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A Ubiquitin Stress Response Induces Altered Proteasome Composition

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SUMMARY

Ubiquitin-dependent protein degradation is essential for cells to survive many environmental stresses. Thus, it may be necessary to buffer ubiquitin and proteasome pools against fluctuation. Proteasome levels are tightly regulated, and proteasome deficiency stimulates a stress response. Here we report a novel pathway of cellular response to ubiquitin depletion. Unlike proteasome stress, ubiquitin stress does not upregulate proteasome abundance. Instead, ubiquitin stress alters proteasome composition. The proteasome-associated deubiquitinating enzyme Ubp6, which spares ubiquitin from proteasomal degradation, is induced by ubiquitin deficiency. This enhances loading of proteasomes with Ubp6, thereby altering proteasome function. A catalytically inactive mutant of Ubp6 fails to recycle ubiquitin and also inhibits proteasome function directly, thus inducing both ubiquitin stress and proteasome stress. These results show that homeostatic control of the ubiquitin-proteasome pathway can be achieved through signal-dependent, subunit-specific regulation of the proteasome, and indicate a dual role of Ubp6 in regulating ubiquitin levels and proteasome function.

INTRODUCTION

Ubiquitin is a highly conserved, 76 residue protein which can be conjugated posttranslationally to other proteins via an isopeptide linkage between glycine-76 of ubiquitin and, most typically, the ε-amino group of a lysine within the target protein. Ubiquitin itself contains seven lysines, allowing the formation of ubiquitin-ubiquitin polymers. Posttranslational modification by ubiquitin is utilized in a number of cellular pathways, but the role of ubiquitin in selective, intracellular protein degradation remains its best-understood function. In this pathway, ubiquitin modification serves as the recognition motif for a large multisubunit protease, known as the proteasome, which renders proteins into small polypeptides.

Initial identification of the ubiquitin genes revealed an unusual mode of synthesis. In the budding yeast *S. cerevisiae*, ubiquitin is encoded by four genes. Three of these genes (*UBI1-3*) encode fusions of ribosomal proteins to the C terminus of ubiquitin (Ozkaynak et al., 1987). The final ubiquitin gene, *UBI4*, contains five head-to-tail repeats of ubiquitin (Ozkaynak et al., 1987). In all cases, the generation of free ubiquitin requires the translation of a fusion protein, an unusual event in eukaryotes, followed by posttranslational cleavage of ubiquitin at its C terminus, a reaction carried out by one or more members of a family of proteases known as deubiquitinating enzymes. Transcriptional control of ubiquitin is highly regulated. Under normal growth conditions, the bulk of cellular ubiquitin is provided by *UBI1-3*. Stress conditions, such as heat shock, stimulate induction of *UBI4* (Ozkaynak et al., 1987; Finley et al., 1987). Transcription of ubiquitin genes is stimulated by a wide variety of cellular stresses in addition to heat shock, and is controlled by a number of distinct transcriptional pathways (Watt and Piper, 1997; Simon et al., 1999). The complexity of regulation of ubiquitin biosynthesis likely reflects the myriad roles of ubiquitin in cellular biology, and the need for precise control over ubiquitin levels for cellular homeostasis.

Ubiquitin is among the most physically stable proteins known, and remains properly folded at temperatures up to 85°C and between a pH range of 1 and 13 (Lenkinski et al., 1977). That ubiquitin displays a significant rate of turnover is therefore intriguing. Early studies in mammalian cells observed that under nutrient-rich conditions, the bulk of ubiquitin degradation was nonlysosomal and ATP-dependent (Haas and Bright, 1987; Hiroi and Rechsteiner, 1992). This degradation is mediated by the proteasome, as more recent studies have demonstrated (Ryu et al., 2006). Significant ubiquitin turnover has also been observed in yeast, where the half-life of ubiquitin has been estimated to be less than 2 hr (Hanna et al., 2003). The crucial role of the proteasome in ubiquitin turnover is emphasized by a yeast mutant lacking the *UBP6* gene, which encodes a proteasome-associated deubiquitinating enzyme. In the absence of this protein, the half-life of ubiquitin is dramatically reduced, and *ubp6Δ* cells rapidly become deficient in steady-state ubiquitin levels, a feature which hypersensitizes such cells to a wide variety of chemical and environmental stresses (Leggett et al., 2002; Hanna et al., 2003; Chernova et al., 2003). Ubp6 uses its catalytic activity to remove ubiquitin from proteasome substrates

(Hanna et al., 2006), and some fraction of this ubiquitin, despite its physical stability, is apparently readily unfolded by proteasomes and destroyed along with substrate in the absence of Ubp6.

It has been assumed that the maintenance of cellular ubiquitin levels is regulated primarily at the level of synthesis. We demonstrate here a regulatory pathway that controls cellular levels of ubiquitin by modulating processes of deubiquitination and protein degradation. The cellular abundance of Ubp6 is sensitive to the levels of cellular ubiquitin: under conditions of ubiquitin depletion, Ubp6 abundance increases, and when ubiquitin levels are restored by exogenous expression, Ubp6 levels return to baseline. Ubiquitin-dependent upregulation of Ubp6 results in greater loading of proteasomes with Ubp6, presumably resulting in greater efficiency of ubiquitin recycling at the proteasome. Intriguingly, a catalytically inactive mutant of Ubp6 (*ubp6-C118A*) triggers the induction of Ubp6, owing to cellular ubiquitin deficiency, but in contrast to the *ubp6* null mutant, *ubp6-C118A* also displays a general upregulation of total proteasome levels that is independent of ubiquitin levels, likely reflecting a previously described noncatalytic function of Ubp6 in proteasome inhibition (Hanna et al., 2006). Proteasome upregulation in *ubp6-C118A* mutants is mediated by the transcription factor Rpn4, and loss of proteasome compensation by Rpn4 results in a severe growth defect. However, ubiquitin-dependent induction of Ubp6 appears to be independent of proteasome induction by Rpn4. These results describe an unexpected pathway of cellular regulation, provide evidence for the existence of a cellular ubiquitin sensor, and indicate that despite the sequential and interdependent nature of ubiquitination and proteasome-mediated degradation in the ubiquitin-proteasome system, the pathways controlling ubiquitin and proteasome levels are largely independent.

RESULTS

Ubiquitin-Dependent Regulation of Ubp6 Protein Levels

We recently generated yeast *CEN* plasmids expressing wild-type Ubp6 and a catalytically inactive mutant, Ubp6-C118A, from the *UBP6* promoter (Hanna et al., 2006). Transformation of *ubp6Δ* cells with the wild-type plasmid resulted in approximately wild-type expression levels of the Ubp6 protein (Figure 1A). In contrast, *ubp6Δ* cells harboring the Ubp6-C118A plasmid accumulated significantly higher levels of the mutant protein (Figure 1A; for quantitation, see Figure S1 in the Supplemental Data). Levels of an unrelated protein, the translation initiation factor eIF5A, were constant throughout, indicating the specificity of these effects (Figure 1A).

