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The Catalytic Activity of the Ubp3 Deubiquitinating Protease Is Required for Efficient Stress Granule Assembly in *Saccharomyces cerevisiae*

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The interior of the eukaryotic cell is a highly compartmentalized space containing both membrane-bound organelles and the recently identified nonmembranous ribonucleoprotein (RNP) granules. This study examines in *Saccharomyces cerevisiae* the assembly of one conserved type of the latter compartment, known as the stress granule. Stress granules form in response to particular environmental cues and have been linked to a variety of human diseases, including amyotrophic lateral sclerosis. To further our understanding of these structures, a candidate genetic screen was employed to identify regulators of stress granule assembly in quiescent cells. These studies identified a ubiquitin-specific protease, Ubp3, as having an essential role in the assembly of these RNP granules. This function was not shared by other members of the Ubp protease family and required Ubp3 catalytic activity as well as its interaction with the cofactor Bre5. Interestingly, the loss of stress granules was correlated with a decrease in the long-term survival of stationary-phase cells. This phenotype is similar to that observed in mutants defective for the formation of a related RNP complex, the Processing body. Altogether, these observations raise the interesting possibility of a general role for these types of cytoplasmic RNP granules in the survival of G_0-like resting cells.

The interior of the eukaryotic cell is a highly compartmentalized space. Organelles harbor distinct sets of proteins that often possess related functions, serving to organize the cellular milieu and increase reaction efficiencies. The recent identification of a family of ribonucleoprotein (RNP) granules suggests that this complexity may be greater than previously recognized (1, 2). These RNP granules differ from traditional organelles in that they lack a limiting membrane and form in response to particular cues, including cellular stress and developmental signals (3). Interest in these structures has increased sharply as they have been recently linked to a number of human diseases, including neurodegenerative disorders like amyotrophic lateral sclerosis and spinocerebellar ataxia type II (4, 5). Although the function of many of these granules is unknown, their conservation throughout evolution suggests that they serve an important biological role.

Stress granules are one of the better-characterized instances of these cytoplasmic RNP complexes. They form in response to various types of cellular stress and in quiescent or G_0 cells (3, 6). They are highly dynamic structures, continuously shuttling components between the cytosol and the granule during the stress and disassembling rapidly following its cessation (7). In mammalian cells, stress granule formation is initiated by the translation arrest that occurs upon the stress-triggered phosphorylation of translation initiation factor 2α (eIF2α) (8). Polysomes disassemble, and 48S preinitiation complexes containing naked mRNA accumulate and are bound by mRNA-binding proteins that contain low-complexity domains (8). These domains are thought to contribute to the subsequent aggregation of these preinitiation complexes and thus the formation of stress granules (9–11). These RNP structures have also been shown to contain various signaling molecules, including protein kinases and phosphatases and their associated adaptor and scaffolding partners (12). The sequestration of signaling molecules is one mechanism by which stress granules modulate cell function. For example, under certain conditions, the scaffold protein RACK1 is sequestered in stress granules, thereby preventing activation of a mitogen-activated protein (MAP) kinase, MTK1, required for apoptosis (13). Other studies have found that the recruitment of Raptor, a key component of mammalian target of rapamycin complex 1 (mTORC1), to stress granules limits a TORC1-induced form of programmed cell death (14). Finally, a recent study in *Saccharomyces cerevisiae* has indicated that a significant number of protein kinases and phosphatases are associated with cytoplasmic granules in quiescent cells (2). Collectively, this work has led to the suggestion that these RNP granules might serve as hubs of signal transduction activity in the cytoplasm of the eukaryotic cell.

Although stress granules have been conserved through evolution, the mechanisms that underlie their formation display species- and stress-specific differences. In budding and fission yeast, for example, stress granule formation occurs in an eIF2α-independent manner in response to heat stress or glucose starvation (15–18). In *Schizosaccharomyces pombe*, cyclic AMP (cAMP)-dependent protein kinase (PKA) signaling is required for stress granule formation following heat stress but not hyperosmotic stress (17). In contrast, PKA signaling is not required for stress granule formation in *S. cerevisiae* under any condition tested thus far (6).

Stress granules are linked spatially, compositionally, and func-

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tionally to another type of RNP complex, known as the processing body (P-body) (19, 20). P-bodies are dynamic structures that form during stress and are enriched in nontranslating mRNAs and proteins involved in mRNA processing (21, 22). Although originally believed to be sites of mRNA decay, mRNA turnover has been found to be unaltered in cells lacking P-body foci (23–25). Recent work has also shown that P-bodies contain many proteins that do not have an obvious link to mRNA decay, including many signaling molecules (2). Several components found in stress granules are also evident in P-bodies, and the docking/fusion of these two structures has been reported (19).

Despite recent efforts, our understanding of the biological roles and significance of these cytoplasmic RNP complexes is still incomplete. However, the identification of yeast strains deficient in the formation of P-bodies has proven to be a useful tool in our attempts to understand these structures (24, 26). In particular, this work has identified a role for P-bodies in the long-term survival of stationary-phase cells (26). We are very interested in the biology of quiescent cells and whether stress granules might also have a role in stationary-phase survival. This possibility was raised as a result of recent work showing that stress granules are induced specifically upon the entry into this quiescent state (6). Unfortunately, deletion strains that display a defect in stress granule formation in stationary phase have not been identified. Therefore, in this study we utilized a candidate approach to screen strains in the yeast knockout collection for regulators of stress granule assembly. We identified one protein, Ubp3, whose absence greatly diminished stress granule formation. Ubp3 is a member of the ubiquitin-specific protease family of deubiquitinases (27). Interestingly, its mammalian homologue, USP10, is a constituent and regulator of stress granules, indicating some evolutionary conservation in stress granule biology (28). We present evidence that the effects of Ubp3 on the assembly of these foci require its catalytic activity as well as its interaction with the cofactor Bre5. Furthermore, we demonstrate a correlation between the loss of stress granules and the decreased viability of stationary-phase cells. The latter effect is similar to what is observed with the loss of P-bodies, and we found that a mutant defective for the assembly of both of these RNP granules exhibited a severe synthetic growth defect (26). Altogether, this work identifies a key regulator of stress granule assembly in *S. cerevisiae* and provides insight into the physiological relevance of these RNP structures.

MATERIALS AND METHODS

Yeast strains and growth conditions. *S. cerevisiae* strain BY4741 was the wild-type strain for all experiments described in this report, and the deletion strains used for screening were obtained from the yeast knockout collection (Open Biosystems). The *ubp3*Δ *pat1*Δ strain was generated by first transforming a *URA3*-marked plasmid containing *PAT1-GFP* (where GFP is green fluorescent protein) into the *ubp3*Δ strain. The *PAT1* locus was subsequently disrupted by replacing its coding sequence with the *LEU2* gene. The resulting strain was then streaked on plates containing 5-fluoroorotic acid to select for yeast that had lost the plasmid. Standard *Escherichia coli* and yeast growth conditions were used throughout. The rich yeast medium yeast extract-peptone-adenine-dextrose (YPAD) and the minimal media yeast minimal (YM) medium and synthetic complete (SC) medium have been previously described (29–31). Carbon starvation experiments were performed by transferring cells to yeast extract-peptone-adenine medium lacking glucose for 20 min. Heat stress experiments were performed by incubating cultures at 45°C for 40 min. For the sodium azide experiments, cultures were incubated in 0.5% sodium azide for 30 min. To assess stationary-phase stress granule or P-body formation, cultures were grown in YPAD or SC medium with dextrose with agitation at 30°C for the indicated number of days.

Plasmid construction. The plasmids encoding the Pbp1-mCh (pPHY3831), Pbp4-mCh (pPHY4207), and Edc3-mCh (pPHY4085) fusion proteins were constructed in the pRS406 vector and integrated into the yeast genome. The *LEU2*-marked, high-copy-number *PDE2* plasmid (pPHY2299, originally pTD40a) was provided by Thomas Fox. The *UBP3-GFP* constructs were made by PCR amplification and were subsequently cloned into the pRS415 vector. The Ubp3 variants used in this study were constructed by site-directed mutagenesis using the Geneart site-directed mutagenesis system (Invitrogen). Additional constructs encoding Ubp3- and Ubp3^{C469A}-GFP were generated in the pRS405 vector to allow for integration into the genome. In all constructs, the encoded proteins were tagged with the respective fluorescent reporter at the C terminus.

Fluorescence microscopy. Cells expressing fluorescent protein fusion constructs were grown as indicated, collected by centrifugation, and spotted onto microscope slides as described previously (32, 33). Cells were imaged with a 100×/1.45 numerical aperture Plan-Apo objective lens on an Eclipse Ti inverted microscope (Nikon, Melville, NY) with an Andor Zyla digital camera and the appropriate Nikon HC filter sets. All data represent two or more independent experiments. For quantitation of foci, the data represent a minimum of three replicates with at least 200 cells examined in each. In many experiments, the foci were placed into three different categories: large, bright foci with well-delineated edges; dim, smaller foci but still with well-defined edges; and finally, dim but with a more amorphous appearance that we refer to as diffuse foci in this report. Merged images were created with the ImageJ software package.

