Pak1, and Snf1, colocalized with PSGs, and none of them were identified in our mass spectrometry analysis of cross-linked PSGs. However, it is interesting to note that the phenotypes of quiescent yeast cells lacking either kinase granules or PSGs are similar. Both displayed reduced fitness in chronological aging, suggesting that the global reorganization of various enzymes into cytosolic foci is beneficial for the longevity and reversibility of quiescence (Shah et al., 2014).
the diauxic shift from glycolysis to gluconeogenesis (Zampar et al., 2013). Dnm1, a short-lived GTPase regulating mitochondrial fission during diauxic shift, was identified as well. The half-life of Dnm1 depends on Blm10 (Tar et al., 2014), linking two hit proteins of our high-throughput analysis.

All hit genes listed in the complete set of data (see the Supplemental Material) showed biological variability with regard to their impact on PSG formation. Thus we focused our efforts on those genes, in which the sequestration of CP and RP into PSGs was almost abrogated. These genes, namely \textit{UBP6} and \textit{UBI4}, encode proteins regulating ubiquitin concentration. Disturbed PSG formation as displayed by \textit{ubp6}\(\Delta\) and \textit{ubi4}\(\Delta\) mutants correlated with significantly decreased levels of free ubiquitin, which below a yet unknown threshold abrogates PSG formation. Ubp6, an ubiquitin-specific hydrolase associated with the RP base, aids to replenish the pool of free ubiquitin and modifies protein substrates by trimming branched polyubiquitin chains (Crosas et al., 2006; Hanna et al., 2006), specifically by catalyzing the removal of supernumerary polyubiquitin chains (Lee et al., 2016). Its activity contributes to PSG formation, supporting our assumption that proteasomes devoid of branched polyubiquitin chains are preferentially sequestered into PSGs. Mutations in Rpn11, the RP lid subunit responsible for the removal of ubiquitin moieties from polyubiquitinylated substrates, lead to nuclear retention of proteasomes and impaired PSG formation (Saunier et al., 2011).

The CCAAT transcription factor complex is a global regulator of respiratory gene expression that is repressed by glucose. Our high-throughput imaging screen identified Hap subunits of the CCAAT transcription factor, which mirrors metabolic changes during the diauxic shift from glycolysis to gluconeogenesis (Zampar et al., 2013). Dnm1, a short-lived GTPase regulating mitochondrial fission during diauxic shift, was identified as well. The half-life of Dnm1 depends on Blm10 (Tar et al., 2014), linking two hit proteins of our high-throughput analysis.

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which facilitates CP sequestration into the PSG and its reimport into the nucleus upon exit from quiescence. Mono-ubiquitin is a crucial component for the formation of PSGs. Its overexpression, especially if ubiquitin cannot be conjugated to a growing polyubiquitin chain, induces premature PSG formation in proliferating cells.

Interestingly, proteasome inhibition and conditional mutations within the proteasome cause cell cycle arrest and the formation of reversible proteasome granules, named JUNQ (Kaganovich et al., 2008), close to the nuclear envelope. These observations support our model that proteasomes with polyubiquitin chains and inhibited during degradation remain stuck at the nuclear envelope.

None of our hit proteins, except proteins of the ubiquitin–proteasome system, colocalized with the PSGs. This is consistent with other work showing that most cytosolic foci induced by thermal, chemical, and nutritional stress are not composed of multisubunit complexes. From a selection of ~800 cytosolic proteins, nearly 200 proteins reside in irreversible foci and do not colocalize with enzymes participating in the same metabolic pathway. Mass spectrometry analysis of these foci did not yield peptides from proteasomal subunits (O’Connell et al., 2014), but rather peptides overlapping with those detected in our untagged cells used as control (Guerrero et al., 2006).

Stress granules, as analyzed in an approach similar to ours (Wallace et al., 2015; Jain et al., 2016), did not contain proteasomal components, further supporting the conclusion that PSGs are unique reservoirs of ubiquitin and proteasomes, which according to our dSTORM analysis are densely packed into granules. This reservoir of key components of the ubiquitin–proteasome system can be instantaneously mobilized with the resumption of growth. PSGs dissolve, and mature proteasomes are translocated within minutes into the nucleus to degrade polyubiquitinylated proteins. The synthesis of new proteins and degradation of short-lived proteins (e.g., cell cycle regulators) resume with the exit from quiescence. In contrast to proliferating cells with high ATP levels, quiescent cells experience an austerity budget, and protein homeostasis is adjusted to the economic challenge of decreased ATP levels. Therefore we assume that quiescence lasts with less degradation of polyubiquitinylated proteins. Protein synthesis is stalled, and fewer short-lived proteins accrue as polyubiquitinylated substrates.