Two related functions have been ascribed to the catalytic activity of Ubp6: Ubp6 gradually removes ubiquitin from proteasome-bound ubiquitin conjugates, and it prevents or minimizes degradation of ubiquitin by the proteasome. The degradation of ubiquitin by *ubp6Δ* proteasomes presumably reflects that substrates not subject to deubiquitination by Ubp6 can be unfolded and translocated into the degradation-competent core particle of the proteasome with at least some ubiquitin still attached, resulting in degradation of both substrate and ubiquitin components of the conjugate (Leggett et al., 2002; Chernova et al., 2003; Hanna et al., 2006). Failure to regenerate ubiquitin in the absence of Ubp6 results in deficient ubiquitin content in *ubp6Δ* cells, giving rise to hypersensitivity to a variety of chemical, environmental, and genetic stresses (Chernova et al., 2003; Hanna et al., 2003). Because ubiquitin recycling by Ubp6 requires its catalytic function, *ubp6-C118A* mutants largely recapitulate the ubiquitin deficiency of the null mutant (Figure 1A). We therefore hypothesized that accumulation of the Ubp6-C118A protein might reflect a cellular response to ubiquitin deficiency. This model predicts that a correction of ubiquitin levels should restore Ubp6-C118A levels to normal, even without restoration of Ubp6's catalytic activity. Indeed, when ubiquitin levels were increased by expressing a synthetic ubiquitin gene in this strain, the difference in abundance of Ubp6 and Ubp6-C118A levels was minimized (Figure 1B). The plasmid-borne ubiquitin expression construct was chosen to correct ubiquitin levels without dramatically overexpressing ubiquitin (see Experimental Procedures), thus enhancing the likelihood of identifying regulatory pathways that operate in a physiological fluctuation range of ubiquitin.

Ubiquitin is conjugated posttranslationally to other proteins, but is also known to bind a variety of proteins in a noncovalent fashion. To determine whether conjugation of ubiquitin was necessary for the downregulation of Ubp6-C118A levels, we utilized a mutant of ubiquitin lacking its final two glycine residues, which are required for conjugation (hereafter referred to as ubiquitin-desGlyGly; Arnason and Ellison, 1994). In contrast to results obtained with wild-type ubiquitin, ubiquitin-desGlyGly was unable to restore levels of Ubp6-C118A to those seen with wild-type Ubp6 (Figure 1C). The inability of ubiquitin-desGlyGly to influence Ubp6 levels could not be attributed to low expression levels of the mutant ubiquitin. Indeed, when ubiquitin-desGlyGly was expressed in *ubp6Δ* and *ubp6-C118A* cells, the resulting total free ubiquitin content was comparable to that of endogenous ubiquitin in a wild-type cell (Figure 1C). These data suggest that ubiquitin conjugation is required for the pathway of ubiquitin-dependent regulation of Ubp6, and the implications of this finding are discussed in more detail below.

Endogenous Ubp6 Is Regulated by Ubiquitin Levels

We next sought evidence that the ubiquitin-dependent regulation of Ubp6-C118A reflected a physiological cellular response. Were this the case, one would expect an induction of wild-type, endogenous Ubp6 in response to cellular ubiquitin deficiency that does not derive from impaired Ubp6 function. To test this model, we employed two independent ubiquitin-deficient yeast mutants: *doa4Δ* and *ubi1-3Δ* (Swaminathan et al., 1999; Hanna et al., 2003;

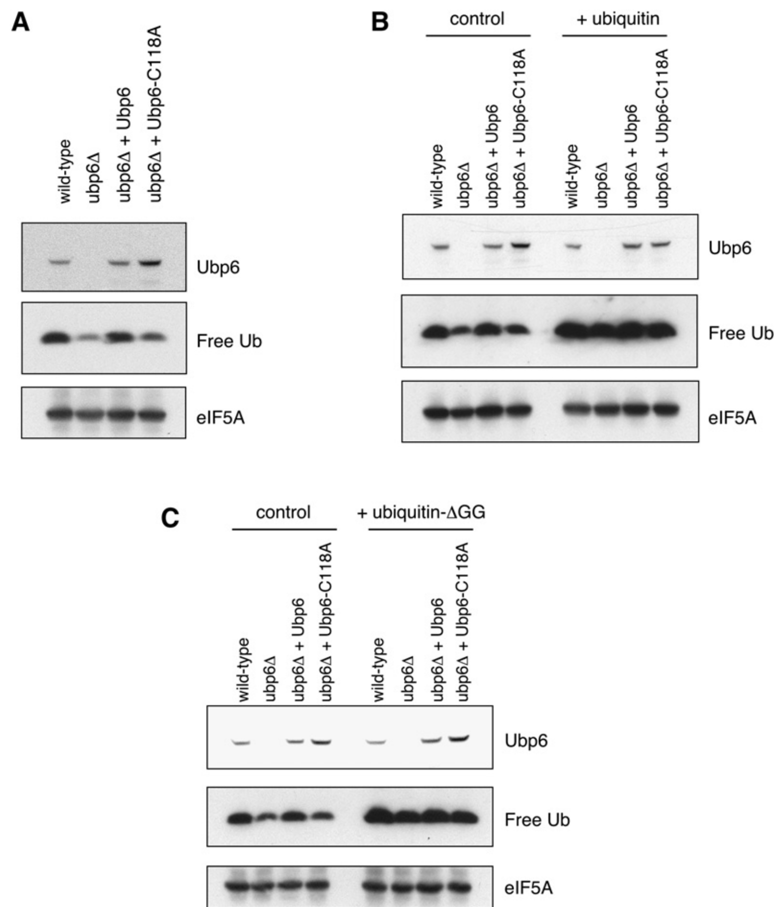

Figure 1. Ubiquitin-Dependent Regulation of Ubp6

(A) Cellular levels of Ubp6 and Ubp6-C118A proteins, as determined by immunoblotting. Middle panel, free ubiquitin; lower panel, eIF5A, which serves as a loading control. Strains: sJH152–sJH155.

(B) Cellular levels of Ubp6 and Ubp6-C118A after ubiquitin overexpression. Strains: sJH197–sJH204.

(C) As in (B), except that the ubiquitin expression plasmid encoded a ubiquitin mutant (ubiquitin-desGlyGly). Strains: sJH269–sJH276.

Figure 2A). *DOA4*, like *UBP6,* encodes a deubiquitinating enzyme. However, the ubiquitin deficiency in *doa4Δ* mutants is suppressed by mutations in vacuolar proteases, suggesting that the site of aberrant ubiquitin degradation is the vacuole (Swaminathan et al., 1999), rather than the proteasome, as seen for *ubp6Δ* (Hanna et al., 2003; Chernova et al., 2003). The *ubi1-3Δ* mutant lacks three of the four ubiquitin genes, but the ribosomal portions of these fusion proteins are supplied on plasmids, as they are essential for viability (see Table S1 in the Supplemental Data). Upon examination of the *doa4Δ* and *ubi1-3Δ* mutants, we observed increased levels of endogenous Ubp6 protein (Figure 2A).

To confirm that increased Ubp6 levels in the *ubp6-C118A, doa4Δ,* and *ubi1-3Δ* mutants was due specifically to the ubiquitin deficiency common to these mutants, and not to some unappreciated common characteristic of the three strains, we employed a yeast mutant in which all endogenous ubiquitin coding elements have been removed and replaced with a single galactose-inducible ubiquitin gene. This strain allows for precise shut-off of ubiquitin synthesis by switching the culture from a galactose-containing medium to one containing glucose. As described previously, cellular ubiquitin levels are rapidly depleted in this strain upon cessation of ubiquitin synthesis, reflecting ubiquitin turnover (Hanna et al., 2003; Figure 2B). In contrast, as ubiquitin levels declined over the course of the experiment, Ubp6 levels rose (Figure 2B). Again, levels of an unrelated control protein, eIF5A, showed no corresponding increase, indicating the specificity of the Ubp6 response. Together, the data of Figure 1 and Figure 2 indicate a robust cellular pathway for regulating Ubp6 that appears to respond precisely and specifically to the amount of ubiquitin in the cell. Ubiquitin deficiency, whether it arises through defects in biosynthesis or through aberrant degradation by either the proteasome or the vacuole, appears to be a sufficient signal for upregulation of Ubp6.