Protein analysis. Protein samples were prepared for Western blotting by a glass bead lysis method, separated on a 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes as described previously (34–36). The membranes were probed with the appropriate primary and secondary antibodies, and immunoreactive bands were detected using the Supersignal chemiluminescent substrate (Pierce).

Stationary-phase viability assays. Cells were grown in YM-glucose minimal medium for the indicated number of days, collected by centrifugation, and resuspended in water at a concentration of 5 optical density at 600 nm (OD_{600}) units/ml. Fivefold dilutions of these cultures were spotted onto plates containing the same medium and incubated at 30°C for 2 to 3 days.

Thermal resistance assays. Strains grown for 4 days at 30°C in YM-glucose minimal medium were subjected to a 20-min heat shock at 50°C as described previously (29). Dilutions of the cultures, taken before and after the heat shock, were plated on minimal medium and incubated at 30°C for 2 to 3 days to allow colony formation. The relative survival rate was determined by comparing the numbers of CFU present before and after the heat shock.

Assessing protein translation rates. Yeast cultures were grown in YM medium to an OD_{600} of 0.4 to 0.6/ml. One OD_{600} equivalent of cells was resuspended in 300 μl of medium lacking methionine and incubated for 20 min at 30°C. To induce heat stress, the cells were incubated at 45°C for up to 40 min; 100 μCi of Tran^{35}S-Label (MP Biomedicals) was added to the culture for the final 5 min of the heat stress. The incorporation of [^{35}S]methionine-cysteine was stopped by adding 300 μl of ice-cold 1.2 mg/ml methionine in 20% trichloroacetic acid (TCA) and incubating on ice for at least 10 min. The protein precipitate was filtered using GF/C filters and washed once with 10 ml ice-cold TCA and twice with 10 ml 95% ethanol. Filters were dried and counted in a liquid scintillation counter.

RESULTS

Identifying regulators of stress granule formation in stationary phase. We recently found that stress granules are induced specifically when *S. cerevisiae* cells enter into stationary phase (6). To better understand the significance of this induction, we set out to

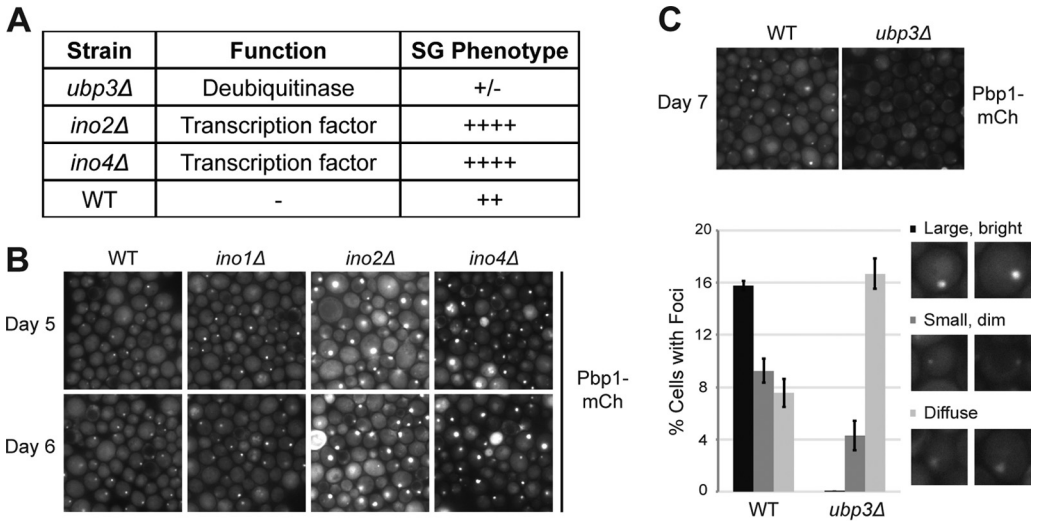

FIG 1 Identification of regulators of stress granule formation in stationary-phase cells. (A) A candidate screen for regulators of stationary-phase stress granule assembly yielded three strains that exhibited either enhanced or diminished granule formation. The relative effects of each mutation on focus number and/or size are indicated in the "SG Phenotype" column. (B) Enhanced stress granule formation was observed in the *ino2*Δ and *ino4*Δ strains. Pbp1-mCh foci were analyzed in the indicated strains after 5 or 6 days of growth in the rich medium YPAD. (C) Strains lacking the Ubp3 protein were defective for stress granule formation. Wild-type and *ubp3*Δ strains were grown for 7 days in rich medium, and the localization of the Pbp1-mCh reporter was assessed by fluorescence microscopy. Representative fields of cells are shown in the top panels, and the quantitation of these data is presented below. The foci were placed into three categories depending upon the intensity of the fluorescence signal and the size and density of the focus. Examples of each of the three categories of foci are shown in the inset images. The data represent the averages for three replicates, where *n* is >300 for each.

identify mutants that were defective for stress granule formation in these quiescent cells. In particular, we used a candidate approach to screen strains in the yeast knockout collection for regulators of this stationary-phase assembly process. Thirty-seven strains were initially chosen based on reported physical and genetic interactions with the stress granule components Pbp1 and Pab1. These interactions were obtained either from the *Saccharomyces* Genome Database website or were uncovered in synthetic genetic array (SGA) screens performed in the laboratory of Charlie Boone (37). The latter data were graciously provided before publication. An additional 22 strains were subsequently examined based on the findings from this initial screen. For this analysis, the chromosomally encoded Pbp1 (or Pab1) protein was tagged at its C terminus with the fluorescent reporter mCherry (mCh). Fluorescence microscopy was then used to assess stress granule formation in cells that were grown in the rich medium YPAD for 5 to 7 days. We have shown previously that cells are beginning to enter stationary phase under these conditions and that stress granules are forming at this time (6). The complete list of strains tested is shown in Table S1 in the supplemental material.

In total, this analysis identified three deletion strains that displayed marked effects on stress granule formation (Fig. 1A). Two of these, the *ino2*Δ and *ino4*Δ strains, exhibited a significant increase in stress granule size in stationary-phase cells (Fig. 1B). The Ino2 and Ino4 proteins form a heteromeric complex that functions as a transcription activator (38). One of the primary targets of this complex is the *INO1* locus that encodes an inositol-3-phosphate synthase (38). However, we found that stress granule formation was not enhanced in the *ino1*Δ strain, suggesting that the observed effects on these granules are due to other targets of the Ino2/Ino4 complex (Fig. 1B). Finally, the *ubp3*Δ strain displayed a diminished ability to form stress granules in stationary-phase cells (Fig. 1C). *UBP3* encodes a ubiquitin-specific protease that is part of a large family of such enzymes that act to remove ubiquitin from particular sets of ubiquitinated protein targets (27). The remainder of this report will focus on the role of Ubp3 in promoting stress granule formation.

A time course analysis showed that Pbp1-mCh foci were evident in wild-type cells as early as day 4 and were maintained through day 18 (the latest time point tested). However, in the *ubp3*Δ strain the appearance of Pbp1-mCh foci was delayed, and once formed, the foci were smaller, less intense, and present in a smaller percentage of cells than in wild-type cells (Fig. 2A). For example, on day 7 nearly 16% of wild-type cells contained large, bright Pbp1-mCh foci compared to 0% of *ubp3*Δ cells (Fig. 1C). Moreover, defined foci (comprising large, bright foci and small, dim foci) were observed in 25% of wild-type cells but in only 4% of *ubp3*Δ cells. A Western blot analysis indicated that Pbp1-mCh levels were similar in wild-type and *ubp3*Δ cells in both log-phase and day 5 cultures (Fig. 2B). Thus, the decrease in focus formation did not appear to be due to reduced levels of Pbp1-mCh expression. A similar decrease in focus formation was observed when Pbp1 was tagged with the monomeric enhanced citrine fluorescent protein (mECitrine), suggesting that the observed aggregation was independent of the particular tag used (Fig. 2C). To determine whether the observed effects were specific for Pbp1, the localization of two additional stress granule proteins, Pbp4 and Pab1, was assessed in wild-type and *ubp3*Δ cells. The resulting foci for both of these mCh-tagged proteins were diminished in the *ubp3*Δ strain in a manner similar to that observed for Pbp1 (Fig. 2D; data not shown). Finally, the stress granules that did form in *ubp3*Δ mutants exhibited disassembly kinetics similar to those of the foci present in wild-type cells (Fig. 2E). These results are therefore consistent with Ubp3 having a general role in the assembly of stress granule foci in stationary-phase cells.