It still remains a mystery how proteasomes and ubiquitin separate in PSGs in the aqueous environment of the cytoplasm. Recent studies show that the cytoplasmic fluidity changes between the metabolically active and dormant state of cells. Accordingly, the motion of intracellular components may become disproportionally constrained with increasing size during the transition from proliferation to quiescence.

**FIGURE 8:** Model of PSG organization and proteasome dynamics between proliferation and quiescence. In proliferating cells in which the ATP level is high, proteasome holo-enzymes mainly occur in the nucleus and are engaged in the degradation of polyubiquitinylated proteins. During transition to quiescence, glucose is depleted, the ATP level declines, and fewer proteins might be degraded in the nucleus. Proteasomes migrate to the nuclear envelope and exit the nucleus, most likely through nuclear pores. PSGs are formed and move as membrane-less entities through the cytoplasm. Because proteasome holo-enzymes are less stable at low ATP concentration, RP and CP might be separately stored within PSGs. RP, CP, and mono-ubiquitin are major constituents of the PSGs. With the addition of glucose, cells receive the signal to resume growth. The ATP level increases, and PSGs dissolve within a few minutes. Proteasomes are rapidly relocated to the nucleus, where ubiquitin-dependent proteolysis of short-lived proteins promotes cell cycle progression.

et al., 2013). Furthermore, the lack of Hul5, which works as an antagonist of Ubp6 by elongating polyubiquitin chains (Finley, 2011), leads to proteasomes loaded with polyubiquitin chains, their retention in the nucleus, and consequently, reduced PSG formation (van Deventer et al., 2015).

On the basis of these findings we propose the following model in which proteasomes loaded with polyubiquitin chains are mainly localized in the nucleus, where a major need for proteasomal protein degradation seems to be required during cell proliferation. The release of polyubiquitin chains and the presence of mono-ubiquitin trigger nuclear export and the sequestration of proteasomes into PSGs (Figure 8). The decline of ATP in quiescence impacts the stability of proteasome holo-enzymes, suggesting that proteasomes do not exist as holo-enzymes within PSGs. They are rather dissociated into RP and CP with a significant fraction bound to Blm10,
(Parry et al., 2014; Munder et al., 2016). However, PSG organization may be driven not only by extrinsic changes in the solvent structure but also by intrinsic features of the solute protein. Proteins that tend to undergo liquid-liquid phase separations in an aqueous environment are often intrinsically disordered (Marsh and Forman-Kay, 2010; Brangwynne, 2013). How proteasomes follow this concept of liquid-liquid phase separation is to be tested, because their subunits are folded proteins with only a few intrinsically disordered domains (Auderheide et al., 2015). Intrinsically disordered proteins are even at risk of being degraded by the proteasomes (Liu et al., 2003) if they serve as PSG scaffolds. Future reconstitution assays of PSGs using CP and RP components and ubiquitin followed by biophysical investigations of protein conformations will shed light into molecular dynamics of proteasomes, which seem to exist either as freely diffusible protein complexes or as condensed particles in a liquid droplet.

**MATERIALS AND METHODS**

**Automated image acquisition**

PCR-based homologous recombination (Tong et al., 2001) was used to create the query strain expressing Pre2-GFP, RPL39pr-tdTomato, and Hta2-mCherry. The query strain was mated to the collection of yeast deletion strains by replica pinning (Giaever et al., 2002). Diploids were selected and sporulated using standard selection strategies (Tong et al., 2001). The genesΔ haploids expressing Pre2-GFP, RPL39pr-tdTomato, and Hta2-mCherry were isolated through successive pinning onto selective media lacking histidine, uracil, leucine, arginine, and lysine, but supplemented with canavanine, S-(guanidoethyl)-L-cysteine hydrochloride, genetin, and nourseothricin. Then cells were transferred into liquid YPD by using the RAININ Liquidator96 manual benchtop pipetting system (Metller Toledo). Antibiotics were omitted, as they impact the growth to stationary phase. Cells were grown overnight in 96-well plates covered with Breathe Easy Sealing Tape (E&K Scientific) before they were diluted by 1:3 in 50 µl YPD in 96–deep well plates and incubated for 5 d at a 30° angle, 220 rpm, and 30°C. For optimization of cell density and elimination of autofluorescence of aged YPD for image analysis, each subculture was diluted in sterile water at 1:100 and transferred into Greiner 96-well plates. The optical density at 600 nm (OD600) was measured using the spectrophotometer Synergy2 (BioTek). Gen5 software was used to calculate the average cell density, and adjusted volumes were transferred to a 384-well CarCloR glass slide (PerkinElmer; 6007558) for high-throughput imaging with the Evotec Opera microscope, an automated spinning-disk confocal microscope equipped with a 60x/1.2 NA water-immersion objective (Perkin Elmer-Cetus). GFP was detected at 488-nm laser excitation and with 520/535-nm emission filters. mCherry and tdTomato (red fluorescent protein [RFP]) were detected at 561-nm laser excitation and with 520/535-nm emission filters. tdTomato cannot be visualized by using these settings and can only be viewed with the Evotech high-throughput microscope.