A third deubiquitinating enzyme, Ubp14, was of interest because of its known role in ubiquitin homeostasis. In contrast to *ubp6Δ* and *doa4Δ* mutants, *ubp14Δ* mutants are not significantly deficient in either free or total ubiquitin; rather, *ubp14Δ* mutants accumulate unanchored polyubiquitin species (Amerik et al., 1997; see also Figure S2). We examined Ubp6 levels in whole-cell extracts of these mutants, and we were unable to detect significant induction of the Ubp6 protein (Figure S2).

In addition to *ubp6Δ*, other yeast mutants, including *ufd3Δ* and as described above *doa4Δ*, are known to be deficient in ubiquitin (Swaminathan et al., 1999; Johnson et al., 1995). We therefore sought to determine whether Doa4 or Ufd3 might be subject to a similar pathway of

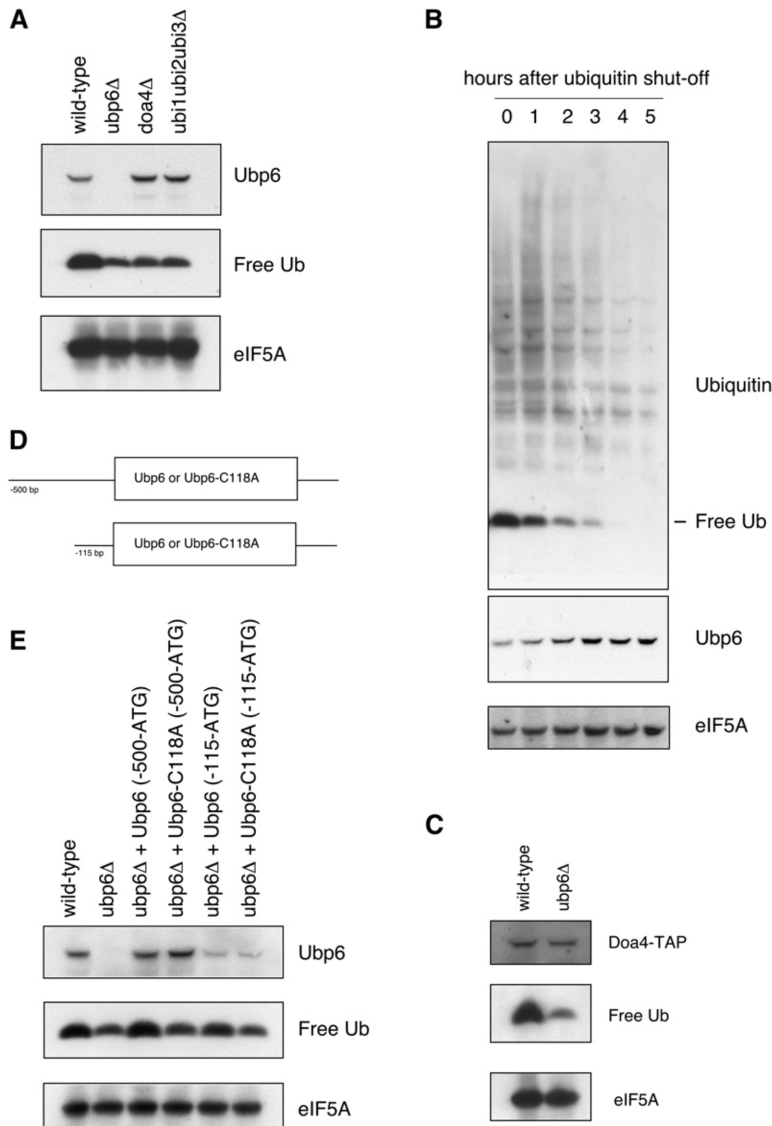

Figure 2. Endogenous Ubp6 Is Regulated by Ubiquitin Levels

(A) Cellular levels of endogenous Ubp6 in the ubiquitin-deficient mutants *doa4Δ* and *ubi1-3Δ*. Middle panel, free ubiquitin; lower panel, eIF5A, which serves as a loading control.

(B) Cellular levels of endogenous Ubp6 after cessation of ubiquitin synthesis.

(C) Cellular levels of TAP-Doa4 in response to ubiquitin deficiency conferred by deletion of *UBP6.* TAP-tagged constructs were detected by immunoblotting, using a colorimetric assay involving horseradish peroxidase and antibodies to peroxidase.

(D) Schematic of Ubp6 expression constructs used in (E).

(E) Cellular levels of free ubiquitin are affected by reduction in Ubp6 levels. Upper panel, Ubp6; lower panel, eIF5A, which serves as a loading control. Strains: sJH152–sJH155, sJH285–sJH286.

ubiquitin-dependent regulation. We utilized strains bearing TAP-tagged versions of Doa4 and Ufd3, respectively, and rendered these strains ubiquitin deficient by deletion of *UBP6*. However, we were unable to detect ubiquitin-dependent induction of either TAP-Doa4 (Figure 2C) or TAP-Ufd3 (data not shown), indicating some degree of specificity of the ubiquitin-dependent pathway of Ubp6 induction.

Modulation of Ubiquitin Levels by Changes in Cellular Abundance of Ubp6

The preceding data demonstrate that Ubp6 levels respond sensitively to cellular ubiquitin levels. Whereas previous work has demonstrated that the presence or absence of Ubp6 dramatically affects ubiquitin levels (Leggett et al., 2002; Chernova et al., 2003), we also sought to determine whether incremental changes in Ubp6 levels could in turn affect cellular ubiquitin abundance. We artificially reduced expression of Ubp6 by removing a large portion of its promoter sequence (Figure 2D). Such promoter truncation also abolished ubiquitin-dependent upregulation of Ubp6 (Figure 2E). When we examined ubiquitin levels in these strains, we observed that reduction in Ubp6 levels was matched with corresponding reduction in free and total ubiquitin levels (Figure 2E, and data not shown).

Ubiquitin-Dependent Upregulation of Ubp6 Is Mediated via Control of Gene Expression

An increase in abundance of Ubp6 protein could be achieved through either an increase in synthesis or a decrease in degradation. To address this point, we followed *UBP6* RNA in cells responding to ubiquitin depletion. We utilized the *doa4Δ* and *ubi1-3Δ* mutants from Figure 2 and observed a significant increase in wild-type *UBP6* RNA levels in both of these mutants, as determined by northern

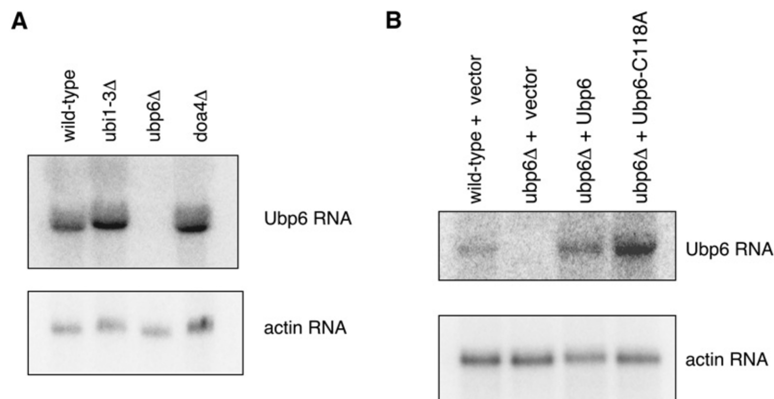

Figure 3. Ubiquitin-Dependent Induction of Ubp6 Is Transcriptional

(A) Cellular levels of *UBP6* RNA in the ubiquitin-deficient mutants, *doa4Δ* and *ubi1-3Δ*, as determined by northern blotting. Actin RNA serves as a loading control.