To confirm that the defect in stress granule formation was

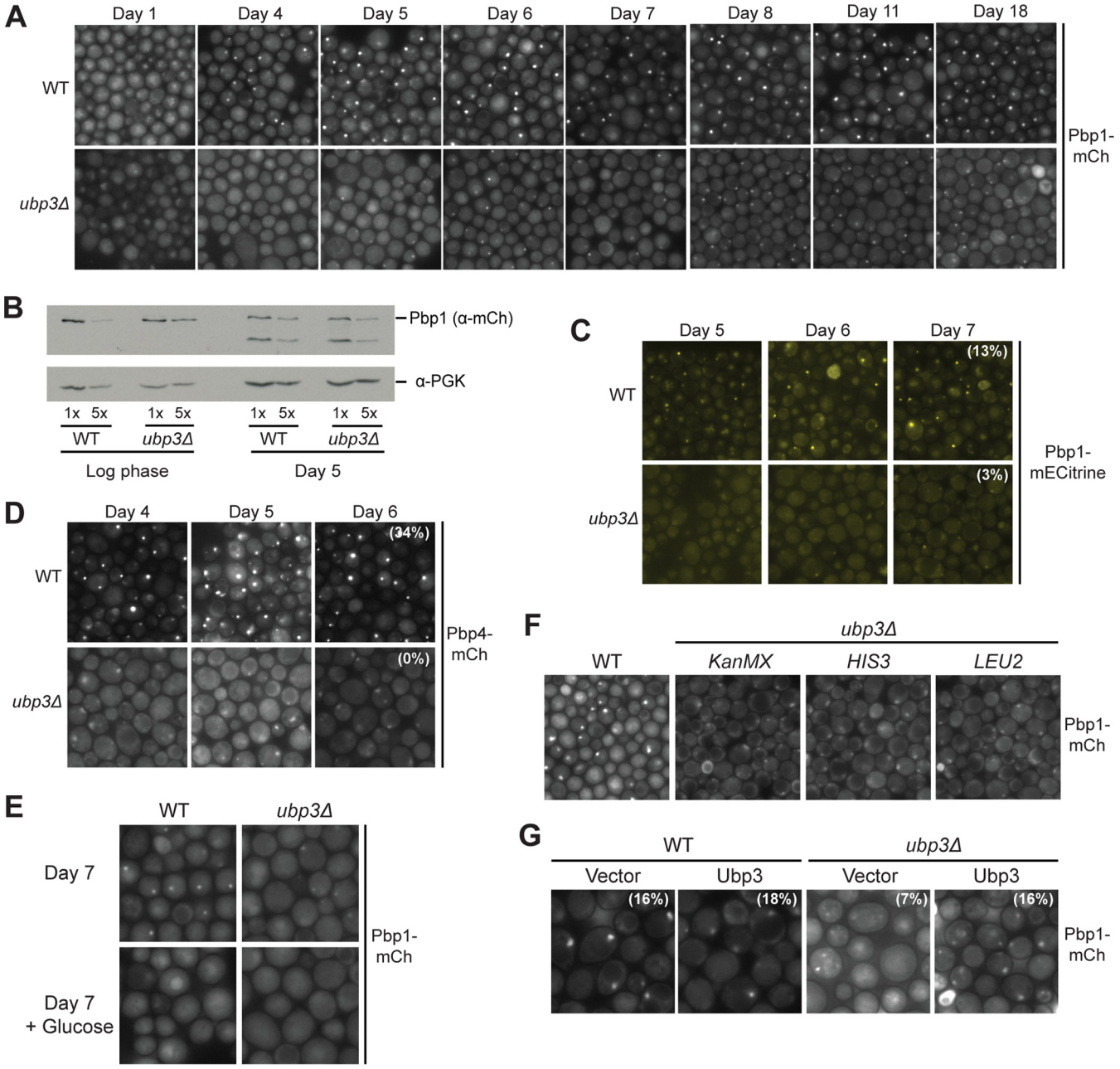

FIG 2 Stationary-phase stress granule formation was defective in a *ubp3*Δ strain. (A) Stress granule formation was assessed in wild-type (WT) and *ubp3*Δ cells after the indicated days of growth in rich medium. (B) Pbp1-mCh levels were assessed by Western blotting with wild-type and *ubp3*Δ cell extracts prepared from either log-phase or day 5 cultures grown in the rich medium YPAD. Cell lysates were diluted as indicated (1× or 5×). The levels of phosphoglycerate kinase (PGK) served as a loading control. (C, D) The subcellular localization of the stress granule reporters Pbp1 tagged with the monomeric enhanced citrine fluorescent protein (mECitrine) (C) or Pbp4-mCh (D) was assessed by fluorescence microscopy in wild-type and *ubp3*Δ cells grown for 4 to 7 days in rich medium. The fraction of cells with large, bright foci, as depicted in Fig. 1C, is indicated. (E) Disassembly of stationary-phase stress granules following glucose readdition. The indicated strains were grown in rich medium for 7 days to allow stress granule formation and were then transferred to fresh medium containing 2% glucose. The Pbp1-mCh foci were analyzed by fluorescence microscopy after a 15-min incubation at 30°C. (F) The stress granule assembly defect was observed in multiple *ubp3* deletion strains. Strains carrying the indicated *ubp3* deletion alleles were grown in rich medium, and stress granule formation was assessed on day 7 of growth. (G) The expression of Ubp3-GFP rescued the stress granule assembly defect associated with the *ubp3*Δ mutant. Wild-type and *ubp3*Δ strains were transformed with either the vector control or a single-copy plasmid expressing Ubp3-GFP from its endogenous promoter. Cells were grown for 6 days in minimal medium, and the frequency of Pbp1-mCh foci was assessed by fluorescence microscopy. The fraction of cells with a focus is indicated. In all experiments, the data shown represent the averages for 3 replicates, where *n* is >200 cells for each.

due to the absence of Ubp3, additional deletions strains in which the *UBP3* locus was disrupted by replacing its coding sequences with either the *HIS3* or the *LEU2* genes were generated. Pbp1-mCh focus formation was similarly diminished in both of these deletion strains (Fig. 2F). Finally, we found that the expression of Ubp3 from a plasmid rescued the observed defect in Pbp1-mCh focus formation observed in the original *ubp3*Δ strain (Fig. 2G). Altogether, these data suggested that

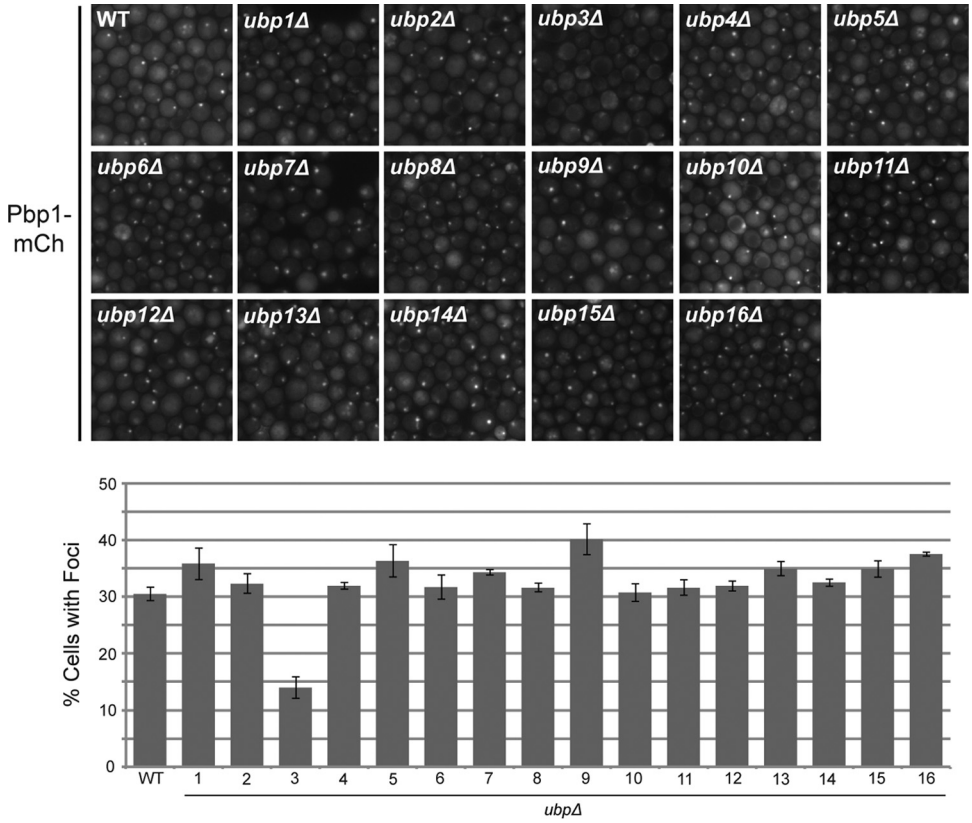

FIG 3 Ubp3, but not other ubiquitin-specific proteases, has a role in the formation of stationary-phase stress granules. Wild-type (WT) or single-deletion strains of the ubiquitin-specific protease (Ubp) family containing the integrated Pbp1-mCh reporter were grown for 7 days in rich medium and then analyzed by fluorescence microscopy. Quantitation of the microscopy data is shown in the graph below, in which the percentage values represent the fraction of cells with foci belonging to any of the categories indicated in Fig. 1C. The data represent the averages for 3 replicates, where *n* is >400 cells for each.

the Ubp3 protein plays an important role in the regulation of stress granule formation.