The resumption of growth after quiescence was induced by adding fresh YPD, and the protein dynamics were immediately monitored by fluorescence microscopy within a time frame of 10–30 min, as described previously (Weber et al., 2013).

**dSTORM sample preparation, imaging, and analysis**

Cross-linked PSGs were isolated from wild-type cells expressing Rpn1-GFP and stained with an anti-GFP single-domain antibody (ChromTek gt-250) conjugated with Alexa Fluor 487. Single-molecule localization microscopy was performed on a home-built super-resolution epifluorescence/TIRF microscope comprising an Olympus IX-83 inverted microscope with a 100x/1.4 NA oil-immersion objective (Olympus UPlanApo 100x/W1.40), a 100-mW, 488-nm DPSS laser (Ultralasers); and a Cy5 filter set (Chroma). Imaging was performed with laser configuration in a highly inclined and laminated optical sheet (HiLo). A cylindrical lens (LK100L1-A f = 1000, Thorlabs) was placed in the emission path between the microscope body and the camera to induce asymmetry in the x and y focal planes, allowing for estimation of the z-position of single-molecule localizations via the astigmatism method (Huang et al., 2008). Five thousand frames were captured for each sample at a sampling rate of 50 Hz. Images were acquired in MicroManager (v.1.4.23) using a Photometrics QuantEM EMCCD (multiplier gain = 100). Raw data were processed and superresolution reconstructions were generated with the ImageJ plug-in ThunderSTORM using a maximum-likelihood estimation and multi-emitter fitting for subpixel localizations. Three-dimensional surface renderings were generated with VisIt (Lawrence Livermore National Laboratory).

**Yeasts strains used in this study**

All the strains used in the high-throughput array are isogenic to BY4741. The relevant genotypes are listed in Supplemental Table S4. GFP, tdTomato, and mCherry fusions were constructed using one-step PCR-mediated homologous recombination (Longtine et al., 1998). All genes listed in Supplemental Table S1 were confirmed to be chromosomally deleted in the respective mutant by using PCR. The expression of fluorescent protein-labeled proteins was confirmed by Western blot analysis. Antibodies against Rpn1 and Rpn2 were kindly provided by Michael Glickman (Technion, Haifa, Israel), against Rpt1 and Rpt6 by Carl Mann (University of Paris-South, France), and against Tim23 by Angus McQuibban (University of Toronto, Canada). The source of anti-Blm10, Kar2 and Rpn5 antibodies is described previously (Weber et al., 2013). Monoclonal mouse antibodies against GFP and mCherry were purchased from Clontech, against RGS-His from Qiagen, polyclonal rabbit antibodies against RPS6, Histone H3, and anti-GAPDH-HRP conjugates were from Abcam and antibodies against ubiquitin (BML-UG9511) from Enzo. Before blocking and adding antibodies blots were always stained with either Amido Black or Ponceau S to test for equal protein load.
Proteomic analysis of PSGs in quiescent yeast cells

The affinity purification of HBH- and GFPS-labeled CP and the enrichment of PSGs from formaldehyde cross-linked cells are described in detail in the Supplemental Material. The proteomic analysis was performed according to previous protocols (Guerrero et al., 2006; Kaake et al., 2010). CUP1-induced overexpression of His-tagged versions of ubiquitin was performed as described previously (Finley et al., 1994). Cells transformed with an episomal plasmid were grown on selective synthetic complete medium. Copper induction of protein expression was done in YPD.

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