(B) Cellular levels of *UBP6* and *UBP6-C118A* RNA. Strains: sJH152–sJH155.

blotting (Figure 3A). Consistent with these findings, when we employed the *ubp6-C118A* mutant, which is ubiquitin-deficient by virtue of the loss of Ubp6 catalytic activity, we observed increased *ubp6-C118A* RNA levels as compared with either a wild-type strain or a *ubp6Δ* strain complemented with the wild-type Ubp6 plasmid (Figure 3B; for quantitation, see Figure S3). These data indicate that the observed ubiquitin-dependent upregulation of Ubp6 protein is mediated, at least in part, at the RNA level.

We next sought to determine whether a decrease in the rate of degradation of the Ubp6 protein could also contribute to an increase in Ubp6 levels. To this end, we developed a yeast strain in which the endogenous *UBP6* promoter has been replaced by the *GAL1* promoter. This allows for precise shut-off of Ubp6 synthesis, analogous to the ubiquitin shut-off described in the experiment of Figure 2B. After cessation of Ubp6 synthesis, we were unable to observe a significant decrease in Ubp6 abundance over a 3 hr time course, indicating that the Ubp6 protein is relatively stable (data not shown). Additionally, studies of the human ortholog of Ubp6, known as Usp14, did not reveal significant turnover of that protein (Borodovsky et al., 2001).

Upregulation of Ubp6 Increases Proteasomal Ubp6 Content

It is thought that the major known functions of Ubp6 are carried out at the proteasome because deletion of Ubp6's proteasome binding domain (known as the Ubl domain) recapitulates the null phenotype in every context studied to date (Leggett et al., 2002; Chernova et al., 2003; Hanna et al., 2006). Furthermore, because Ubp6 is dispensable for the overall structural integrity of the proteasome, its abundance on the proteasome could in principle be varied in response to cellular demands. We therefore sought to determine whether increases in total cellular Ubp6 levels resulted in corresponding increases in the amount of Ubp6 on proteasomes. Proteasomes were affinity purified (Leggett et al., 2002) from the ubiquitin-deficient *doa4Δ* mutant, and compared to those of wild-type yeast. The overall electrophoretic profiles of the two proteasome preparations were similar (Figure 4A). It is difficult to visualize Ubp6 by standard Coomassie staining because it comigrates electrophoretically with the proteasome subunit Rpn3. However, when we examined the abundance of Ubp6 in these preparations by immunoblotting, we detected an increased amount of Ubp6 in proteasomes from the *doa4Δ* strain (Figure 4B). This difference was specific for Ubp6, as levels of a different proteasome subunit, Rpn12, were comparable between preparations (Figure 4B).

We next examined proteasomes purified from a strain expressing Ubp6-C118A. When compared with proteasomes from either a wild-type strain or a *ubp6Δ* strain complemented with a wild-type Ubp6 plasmid, proteasomes from the *ubp6-C118A* mutant displayed a greater amount of Ubp6 protein (Figure 4C; for quantitation, see Figure S4). Again, this result was specific for Ubp6, as other proteasome subunits, such as Rpn12, were not similarly affected (Figure 4C). In the case of Ubp6-C118A, the increase in Ubp6 association with the proteasome appeared to be greater than that associated with proteasomes from the *doa4Δ* strain. The reason for this difference remains unclear. Because the Ubp6-C118A protein is devoid of catalytic activity, its increased accumulation on proteasomes might reflect a futile and thus more persistent cellular response to restore ubiquitin levels.

The data of Figures 4B and 4C indicate that proteasomes from wild-type cells are not saturated with Ubp6. Consistent with this model, when matrix-immobilized purified proteasomes from wild-type cells were challenged with an excess of purified, bacterially produced Ubp6, levels of proteasome-bound Ubp6 increased significantly, with the increase comparable to that seen in Figure 4C (data not shown). Whether the extent of proteasome loading by Ubp6 is controlled by means other than *UBP6* gene expression remains to be evaluated.

Subunit-Specific Regulation of the Proteasome

In yeast, the synthesis of proteasome subunits, over 30 in total, is thought to be coordinated through a specific pathway of transcriptional control. All known proteasome genes, including *UBP6*, contain a *cis*-acting upstream sequence of eight nucleotides known as the Proteasome-Associated Control Element (PACE) (Mannhaupt et al., 1999; Leggett et al., 2002). This regulatory element is

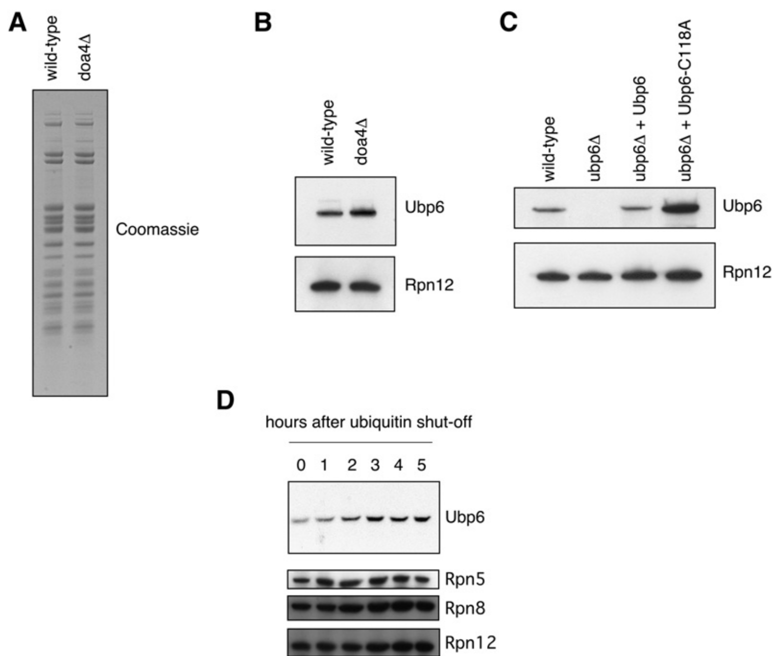

Figure 4. Induction of Ubp6 Increases Proteasomal Ubp6 Content

(A) Coomassie blue staining of proteasomes purified from wild-type and ubiquitin-deficient *doa4Δ* strains.

(B) Proteasomes from (A) were analyzed by immunoblotting with antibodies against Ubp6 (upper panel) and proteasome subunit Rpn12 (lower panel).

(C) Proteasomal abundance of Ubp6 and Ubp6-C118A. Proteasomes were purified and analyzed by immunoblotting, as in (B). Strains: sJH175–sJH178.

(D) Cellular levels of Ubp6 and proteasome subunits Rpn5, Rpn8, and Rpn12 after cessation of ubiquitin synthesis. The Ubp6 panel is taken from the experiment of Figure 2B.

recognized by the zinc-finger transcriptional activator Rpn4, which is itself short-lived by virtue of its rapid destruction by the proteasome (Xie and Varshavsky, 2001). This system is thought to maintain an adequate level of proteasome function via a negative feedback circuit. Under conditions of adequate proteasome function, Rpn4 is rapidly degraded and thus present at a low level of abundance. When proteasome function is compromised, Rpn4 is stabilized and promotes proteasome synthesis via transcription of proteasome genes. The increase in proteasome function in turn leads to destruction of the previously stabilized Rpn4 protein, thereby maintaining proteasome homeostasis (Xie and Varshavsky, 2001).