Ubp3, but not other ubiquitin-specific proteases, was required for stationary-phase stress granule formation. In *S. cerevisiae*, there are 16 members of the ubiquitin-specific protease (Ubp) family, none of which are essential for viability (39). The homology among these Ubp proteins is limited to the catalytic core domain with each containing rather distinct N- and C-terminal extensions of this core. To determine whether any of these other Ubp members might influence stress granule assembly, strains lacking each of these proteins were assessed for Pbp1-mCh foci formation in stationary phase. In each case, we found that these *ubp* deletion strains contained Pbp1 foci that were indistinguishable from those found in the wild-type strain (Fig. 3). These data therefore suggested a specific role for Ubp3 in stress granule formation.

The catalytic activity of Ubp3 and its interaction with Bre5 were needed for stationary-phase stress granule formation. Ubp3 and the other Ubp enzymes act primarily as deubiquitinating cysteine proteases in the cell. Their main function is to remove either mono- or polyubiquitin modifications from particular sets of target proteins (39). To test whether the catalytic activity of Ubp3 was important for stress granule formation, we examined the localization of the Pbp1-mCh reporter in three strains known to possess diminished levels of Ubp3 activity. The first of these expresses a variant protein, $Ubp3^{C469A}$, where a cysteine residue critical for catalytic activity has been replaced with an alanine (40). In this strain, we observed an almost complete lack of the bright foci that were found in 14% of the wild-type cells (Fig. 4A). The number of total foci (including dim, diffuse, and intense puncta) was also lower by approximately one-third than the number in wild-type control. A similar result was seen with the second strain, the *bre5*Δ strain, which lacks a key protein cofactor, Bre5, important for Ubp3 activity (40). This interaction between Ubp3 and Bre5 has been conserved through evolution (41). Essentially no bright or intense Pbp1-mCh foci were observed in this strain after 5 days of growth in the rich medium YPAD (Fig. 4B). Finally, the third strain encodes a Ubp3 variant in which four residues in the Bre5 interaction domain ($L_{208}FIN_{211}$) have been replaced with alanines. The resulting protein, $Ubp3^{LFIN\text{-}AAAA}$, is unable to interact efficiently with Bre5 and thus exhibits decreased levels of catalytic activity (42). For the last experiment, we introduced single-copy plasmids that encoded either the wild-type Ubp3 or the $Ubp3^{C469A}$ or $Ubp3^{LFIN\text{-}AAAA}$ variants into a *ubp3*Δ strain. These strains were grown for 6 days in SC-glucose minimal medium, and the numbers of bright Pbp1-mCh foci were determined by fluorescence microscopy. Consistent with the above results, we found that the number of foci was diminished to a similar degree in the strains expressing either $Ubp3^{C469A}$ or $Ubp3^{LFIN\text{-}AAAA}$ (Fig. 4C). As reported previously, the fraction of cells that possessed stress granules in minimal media was substantially lower than that observed in rich growth media (6). The expression levels of both of

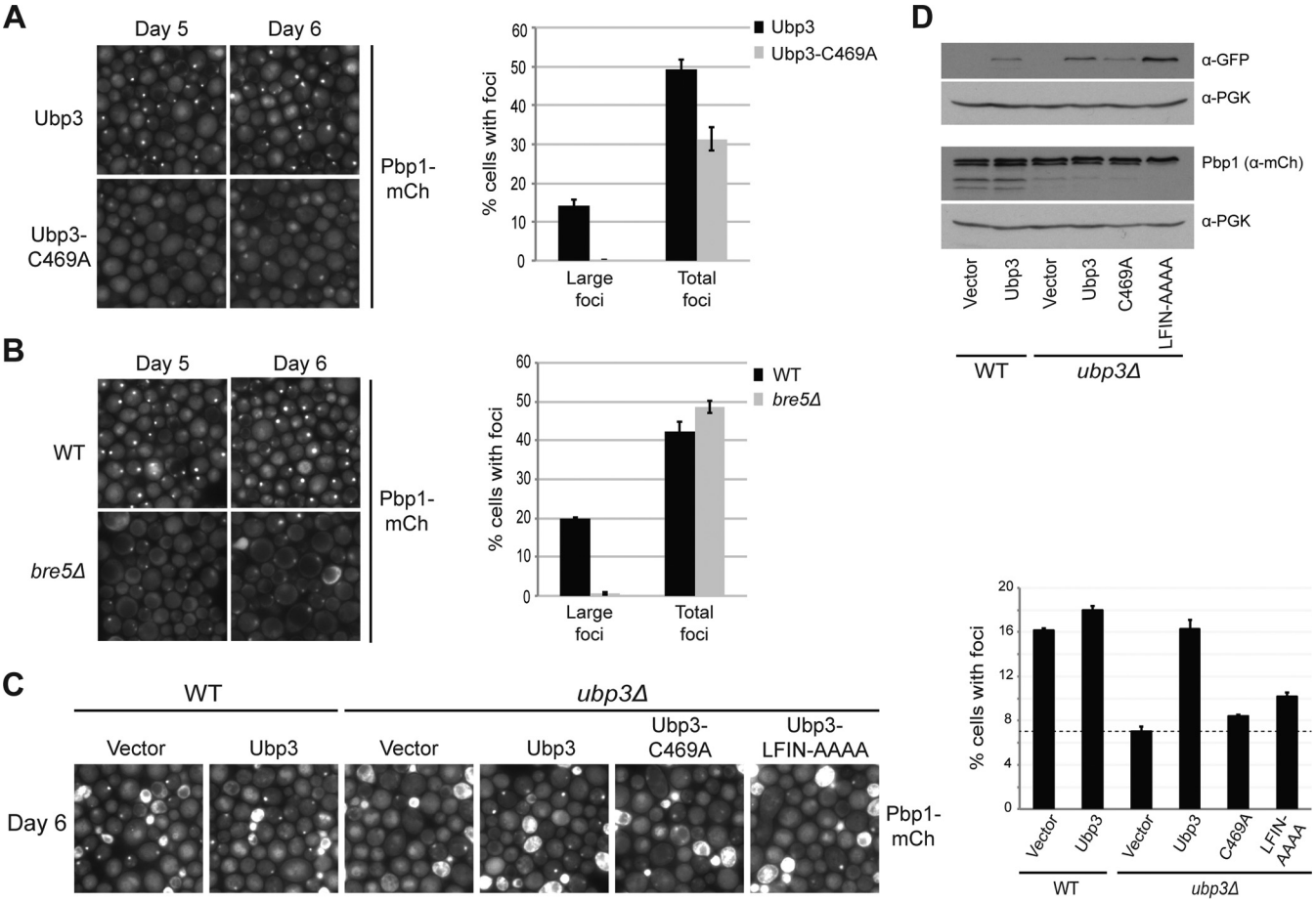

FIG 4 Ubp3 catalytic activity and the interaction with Bre5 were needed for efficient stationary-phase stress granule assembly. (A, B) Pbp1-mCh focus formation was assessed by fluorescence microscopy in cultures grown for 5 or 6 days in rich medium. The cells either expressed the catalytically inactive variant Ubp3^{C469A} (A) or were lacking the Ubp3 cofactor Bre5 (B). Ubp3 and Ubp3^{C469A} were tagged with GFP in order to confirm expression by Western blotting (not shown). The fraction of cells with large bright and total Pbp1-mCh foci is indicated in the graphs to the right of the microscopy images. (C) Expression of a Ubp3 variant defective for the interaction with Bre5, Ubp3$^{LFIN\text{-}AAAA}$, was unable to rescue the stress granule assembly defect associated with the *ubp3*Δ mutant. Wild-type and *ubp3*Δ strains expressing the indicated Ubp3 variants from a single-copy plasmid were grown for 6 days in minimal media. The fraction of cells with Pbp1-mCh foci is indicated in the accompanying graph. The data shown represent the averages for 3 replicates, where *n* is >200 cells for each. Cells with a strong, delocalized fluorescence were dead or dying and were not counted in any of the experiments. (D) The expression levels of the different Ubp3 variants in panel C were assessed by Western blotting with an anti-GFP antibody. The levels of the Pbp1-mCh reporter were also determined. PGK served as a loading control in these experiments.

these Ubp3 variants were similar to that of the wild-type Ubp3, and the Pbp1 reporter levels were similar in all strains (Fig. 4D). Thus, altogether these data indicated that the catalytic activity of Ubp3 is required for efficient stress granule formation in stationary-phase cells.