The data from Figures 4B and 4C suggest a novel pathway of proteasome regulation inasmuch as the stoichiometry of Ubp6 on proteasomes changed relative to that of other subunits. In contrast to the concerted regulation of the proteasome as a single entity by Rpn4, it appears that proteasome function can be modulated by specific regulation of a single proteasome component. To substantiate this idea, we again utilized the technique of ubiquitin shut-off to determine whether the observed changes in cellular Ubp6 levels reflected a Ubp6-specific response or whether they were simply a part of a larger program of proteasome regulation. As shown in Figure 4D, the dramatic increase in Ubp6 levels that occurs as ubiquitin levels decline is not accompanied by corresponding increases in the cellular levels of three other proteasome subunits, Rpn5, Rpn8, and Rpn12. Supporting this conclusion, the steady-state levels of proteasome components other than Ubp6 also remained unchanged in ubiquitin-deficient strains such as *ubp6Δ* (see Figure 5A, below; Hanna et al., 2003; 2006) and *doa4Δ* (Figure S5). An independent study of *doa4Δ*, furthermore, did not detect significant induction of proteasome gene transcription or stabilization of Rpn4 protein, two hallmarks of proteasome stress (London et al., 2004). Therefore, the stress associated with ubiquitin deficiency does not signal a general proteasome stress response, but is addressed through a novel pathway of proteasome subunit-specific regulation.

Proteasome Stress in the *ubp6-C118A* Mutant

In the course of analyzing the *ubp6-C118A* mutant, we noticed that the cellular levels of proteasome subunits such as Rpn8 were elevated in comparison with those of a wild-type strain (Figure 5A). We previously reported that Ubp6 possesses both a catalytic deubiquitinating activity and a noncatalytic activity, the latter appearing to inhibit the proteasome directly (Hanna et al., 2006). It seems likely that increased proteasome levels in the *ubp6-C118A* mutant reflect this noncatalytic inhibition of the proteasome, especially as proteasome levels were not elevated in the *ubp6* null mutant (Figure 5A). Furthermore, whereas ubiquitin supplementation could restore the abundance of the Ubp6-C118A protein to wild-type levels, proteasome levels remained elevated in the *ubp6-C118A* mutant, even after complete ubiquitin restoration (Figure 5A). Thus, proteasome induction in the *ubp6-C118A* mutant represents a cellular response that is independent of both ubiquitin levels and the catalytic function of Ubp6.

To further substantiate proteasome induction in the *ubp6-C118A* mutant, we employed a more sensitive and quantitative assay of cellular proteasome levels. suc-LLVY-AMC is a fluorogenic peptide substrate of the proteasome that can be degraded in a manner independent of ubiquitin and the unfolding activities of the proteasome. Thus, even under conditions in which the capacity of the

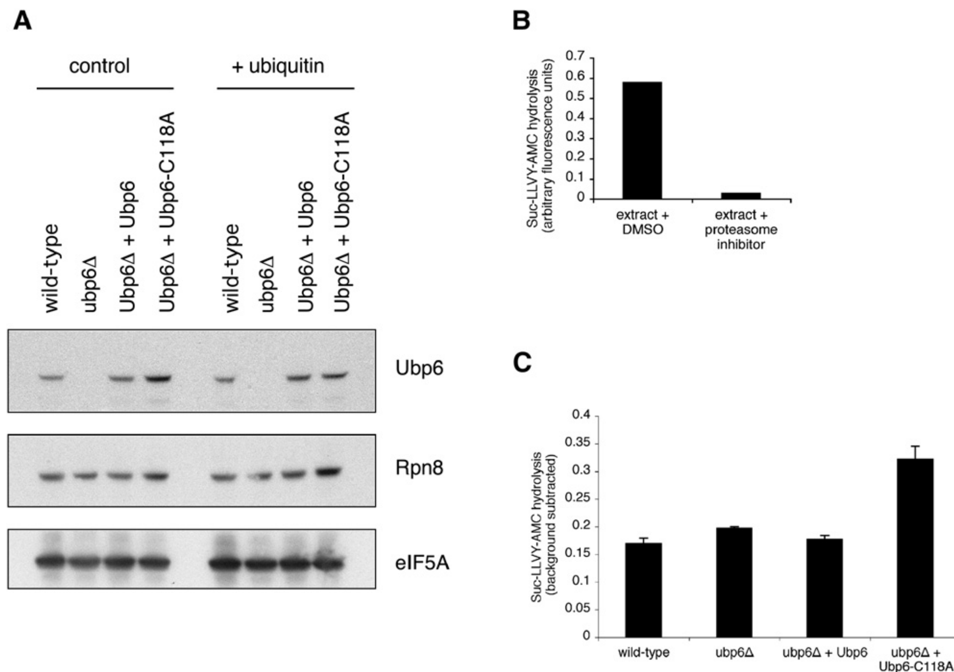

Figure 5. Proteasome Stress in the *ubp6-C118A* Mutant

(A) Cellular levels of proteasome subunit Rpn8 in wild-type, *ubp6Δ,* and *ubp6-C118A* strains (middle panel). Cellular levels of Ubp6 (top panel) are shown for reference. eIF5A (lower panel) serves as a loading control. Strains: sJH197–sJH204.

(B) suc-LLVY-AMC hydrolysis by wild-type whole-cell extracts pretreated with DMSO (control) or the proteasome inhibitor clasto-lactacystin-β-lactone (200 μM, dissolved in DMSO).

(C) suc-LLVY-AMC hydrolysis by whole-cell extracts (5 μg) from wild-type, *ubp6Δ,* and *ubp6-C118A* strains. The standard errors from an experiment carried out in duplicate are shown. Strains: sJH152–sJH155.

proteasome to degrade ubiquitinated proteins is compromised, such as is likely the case for the *ubp6-C118A* mutant, suc-LLVY-AMC can be used to assay proteasome activity and, by extension, proteasome levels. Indeed, suc-LLVY-AMC has been previously used in this way in whole-cell extracts to demonstrate decreased proteasome abundance in the *rpn4Δ* mutant (London et al., 2004).

To be certain that the suc-LLVY-AMC assay faithfully reported proteasome activity and not the activity of other factors capable of cleaving this peptide, we first determined the fraction of suc-LLVY-AMC hydrolysis in a whole-cell extract that could be attributed to proteasomes. Addition of the proteasome inhibitor clasto-lactacystin-β-lactone eliminated more than 95% of suc-LLVY-AMC hydrolysis (Figure 5B), indicating that this assay can faithfully report on proteasome activity. We next examined the *ubp6-C118A* mutant directly. Wild-type and *ubp6Δ* strains showed comparable activity against the peptide substrate; in contrast, strains expressing Ubp6-C118A displayed a strong increase in activity against suc-LLVY-AMC (Figure 5C), consistent with the immunoblotting data from Figure 5A. Additionally, neither Ubp6 (Hanna et al., 2006) nor Ubp6-C118A (Figure S6) had any effect on suc-LLVY-AMC hydrolysis activity by purified proteasomes, indicating that increased suc-LLVY-AMC hydrolysis in whole-cell extracts of *ubp6-C118A* mutants was unlikely to be due to proteasome activation by Ubp6-C118A. Thus, a noncatalytic function of Ubp6, most likely proteasome inhibition, engenders a robust compensatory proteasome stress response by the cell. In addition, these results imply that the previously reported noncatalytic inhibition of the proteasome by Ubp6-C118A in vivo is compensated and therefore likely to represent an underestimate of the true proteasome inhibitory capacity of Ubp6 (Hanna et al., 2006).

Proteasome Compensation in the *ubp6-C118A* Mutant Is Mediated by Rpn4

Given the known role of Rpn4 in proteasome regulation, especially in response to stress, we examined how the loss of Rpn4 would affect the *ubp6-C118A* mutant. When *ubp6-C118A* was introduced into an *rpn4Δ* mutant, a dramatic defect in growth was observed (Figure 6A). The *rpn4Δ ubp6Δ* mutant alone showed no such growth defect, indicating that the behavior of the *rpn4Δ ubp6-C118A* mutant could not be attributed to ubiquitin deficiency or loss of the enzymatic activity of Ubp6 (Figure 6A). Importantly, in an *RPN4*-positive genetic background, the *ubp6Δ* and *ubp6-C118A* mutants showed comparable levels of growth (Figure 6A), suggesting that the less marked phenotype of the *ubp6-C118A* mutant under nonstress

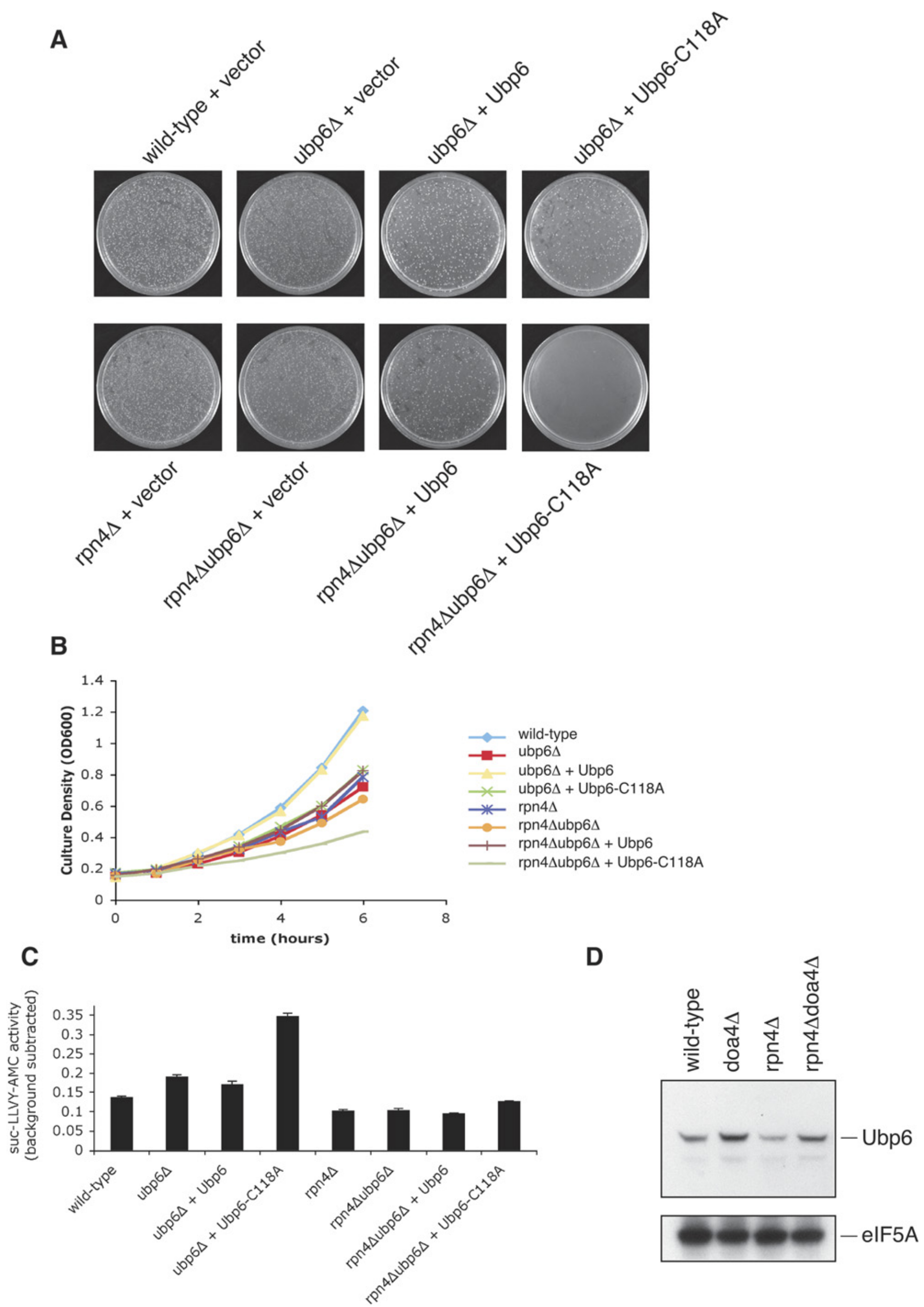

Figure 6. Rpn4-Mediated Cellular Compensation in the *ubp6-C118A* Mutant

(A) Primary transformants of the indicated strains were plated, grown for 2 days at 30°C, and photographed. Strains: sJH261–sJH268.

(B) Liquid media growth curves for the strains shown in (A). (After 4–5 days of growth, *rpn4Δ ubp6-C118A* do form colonies, albeit smaller colonies than any of the other seven strains shown.)

(C) suc-LLVY-AMC hydrolysis by whole-cell extracts (10 μg) prepared from the strains shown in (A) and (B). The standard errors from an experiment carried out in duplicate are shown.

(D) Cellular levels of endogenous Ubp6 in an *rpn4Δ doa4Δ* mutant. eIF5A serves as a loading control in the lower panel. Strains: SUB62, sJH213, 3716, and sJH218.

conditions is achieved only after significant cellular compensation.

The *rpn4Δ ubp6-C118A* strain shown in Figure 6A, if allowed to grow longer, will eventually form colonies, although smaller ones than those of the other strains shown. We collected such colonies and measured their growth rates in liquid media. In an *RPN4*-positive background, the *ubp6Δ* and *ubp6-C118A* mutants displayed similar rates of growth. In contrast, in the *rpn4Δ* background, the rate of growth of the *ubp6-C118A* mutant was significantly slower than that of the *ubp6Δ* (Figure 6B), just as in Figure 6A. This disparity in growth rates might represent an underestimate of the difference, because it cannot be excluded at present that upon propagation the *rpn4Δ ubp6-C118A* transformants adapt to cellular stress imposed by those combined mutations through unknown mechanisms.

It remained unclear whether the difference in growth rates between the *ubp6-C118A* and *rpn4Δ ubp6-C118A* mutants reflected true compensation by Rpn4 for deficient proteasome activity due to the presence of Ubp6-C118A or whether a uniform reduction of proteasome levels by deletion of *RPN4* simply reduced proteasome activity in the *ubp6-C118A* mutant below a critical threshold. To distinguish between these possibilities, we examined suc-LLVY-AMC activities in these strains. In an *RPN4*-positive background, we again noticed a strong increase in peptide hydrolysis in the presence of Ubp6-C118A; in contrast, when *RPN4* was deleted, not only did all strains display a lower basal level of proteasome activity, but the compensatory increase in suc-LLVY-AMC hydrolysis in the *ubp6-C118A* mutant was almost completely abrogated (Figure 6C). Thus, Rpn4 is responsible for compensatory proteasome induction in response to Ubp6-C118A.

Our data describe two cellular pathways of regulation within the ubiquitin-proteasome system, one controlling ubiquitin levels and the other controlling proteasome levels. We wished to determine whether these two pathways were controlled by the same factor or by different factors. Rpn4 mediates proteasome synthesis in the *ubp6-C118A* mutant. We therefore sought to determine whether Rpn4 might also modulate ubiquitin-dependent induction of Ubp6, especially as Ubp6 contains a putative Rpn4 binding site in its promoter (Leggett et al., 2002). As shown in Figure 2, ubiquitin deficiency arising from deletion of the *DOA4* gene is sufficient to elevate Ubp6 protein levels. We therefore constructed an *rpn4Δ doa4Δ* double mutant, which is significantly ubiquitin deficient compared with the *rpn4Δ* mutant alone (data not shown). Overall levels of Ubp6 were depressed in both the *rpn4Δ* and *rpn4Δ doa4Δ* mutants, as expected, but considerable compensatory induction of Ubp6 was observed in the double mutant (Figure 6D). This result indicates that Rpn4 is not necessary for the ubiquitin-dependent pathway of Ubp6 regulation. Rather, it seems likely that the two pathways of cellular regulation that control ubiquitin and proteasome levels, respectively, are carried out by distinct systems of gene regulation (see Figure 7).