Ubp3 was required for heat stress- and sodium azide-induced stress granule formation. To test whether Ubp3 has a more general role in regulating stress granule assembly, we examined granule formation in response to two additional conditions known to induce stress granules: heat stress and sodium azide treatment (15, 43). For the former condition, wild-type and *ubp3*Δ cells were exposed to 45°C for 40 min and the Pbp4-mCh foci formed were visualized by fluorescence microscopy. Significantly fewer foci were observed in the *ubp3*Δ cells and the foci present tended to be less intense than those observed in the wild-type cells (Fig. 5A). This assembly defect was not due to a failure to arrest translation, as the rate of protein synthesis in the *ubp3*Δ mutant declined with kinetics similar to those of the wild type during the heat stress (Fig. 5B). A similar deficiency in stress granule assembly was observed when wild-type and *ubp3*Δ cells were exposed to 0.5% sodium azide for 30 min (Fig. 5C). In the absence of Ubp3, Pbp1-mCh focus formation was reduced and the foci formed were more diffuse than those observed in the wild-type control. Altogether, these data suggested that Ubp3 is generally required for the efficient assembly of stress granules in *S. cerevisiae*.

The Ubp3 protein was associated with stress granules. Although the above-described data indicated that Ubp3 regulates stress granule formation, it was not known whether this protein was associated with these RNP granules in *S. cerevisiae*. Therefore, we examined the localization of a Ubp3-GFP fusion protein following both an exposure to heat stress and the entry into stationary phase. For the former condition, we found that this Ubp3-GFP protein was indeed present in stress granules after a 40-min exposure to 45°C (Fig. 5D). However, the situation was more complicated for the latter, as Ubp3-GFP foci were not observed at any of the times examined during the growth into stationary phase (Fig. 5E). This analysis included day 7, a time when stress granules were

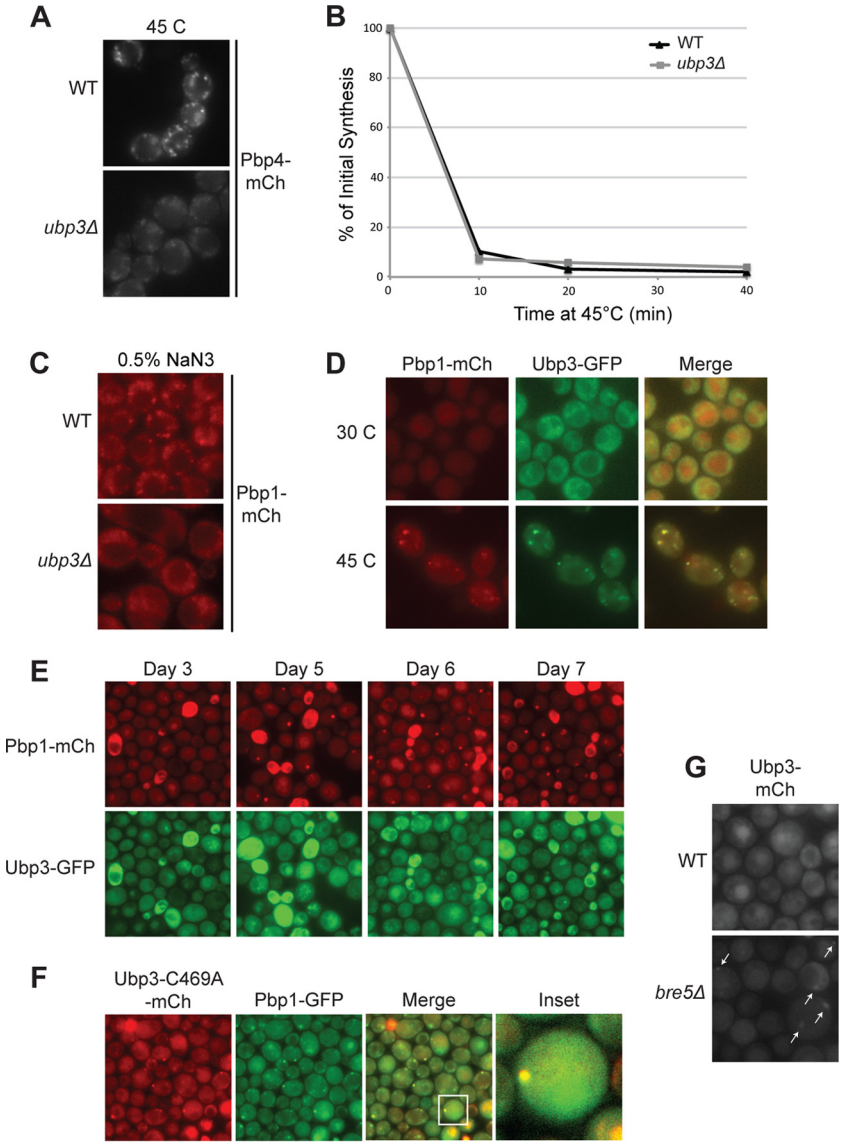

FIG 5 Ubp3 was associated with stress granules. (A) Ubp3 was required for stress granule formation in response to a heat stress. Wild-type and *ubp3*Δ cells were grown to mid-log phase in rich medium and then incubated at 45°C for 40 min. For the panels in this figure, stress granule formation was monitored by assessing the subcellular localization of an integrated Pbp1- or Pbp4-mCh reporter, as indicated. (B) *ubp3*Δ mutants exhibited a normal decrease in protein translation rates in response to a heat shock stress. Wild-type and *ubp3*Δ cells were grown to mid-log phase and then incubated at 45°C for up to 40 min. The relative rates of [^{35}S]methionine and [^{35}S]cysteine incorporation into TCA-precipitable material were determined as described in Materials and Methods. The graph shows the relative rates of incorporation after the indicated times of heat shock. (C) Ubp3 was required for stress granule formation in response to sodium azide. Wild-type and *ubp3*Δ cells were grown to mid-log phase in rich medium and then incubated with 0.5% sodium azide for 30 min. (D) Ubp3 was associated with heat stress-induced stress granules. Wild-type cells expressing Ubp3-GFP from a chromosomally integrated construct were grown to mid-log phase in rich medium and then incubated at either 30°C or 45°C for 40 min. (E) The wild-type Ubp3 was not detected in the stress granules formed in stationary-phase cells. Wild-type cells expressing Ubp3-GFP and Pbp1-mCh were grown for the indicated days in rich medium, and focus formation was assessed by fluorescence microscopy. (F) The catalytically inactive variant Ubp3^{C469A} was associated with stress granules in stationary phase. (G) A wild-type Ubp3-mCh protein formed dim cytoplasmic foci in *bre5*Δ cells. Wild-type and *bre5*Δ cells expressing Ubp3-mCh from a chromosomally integrated construct were grown for 9 days in YPAD rich medium and then visualized by fluorescence microscopy.

readily apparent in the cells. In contrast, the catalytically inactive Ubp3^{C469A} protein was found to be associated with stress granules in stationary-phase cells (Fig. 5F). Moreover, the wild-type Ubp3 was found to be associated with foci in *bre5*Δ cells, although these foci were less defined than those formed by the Ubp3^{C469A} protein (Fig. 5G). A possible interpretation of these data is that the wild-type Ubp3 is only transiently associated with stress granules, perhaps through an interaction with its ubiquitinated target. The removal of the ubiquitin moiety could then lead to Ubp3 disassociation from the granule. In this model, the catalytically inactive Ubp3 might be expected to exhibit a more prolonged association with these granules than the wild-type protein. In any case, the data here indicate that Ubp3 is found to be associated with stress granules under at least some stress conditions.

P-body formation was delayed in a *ubp3*Δ strain. The P-body is an RNP complex that has been linked spatially, compositionally,

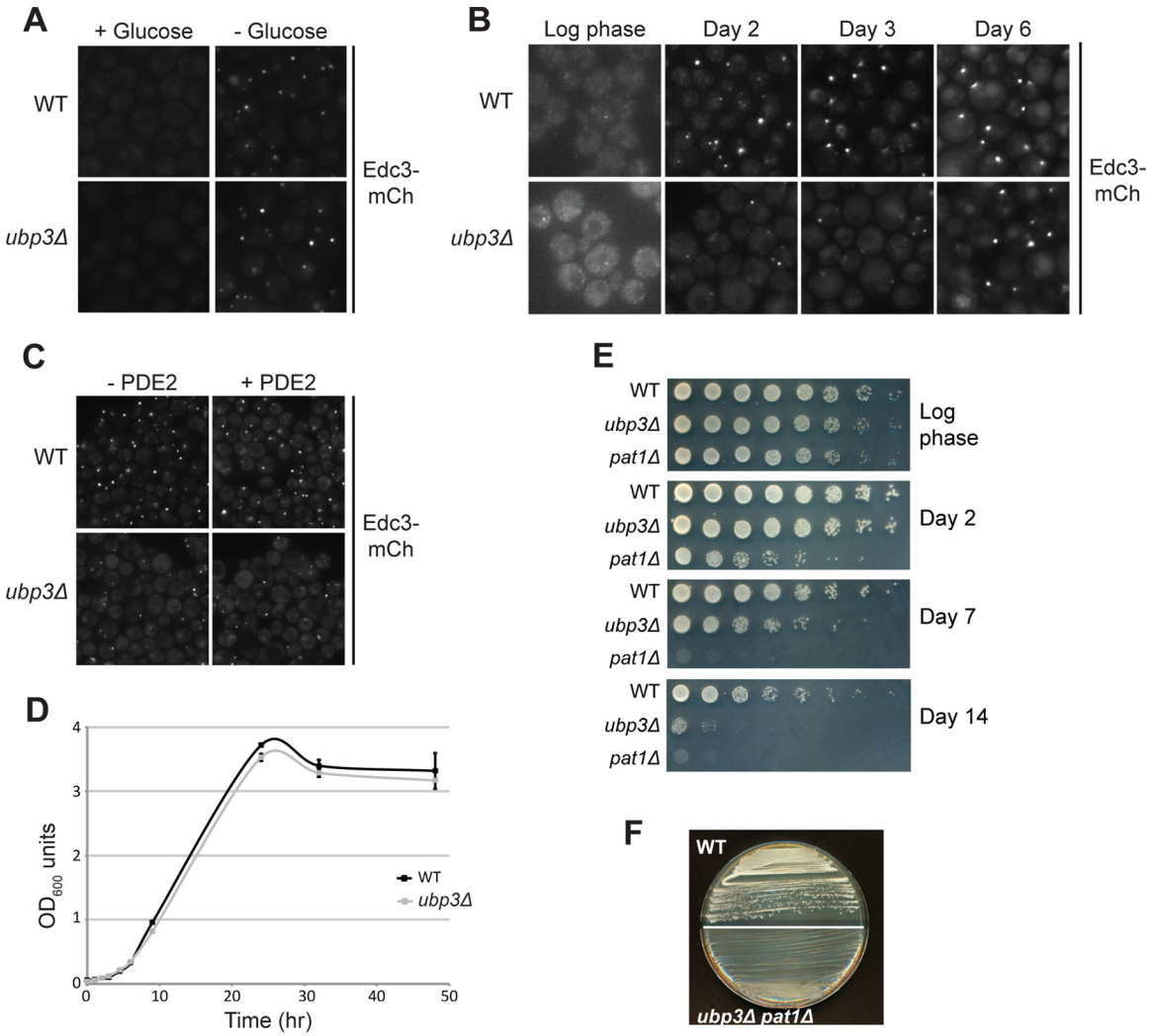

FIG 6 The *ubp3*Δ mutant exhibited a diminished ability to survive during the stationary phase of growth. (A) P-body formation in response to an acute starvation for glucose occurred normally in the *ubp3*Δ mutant. Wild-type and *ubp3*Δ cells were grown to mid-log phase in rich medium and then transferred to the same medium lacking glucose for 20 min. The formation of Edc3-mCh foci was assessed by fluorescence microscopy. (B, C) P-body assembly was delayed in a *ubp3*Δ strain in response to a more gradual depletion of glucose. Wild-type (WT) and *ubp3*Δ cells were grown for up to 6 days in rich medium. The subcellular localization of the P-body marker Edc3-mCh was analyzed by fluorescence microscopy in the absence (B) or presence (C) of Pde2 overexpression. (D) Growth curves for the wild-type and *ubp3*Δ strains in minimal medium are shown. (E) Ubp3 and/or stress granules were required for the long-term survival of stationary-phase cells. Serial dilutions of the indicated strains were plated following culture growth for up to 14 days in a minimal medium. The plates were then photographed after 2 to 3 days of incubation at 30°C. (F) The *ubp3*Δ *pat1*Δ double mutant exhibited a severe growth defect. The indicated strains were streaked onto rich medium and incubated for 2 days at 30°C.

and functionally to stress granules (19). Here, we set out to test whether the loss of Ubp3 had any effect upon P-body formation. For these experiments, we used an Edc3-mCh protein as a reporter for P-bodies and examined granule formation in response to both an acute and a gradual deprivation of glucose, as described previously (6). For the former, log-phase cells were transferred to a medium that lacked glucose for 20 min and P-body foci were then visualized by fluorescence microscopy. Under these conditions, we observed no significant difference in the Edc3-mCh foci formed in wild-type and *ubp3*Δ cells (Fig. 6A). However, we did see a difference with the latter regimen as P-body focus formation was delayed in the *ubp3*Δ cells (Fig. 6B). Under the standard growth conditions used here, the cells would have depleted most of the glucose in the medium within the first day of growth. This depletion of glucose is responsible for the P-body induction that is observed at this time (6). However, we found here that Edc3-mCh foci did not form appreciably until after 2 to 3 days of growth in the *ubp3*Δ cultures (Fig. 6B). At later times, the number and intensity of the P-body foci were similar in the wild-type and *ubp3*Δ cells. It is important to point out here that our previous work has shown that stress granule formation occurs independently of P-bodies in stationary-phase cultures. In particular, stress granules formed normally in mutants that had severe defects in P-body assembly (6).

We have previously shown that the formation of P-bodies is inhibited by the PKA pathway in *S. cerevisiae* (26). Since Ubp3 has been found to act as a negative regulator of PKA signaling (44), the delay in P-body formation observed in the absence of Ubp3 could be due to the removal of this inhibitory input. If this is indeed so, we reasoned that the overexpression of the cAMP phosphodies-

terase Pde2 might reverse this effect. Therefore, we introduced a *PDE2* high-copy-number plasmid that we have used previously to lower PKA activity (26, 45, 46). However, the presence of this plasmid had no effect on P-body assembly in the *ubp3*Δ cells (Fig. 6C). Therefore, the Ubp3 effects on P-body formation may be independent of its effects on the PKA signaling pathway.

The absence of Ubp3 was correlated with a diminished level of cell survival during the stationary phase of growth. To examine the physiological significance of stress granules during stationary phase, we assessed wild-type and *ubp3*Δ cell survival during this period of quiescence. For these studies, the strains were grown for the indicated number of days in minimal medium, and serial dilutions of the cultures were then plated out. The number of days that the cells remain viable has been referred to as the chronological life span (CLS) and has been used as a model for the study of cellular aging (47). The wild-type and *ubp3*Δ strains exhibited similar growth curves for the culture conditions used here (Fig. 6D). However, we found that the *ubp3*Δ strain exhibited a significantly shorter CLS than the wild-type control, a result consistent with the presence of Ubp3 and/or stress granules being an important factor for stationary-phase survival (Fig. 6E). *ubp3* mutants appeared to enter into stationary phase, as they exhibited the heightened resistance to heat shock typically associated with this resting state (29, 48). In particular, wild-type, *ubp3*Δ, and $ubp3^{C469A}$ cells were all found to exhibit a survival rate of greater than 50% following a 20-min heat shock at 50°C (54%, 73%, and 58%, respectively). In contrast, fewer than 1% of the cells in each log-phase control culture were resistant to this heat stress. Mutants that fail to enter into a normal stationary phase resemble log-phase cells and are also sensitive to this heat shock regimen (29, 48).

The CLS defect in *ubp3*Δ cells was less severe than that observed with *pat1*Δ mutants, which are defective for P-body formation (6, 26). To assess the consequences of compromising both stress granule and P-body assembly, we constructed a *ubp3*Δ *pat1*Δ double mutant as described in Materials and Methods. Interestingly, this strain exhibited a severe growth defect that might suggest a role for these RNP granules during mitotic growth (Fig. 6F). However, it is important to point out that Ubp3 has multiple targets and the growth effects observed here could be due to the failure to deubiquitinate a substrate unrelated to stress granules. Nonetheless, the data here indicate that the Ubp3 protein and/or stress granules are required for the long-term survival of stationary-phase yeast cells.

DISCUSSION

Stress granules are evolutionarily conserved structures that appear to have an important role in the cellular response to stress. The ability to manipulate their formation could yield invaluable insight into precisely how such granules influence cell physiology under these conditions. In this study, we identified a critical role for a particular deubiquitinating protease, Ubp3, in stress granule formation in response to a variety of conditions in *S. cerevisiae*. These conditions include the entry into stationary phase and an exposure to either sodium azide or a heat stress. Both Ubp3 catalytic activity and the presence of a Ubp3 cofactor, Bre5, were required for the efficient assembly of these cytoplasmic granules. Altogether, these results suggest that the deubiquitination of a particular Ubp3 target is generally important for optimal stress granule formation. Finally, we found that the absence of Ubp3, and thus stress granules, results in a significant decrease in viability in stationary-phase cells, much like that observed upon impairment of P-body formation. These studies are therefore consistent with cytoplasmic RNP granules generally having an important role in the long-term survival of quiescent cells.