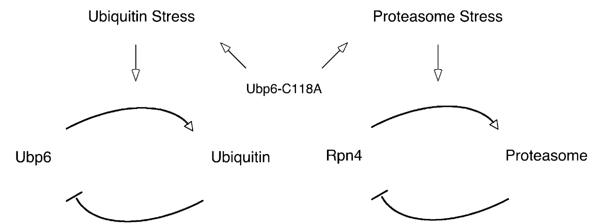

Figure 7. Distinct Cellular Responses to Ubiquitin Stress and Proteasome Stress
Homeostatic control of ubiquitin and proteasome levels, respectively, by pathways which appear to be largely independent. Regulated proteasome synthesis is mediated by the transcription factor Rpn4, which is itself a substrate for degradation by the proteasome. Ubp6 spares ubiquitin from proteasomal destruction, and is itself subject to regulated gene expression by ubiquitin. This is an abbreviated representation of the feedback loop, which must involve additional, so far unknown, components. The mutant *ubp6-C118A* simultaneously induces ubiquitin stress and proteasome stress, highlighting the key role of Ubp6 in regulating both ubiquitin levels and proteasome function.

DISCUSSION

Ubiquitin-Dependent Regulation of Ubp6

Free ubiquitin levels are subject to major environmentally induced fluctuations, even in wild-type cells (Rose and Warms, 1987; Mimnaugh et al., 1997). Thus, the existence of mechanisms that sense and correct transient inactivation of this pathway is a reasonable expectation. Perturbations of ubiquitin levels could compromise cell function in many respects because of the unusual scope of the ubiquitin-dependent regulatory pathways. For example, ubiquitination is critical to the control of processes such as cell cycle regulation, transcription, cell surface receptor regulation, protein quality control, apoptosis, DNA repair, and protein sorting.

Ubiquitin deficiency is known to sensitize yeast cells to a variety of chemical and environmental stresses, including those associated with heat, protein misfolding, DNA damage, exposure to heavy metals, inhibition of translation, and starvation (Finley et al., 1987; Chernova et al., 2003; Hanna et al., 2003). In higher eukaryotes, ubiquitin deficiency is associated with disease in the *ataxia* and the gracile axonal dystrophy (*gad*) mice (Wilson et al., 2002; Saigoh et al., 1999). The former is caused by mutation in murine Ubp6, known as Usp14, while the latter is due to mutation in another deubiquitinating enzyme, Uch-L1, which is also known to regulate ubiquitin levels (Osaka et al., 2003).

The results presented here describe a novel pathway of ubiquitin homeostasis that appears to modulate ubiquitin levels not through regulation of ubiquitin synthesis, but rather through regulation of ubiquitin degradation. The proteasome-associated deubiquitinating enzyme Ubp6 functions to spare ubiquitin from degradation by the proteasome, apparently by releasing ubiquitin from ubiquitin conjugates, which are the major substrates of the proteasome (Leggett et al., 2002). The absence of Ubp6 results in

the rapid depletion of cellular ubiquitin (Leggett et al., 2002). However, even in the presence of Ubp6, ubiquitin displays a significant rate of degradation (Ryu et al., 2006; Hanna et al., 2003), suggesting that the proteasome is not fully efficient in recycling ubiquitin under steady-state conditions. Furthermore, because Ubp6 is not an integral subunit of the proteasome, and thus not required for its structural integrity, the possibility exists that the function of the proteasome could be altered simply by altering the abundance of Ubp6. Considerable evidence now supports this model: when ubiquitin levels decline, either due to decreased synthesis or increased degradation, the abundance of cellular Ubp6 rises. Conversely, elevated levels of Ubp6 associated with ubiquitin deficiency can be reversed simply by restoring ubiquitin. These results emphasize the physiological and regulatory significance of ubiquitin degradation and the crucial role of ubiquitin recycling in maintaining ubiquitin homeostasis.

The experiments presented here point to a new paradigm in the regulation of the ubiquitin pathway: a ubiquitin stress response. The physiologic relevance and efficaciousness of this stress response are suggested by the ubiquitin-suppressible hypersensitivity of *ubp6* null mutants to stresses that are very diverse in nature, and include canavanine, anisomycin, cycloheximide, chloramphenicol, trichodermin, methotrexate, cadmium, 4NQO, and methylmethanesulfonate (Chernova et al., 2003; Hanna et al., 2003). Even in wild-type cells, we have found, surprisingly, that sensitivity to many such treatments involves ubiquitin limitation (Hanna et al., 2003, 2006; Chen and Piper 1995). Thus, the ubiquitin stress response may represent a critical arm of the cellular stress-responsive regulatory system.

The induction of *UBI4* transcription in response to ubiquitin depletion is also known to occur (Swaminathan et al., 1999; London et al., 2004). However, it remains unclear whether or to what extent increased synthesis of new ubiquitin plays a corrective role. According to existing data, conditions that induce *UBI4* also typically repress the primary sources of ubiquitin under favorable conditions, *UBI1-3* (see, for example, Hanna et al., 2003; Finley et al., 1987), and there is little basis to judge the net effect of these opposing regulatory mechanisms. Some evidence suggests that there may indeed be no net effect (Swaminathan et al., 1999). Interestingly, *UBI4* is also known to be induced in proteasome hypomorphs in an Rpn4-dependent manner (London et al., 2004). The significance of this response remains unclear, especially as the overexpression of ubiquitin is actually toxic to at least some proteasome hypomorphs (London et al., 2004).

Subunit-Specific Regulation of the Proteasome

The proteasome, as it was originally defined, consists of a core group of essentially stoichiometric subunits that is highly resistant to dissociation (see, for example, Glickman et al., 1998). The development of affinity purification methods for the proteasome has recently allowed the identification of a number of more loosely associated proteasome components that are typically found at lower abundance compared with the canonical proteasome subunits (Verma et al., 2000; Leggett et al., 2002). In yeast, proteasome synthesis is known to be regulated in a coordinated manner, and the genes for every known proteasome subunit, as well as a number of the loosely associated components such as Ubp6, contain a conserved promoter sequence (PACE) that mediates the transcriptional activation of such genes (Mannhaupt et al., 1999; Leggett et al., 2002). Proteasome synthesis is induced in response to a variety of stimuli that presumably either decrease proteasome function or increase demand for proteasome function. Indeed, loss-of-function mutations in proteasome subunits themselves can potently induce the proteasome stress response (Ju et al., 2004; London et al., 2004). Such responses also occur in metazoans, including mammals, although the *cis*-acting elements and transcriptional mediators remain unidentified (see, for example, Meiners et al., 2003).

In the present work, we report that despite the widespread requirement for ubiquitin in proteasome-mediated degradation, ubiquitin stress does not engender an overt proteasome stress response. Interestingly, these two stress-response pathways must therefore be designed so as to distinguish between protein degradation defects arising from failure to ubiquitinate substrate and those arising from failure to degrade the ubiquitinated from of the substrate. Proteasome induction was not observed in response to ubiquitin depletion arising either from a cessation of ubiquitin synthesis or from accelerated ubiquitin degradation in *ubp6Δ* and *doa4Δ* mutants. Instead, the composition of the proteasome was altered in a more subtle manner through the relative accumulation of Ubp6 on proteasomes.