The absence of Ubp3 altered stress granule formation, in terms of both the number of foci per cell and their physical characteristics. In particular, the majority of stationary-phase foci observed in Ubp3-deficient cells were relatively diffuse aggregates comprised of known stress granule components. These foci are visually distinct from the highly punctate granules that are found in Ubp3-expressing cells. Accordingly, fewer and smaller stress granules have been reported in mouse embryonic fibroblasts lacking the Ubp3 homologue, USP10, following exposure to arsenite or heat stress (28). Although these diffuse aggregates contain stress granule components, it is unclear as to whether they retain any functionality.

Multiple lines of evidence presented here indicate that the catalytic activity of Ubp3 mediates its role in stress granule formation. One possibility is that a target(s) of Ubp3 exists in a ubiquitinated form under nonstress conditions and that this modified protein prevents the aggregation required for stress granule assembly. An exposure to an appropriate stress or the entry into stationary phase could trigger the Ubp3-mediated deubiquitination of this protein, thereby allowing for granule formation. Although it remains to be determined whether ubiquitination can in fact inhibit the aggregation necessary for stress granule formation, several posttranslational modifications are known to regulate similar types of protein behavior. For example, α-synuclein aggregation is modulated by phosphorylation, oxidation, and sumoylation (49). Identification of this target(s) of Ubp3 is an important next step toward understanding the mechanisms controlling stress granule formation. To begin to address this issue, we screened single-deletion strains lacking known Ubp3 substrates for effects on stress granule formation. Unfortunately, no defects were observed in any of these mutants (data not shown; relevant strains are indicated in Table S1 in the supplemental material). Therefore, less-directed approaches are likely needed to identify novel Ubp3 substrates important for the stress granule effects observed here.

In addition to regulating the formation of stress granules, Ubp3 can also localize to these foci. For example, we found that Ubp3 colocalizes with the stress granule marker Pbp1 in heat-stressed cells. A similar association has been observed in *S. pombe* cells exposed to a heat stress (50). Furthermore, USP10 was observed to localize to stress granules in mammalian cells exposed to heat or arsenite stress (28). Although the wild-type *S. cerevisiae* Ubp3 did not form foci in stationary-phase cells, a catalytically inactive variant was associated with stress granules at this time. Thus, Ubp3 may normally associate only transiently with stress granules in quiescent cells, perhaps as a result of an interaction with a granule-localized substrate. In this model, the ubiquitination of this substrate would be inhibitory to stress granule formation. It is currently unclear how Ubp3 is recruited to these granules and what, if any, physiological function Ubp3 might have in this RNP structure. Interestingly, in mammalian cells the localization of USP10 to stress granules unleashes its antioxidant activity by altering its interaction with G3BP1 (the mammalian Bre5) within the focus (28). It is not yet known whether stress granule-localized Ubp3 exhibits a similar function in yeast and what role

this type of activity might have in the survival of stationary-phase cells.

Finally, this study suggests that stress granules, much like P-bodies, may be important for the long-term survival of quiescent cells (26). Specifically, a defect in stress granule formation was correlated here with diminished cell survival in stationary-phase cultures. Although this interpretation is complicated somewhat by the kinetic delay in P-body formation observed in *ubp3*Δ cells, normal P-body numbers were present before these cells began to show a significant defect in overall survival. As a result, we feel that the loss of stress granules is a more likely explanation for the diminished CLS displayed by this mutant. Clearly, additional work is needed to confirm this interpretation and to establish that the Ubp3 effects on this survival are direct consequences of defective stress granule assembly. Nonetheless, the results here raise the interesting possibility of a general role for these types of cytoplasmic RNP granules in the survival of G_0-like resting cells. Determining how these granules influence this biology could shed light on both the underlying requirements for survival at this time and the physiological roles that these RNP structures have in the eukaryotic cell. In turn, these observations could provide insight into the observed link between stress granules and neurodegenerative disease.

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REFERENCES

1. **Thomas MG, Loschi M, Desbats MA, Boccaccio GL.** 2011. RNA granules: the good, the bad and the ugly. Cell Signal **23:**324–334. http://dx.doi.org/10.1016/j.cellsig.2010.08.011.
2. **Shah KH, Nostramo R, Zhang B, Varia SN, Klett BM, Herman PK.** 2014. Protein kinases are associated with multiple, distinct cytoplasmic granules in quiescent yeast cells. Genetics **198:**1495–1512. http://dx.doi.org/10.1534/genetics.114.172031.
3. **Anderson P, Kedersha N.** 2009. RNA granules: post-transcriptional and epigenetic modulators of gene expression. Nat Rev Mol Cell Biol **10:**430–436. http://dx.doi.org/10.1038/nrm2694.
4. **Nonhoff U, Ralser M, Welzel F, Piccini I, Balzereit D, Yaspo ML, Lehrach H, Krobitsch S.** 2007. Ataxin-2 interacts with the DEAD/H-box RNA helicase DDX6 and interferes with P-bodies and stress granules. Mol Biol Cell **18:**1385–1396. http://dx.doi.org/10.1091/mbc.E06-12-1120.
5. **Li YR, King OD, Shorter J, Gitler AD.** 2013. Stress granules as crucibles of ALS pathogenesis. J Cell Biol **201:**361–372. http://dx.doi.org/10.1083/jcb.201302044.
6. **Shah KH, Zhang B, Ramachandran V, Herman PK.** 2013. Processing body and stress granule assembly occur by independent and differentially regulated pathways in Saccharomyces cerevisiae. Genetics **193:**109–123. http://dx.doi.org/10.1534/genetics.112.146993.
7. **Kedersha N, Cho MR, Li W, Yacono PW, Chen S, Gilks N, Golan DE, Anderson P.** 2000. Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. J Cell Biol **151:**1257–1268. http://dx.doi.org/10.1083/jcb.151.6.1257.
8. **Kedersha NL, Gupta M, Li W, Miller I, Anderson P.** 1999. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. J Cell Biol **147:**1431–1442. http://dx.doi.org/10.1083/jcb.147.7.1431.
9. **Gilks N, Kedersha N, Ayodele M, Shen L, Stoecklin G, Dember LM, Anderson P.** 2004. Stress granule assembly is mediated by prion-like aggregation of TIA-1. Mol Biol Cell **15:**5383–5398. http://dx.doi.org/10.1091/mbc.E04-08-0715.
10. **Han TW, Kato M, Xie S, Wu LC, Mirzaei H, Pei J, Chen M, Xie Y, Allen J, Xiao G, McKnight SL.** 2012. Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. Cell **149:**768–779. http://dx.doi.org/10.1016/j.cell.2012.04.016.
11. **Kato M, Han TW, Xie S, Shi K, Du X, Wu LC, Mirzaei H, Goldsmith EJ, Longgood J, Pei J, Grishin NV, Frantz DE, Schneider JW, Chen S, Li L, Sawaya MR, Eisenberg D, Tycko R, McKnight SL.** 2012. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. Cell **149:**753–767. http://dx.doi.org/10.1016/j.cell.2012.04.017.
12. **Kedersha N, Ivanov P, Anderson P.** 2013. Stress granules and cell signaling: more than just a passing phase? Trends Biochem Sci **38:**494–506. http://dx.doi.org/10.1016/j.tibs.2013.07.004.
13. **Arimoto K, Fukuda H, Imajoh-Ohmi S, Saito H, Takekawa M.** 2008. Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. Nat Cell Biol **10:**1324–1332. http://dx.doi.org/10.1038/ncb1791.
14. **Thedieck K, Holzwarth B, Prentzell MT, Boehlke C, Klasener K, Ruf S, Sonntag AG, Maerz L, Grellscheid SN, Kremmer E, Nitschke R, Kuehn EW, Jonker JW, Groen AK, Reth M, Hall MN, Baumeister R.** 2013. Inhibition of mTORC1 by astrin and stress granules prevents apoptosis in cancer cells. Cell **154:**859–874. http://dx.doi.org/10.1016/j.cell.2013.07.031.
15. **Grousl T, Ivanov P, Frydlova I, Vasicova P, Janda F, Vojtova J, Malinska K, Malcova I, Novakova L, Janoskova D, Valasek L, Hasek J.** 2009. Robust heat shock induces eIF2alpha-phosphorylation-independent assembly of stress granules containing eIF3 and 40S ribosomal subunits in budding yeast, Saccharomyces cerevisiae. J Cell Sci **122:**2078–2088. http://dx.doi.org/10.1242/jcs.045104.
16. **Wen WL, Stevenson AL, Wang CY, Chen HJ, Kearsey SE, Norbury CJ, Watt S, Bahler J, Wang SW.** 2010. Vgl1, a multi-KH domain protein, is a novel component of the fission yeast stress granules required for cell survival under thermal stress. Nucleic Acids Res **38:**6555–6566. http://dx.doi.org/10.1093/nar/gkq555.
17. **Nilsson D, Sunnerhagen P.** 2011. Cellular stress induces cytoplasmic RNA granules in fission yeast. RNA **17:**120–133. http://dx.doi.org/10.1261/rna.2268111.
18. **Buchan JR, Parker R.** 2009. Eukaryotic stress granules: the ins and outs of translation. Mol Cell **36:**932–941. http://dx.doi.org/10.1016/j.molcel.2009.11.020.
19. **Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fritzler MJ, Scheuner D, Kaufman RJ, Golan DE, Anderson P.** 2005. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J Cell Biol **169:**871–884. http://dx.doi.org/10.1083/jcb.200502088.
20. **Balagopal V, Parker R.** 2009. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. Curr Opin Cell Biol **21:**403–408. http://dx.doi.org/10.1016/j.ceb.2009.03.005.
21. **Eulalio A, Behm-Ansmant I, Izaurralde E.** 2007. P bodies: at the crossroads of post-transcriptional pathways. Nat Rev Mol Cell Biol **8:**9–22. http://dx.doi.org/10.1038/nrm2080.
22. **Franks TM, Lykke-Andersen J.** 2008. The control of mRNA decapping and P-body formation. Mol Cell **32:**605–615. http://dx.doi.org/10.1016/j.molcel.2008.11.001.
23. **Stoecklin G, Mayo T, Anderson P.** 2006. ARE-mRNA degradation requires the 5′-3′ decay pathway. EMBO Rep **7:**72–77. http://dx.doi.org/10.1038/sj.embor.7400572.
24. **Decker CJ, Teixeira D, Parker R.** 2007. Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in Saccharomyces cerevisiae. J Cell Biol **179:**437–449. http://dx.doi.org/10.1083/jcb.200704147.
25. **Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E.** 2007. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol Cell Biol **27:**3970–3981. http://dx.doi.org/10.1128/MCB.00128-07.
26. **Ramachandran V, Shah KH, Herman PK.** 2011. The cAMP-dependent