The proteasome has traditionally been viewed as a single functional entity in yeast, and proteasome regulation was thought to focus on control of its levels and not the nature of its activity. The data presented here support an alternate model in which proteasome function may be not only increased or decreased, but altered in more sophisticated ways to address variable cellular requirements. It seems less likely that the core stoichiometric subunits of the proteasome could serve such regulatory roles because of their requirement for overall proteasome structure and stability, although the activity of such subunits might still be regulated posttranslationally. In contrast, the reversibility of binding and typically substoichiometric representation of proteasome-associating factors make them ideal candidates for subunit-specific modulation of the proteasome. Ubp6 is clearly utilized by the cell to this end, but we suspect that other proteasome-associating proteins might similarly function as key mediators of proteasome regulation, with their levels being modulated to address other as of yet unappreciated cellular demands.

Evidence for a Ubiquitin Sensor

The precise regulation of Ubp6 in response to ubiquitin levels, and the fact that increases in Ubp6 are achieved,

at least in part, via a program of *UBP6* gene regulation, provide strong evidence for the existence of a cellular component capable of sensing ubiquitin levels. In principle, such a sensor could function either by binding ubiquitin or through covalent conjugation to ubiquitin itself. The data of Figure 1C suggest that ubiquitin sensing requires ubiquitin conjugation. The sensor is likely to be conjugated directly to ubiquitin or to bind a polyubiquitin structure. In the case of direct conjugation, the role of ubiquitin in promoting protein destruction could provide a straightforward negative feedback loop responsive to ubiquitin levels. However, an alternative scenario in which the sensor recognizes the C terminus of unconjugated ubiquitin cannot be excluded.

The identification of the putative ubiquitin sensor, as well other components of the transcriptional pathway regulating ubiquitin-dependent induction of Ubp6, represents a challenge for the future. Until these factors are identified, our understanding of the ubiquitin stress response will remain partial. In particular, defining the relevant *cis*-acting element in the *UBP6* promoter will allow testing of the physiological effects of *UBP6* induction in ubiquitin-deficient cells and facilitate searches for the corresponding transcriptional regulators.

Simultaneous Activation of Ubiquitin and Proteasome Stress Responses by *ubp6-C118A*

We previously reported that Ubp6, in addition to its catalytic deubiquitinating activity, possesses a noncatalytic activity capable of delaying substrate degradation by direct inhibition of the proteasome (Hanna et al., 2006). However, the scope and generality of such noncatalytic inhibition remained unclear. We now show that this noncatalytic inhibition, when divorced from Ubp6's catalytic activity in the *ubp6-C118A* mutant, has profound consequences for the entire ubiquitin-proteasome pathway in vivo. *ubp6-C118A* potently stimulates a proteasome stress response, and proteasome levels in such cells rise substantially. When the ability of cells to compensate for such proteasome inhibition is abrogated by loss of the proteasome stress response factor Rpn4, *ubp6-C118A* mutants suffer a marked reduction in growth rates. Together, these data indicate that the effect of noncatalytic proteasome inhibition by Ubp6 is unlikely to be highly restricted in scope.

The induction of proteasome subunits in the *ubp6-C118A* mutant, as well as the reduction in its growth rate when combined with an *rpn4Δ* mutant, are not observed in the *ubp6Δ* mutant. These results are consistent with our previous assignment of Ubp6 as a proteasome inhibitor, the complete absence of which accentuates proteasome function (Hanna et al., 2006). More importantly, these particular nonequivalencies between the *ubp6-C118A* mutant and both the wild-type and *ubp6Δ* strains suggest that proteasome stress observed in the *ubp6-C118A* mutant derives from the uncoupling of noncatalytic inhibition from catalytic function. This would imply that the presence of distinct catalytic and noncatalytic functions in Ubp6 is not fortuitous. Rather, it appears that the two activities of Ubp6 are functionally related, and that noncatalytic inhibition of the proteasome may subserve Ubp6's catalytic function. When catalytic function is abrogated, the proteasome is subject to persistent noncatalytic inhibition, and may not be able to engage a new substrate until either release from noncatalytic inhibition or dissociation of the stalled substrate from the proteasome. The dissociative pathway could be promoted by substrate deubiquitination, possibly representing a key role of Ubp6's catalytic activity.

The work presented here describes two pathways of cellular regulation, which we have referred to as the ubiquitin stress response and the proteasome stress response, respectively (see Figure 7). The ubiquitin stress response is distinct from the proteasome stress response; in particular, the principal mediator of the proteasome stress response, Rpn4, does not appear to control the ubiquitin stress response. Though such a two-fold stress regulation in the ubiquitin-proteasome pathway has not been anticipated, it is perhaps a logical solution. When ubiquitin levels are dangerously low, it would not be advantageous to increase proteasome levels, as this would likely exacerbate ubiquitin depletion. Moreover, it is intriguing that the degradation of Rpn4 has the rare feature of having a strong ubiquitin-independent component (Xie and Varshavsky, 2001); thus Rpn4 seems to respond rather specifically to the state of the proteasome rather than ubiquitin levels, even though ubiquitin levels might be more subject to fluctuation physiologically (Rose and Warms, 1987; Mimnaugh et al., 1997; Hanna et al., 2003). The *ubp6-C118A* mutant may provide an exceptional case of regulation, where the ubiquitin stress response and proteasome stress response are triggered simultaneously (Figure 7). The joint induction of these pathways reflects the key role of Ubp6 in their regulation. It is possible that, at any given time, only one or the other of the two pathways will be significantly induced under physiologically relevant environmental stresses.

EXPERIMENTAL PROCEDURES

Yeast Strains

See Table S1. Standard techniques were used for strain constructions and transformations. Yeast were cultured at 30°C. YPD medium consisted of 1% yeast extract, 2% Bacto-peptone, and 2% dextrose. YPRafGal consisted of 1% yeast extract, 2% Bacto-peptone, 2% raffinose, and 2% galactose. Synthetic medium consisted of 0.7% Difco Yeast Nitrogen Base supplemented with amino acids, adenine, uracil, and 2% dextrose. Yeast plasmids are described in Table S2. Ubiquitin expression was carried out in the absence of copper induction. Under these conditions, free ubiquitin levels are elevated by a factor of approximately two in the wild-type (see, for example, Figure 1).

Preparation of Whole-Cell Extracts for Immunoblotting

Logarithmically growing cells were harvested and resuspended in 1X-Laemmli loading buffer at a concentration of 2×10^7 cells/50 μl loading buffer. Samples were immediately boiled and analyzed by SDS-PAGE followed by immunoblotting. Wherever possible, blots were stripped and reprobed to minimize error due to sample loading.

For ubiquitin shut-off experiments utilizing the SUB328 strain, cells growing logarithmically in YPRafGal were harvested, washed twice in water, and resuspended in YPD. An equivalent number of cells was collected at the indicated time points and processed as described above.

Analysis of *UBP6* RNA

Total RNA was prepared from logarithmically growing cells, separated by agarose gel electrophoresis, and transferred to a Hybond-N+ membrane (Amersham Biosciences) using the Turboblotter™ System Kit (Whatman Schleicher & Schuell). ^{32}P-dUTP-labeled PCR fragments (600 bp) from the *UBP6* and *ACT1* genes, respectively, were used for blotting. For Ubp6 blots, 25 μg of RNA were analyzed; for actin blots, 5 μg of RNA were analyzed.

Proteasome Purification

Proteasome purification was carried out as previously described (Hanna et al., 2006; Leggett et al., 2002).

suc-LLVY-AMC Assays

Logarithmically growing cells were harvested and resuspended in buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5 mM $MgCl_2$, and 1 mM ATP). Cells were lysed by French press and clarified by centrifugation in an SS-34 rotor for 30 min at 16,000 × g. Extracts were normalized by protein concentration and incubated with suc-LLVY-AMC (100 μM) for 10 min at 30°C. Reactions were stopped by the addition of 1% SDS, and the extent of suc-LLVY-AMC hydrolysis was measured using a fluorimeter.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/129/4/747/DC1/.

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