protein kinase signaling pathway is a key regulator of P body foci formation. Mol Cell **43:**973–981. http://dx.doi.org/10.1016/j.molcel.2011.06.032.
27. **Baker RT, Tobias JW, Varshavsky A.** 1992. Ubiquitin-specific proteases of Saccharomyces cerevisiae. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J Biol Chem **267:**23364–23375.
28. **Takahashi M, Higuchi M, Matsuki H, Yoshita M, Ohsawa T, Oie M, Fujii M.** 2013. Stress granules inhibit apoptosis by reducing reactive oxygen species production. Mol Cell Biol **33:**815–829. http://dx.doi.org/10.1128/MCB.00763-12.
29. **Chang YW, Howard SC, Budovskaya YV, Rine J, Herman PK.** 2001. The rye mutants identify a role for Ssn/Srb proteins of the RNA polymerase II holoenzyme during stationary phase entry in Saccharomyces cerevisiae. Genetics **157:**17–26.
30. **Ramachandran V, Herman PK.** 2011. Antagonistic interactions between the cAMP-dependent protein kinase and Tor signaling pathways modulate cell growth in Saccharomyces cerevisiae. Genetics **187:**441–454. http://dx.doi.org/10.1534/genetics.110.123372.
31. **Kaiser C, Michaelis S, Mitchell A.** 1994. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. **Budovskaya YV, Stephan JS, Deminoff SJ, Herman PK.** 2005. An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase. Proc Natl Acad Sci U S A **102:**13933–13938. http://dx.doi.org/10.1073/pnas.0501046102.
33. **Yeh YY, Wrasman K, Herman PK.** 2010. Autophosphorylation within the Atg1 activation loop is required for both kinase activity and the induction of autophagy in Saccharomyces cerevisiae. Genetics **185:**871–882. http://dx.doi.org/10.1534/genetics.110.116566.
34. **Budovskaya YV, Hama H, DeWald DB, Herman PK.** 2002. The C terminus of the Vps34p phosphoinositide 3-kinase is necessary and sufficient for the interaction with the Vps15p protein kinase. J Biol Chem **277:**287–294. http://dx.doi.org/10.1074/jbc.M109263200.
35. **Budovskaya YV, Stephan JS, Reggiori F, Klionsky DJ, Herman PK.** 2004. The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in Saccharomyces cerevisiae. J Biol Chem **279:**20663–20671. http://dx.doi.org/10.1074/jbc.M400272200.
36. **Deminoff SJ, Howard SC, Hester A, Warner S, Herman PK.** 2006. Using substrate-binding variants of the cAMP-dependent protein kinase to identify novel targets and a kinase domain important for substrate interactions in *Saccharomyces cerevisiae*. Genetics **173:**1909–1917. http://dx.doi.org/10.1534/genetics.106.059238.
37. **Costanzo M, Baryshnikova A, Myers CL, Andrews B, Boone C.** 2011. Charting the genetic interaction map of a cell. Curr Opin Biotechnol **22:**66–74. http://dx.doi.org/10.1016/j.copbio.2010.11.001.
38. **Ambroziak J, Henry SA.** 1994. INO2 and INO4 gene products, positive regulators of phospholipid biosynthesis in Saccharomyces cerevisiae, form a complex that binds to the INO1 promoter. J Biol Chem **269:**15344–15349.
39. **Reyes-Turcu FE, Ventii KH, Wilkinson KD.** 2009. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem **78:**363–397. http://dx.doi.org/10.1146/annurev.biochem.78.082307.091526.
40. **Cohen M, Stutz F, Belgareh N, Haguenauer-Tsapis R, Dargemont C.** 2003. Ubp3 requires a cofactor, Bre5, to specifically de-ubiquitinate the COPII protein, Sec23. Nat Cell Biol **5:**661–667. http://dx.doi.org/10.1038/ncb1003.
41. **Soncini C, Berdo I, Draetta G.** 2001. Ras-GAP SH3 domain binding protein (G3BP) is a modulator of USP10, a novel human ubiquitin specific protease. Oncogene **20:**3869–3879. http://dx.doi.org/10.1038/sj.onc.1204553.
42. **Li K, Ossareh-Nazari B, Liu X, Dargemont C, Marmorstein R.** 2007. Molecular basis for bre5 cofactor recognition by the ubp3 deubiquitylating enzyme. J Mol Biol **372:**194–204. http://dx.doi.org/10.1016/j.jmb.2007.06.052.
43. **Buchan JR, Yoon JH, Parker R.** 2011. Stress-specific composition, assembly and kinetics of stress granules in Saccharomyces cerevisiae. J Cell Sci **124:**228–239. http://dx.doi.org/10.1242/jcs.078444.
44. **Li Y, Wang Y.** 2013. Ras protein/cAMP-dependent protein kinase signaling is negatively regulated by a deubiquitinating enzyme, Ubp3, in yeast. J Biol Chem **288:**11358–11365. http://dx.doi.org/10.1074/jbc.M112.449751.
45. **Howard SC, Budovskaya YV, Chang YW, Herman PK.** 2002. The C-terminal domain of the largest subunit of RNA polymerase II is required for stationary phase entry and functionally interacts with the Ras/PKA signaling pathway. J Biol Chem **277:**19488–19497. http://dx.doi.org/10.1074/jbc.M201878200.
46. **Howard SC, Hester A, Herman PK.** 2003. The Ras/PKA signaling pathway may control RNA polymerase II elongation via the Spt4p/Spt5p complex in Saccharomyces cerevisiae. Genetics **165:**1059–1070.
47. **Kennedy BK.** 2008. The genetics of ageing: insight from genome-wide approaches in invertebrate model organisms. J Intern Med **263:**142–152. http://dx.doi.org/10.1111/j.1365-2796.2007.01903.x.
48. **Werner-Washburne M, Braun E, Johnston GC, Singer RA.** 1993. Stationary phase in the yeast Saccharomyces cerevisiae. Microbiol Rev **57:**383–401.
49. **Beyer K.** 2006. Alpha-synuclein structure, posttranslational modification and alternative splicing as aggregation enhancers. Acta Neuropathol **112:**237–251. http://dx.doi.org/10.1007/s00401-006-0104-6.
50. **Wang CY, Wen WL, Nilsson D, Sunnerhagen P, Chang TH, Wang SW.** 2012. Analysis of stress granule assembly in Schizosaccharomyces pombe. RNA **18:**694–703. http://dx.doi.org/10.1261/rna.030270.111.