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Deubiquitinating Enzyme Ubp6 Functions Noncatalytically to Delay Proteasomal Degradation

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SUMMARY

Ubiquitin chains serve as a recognition motif for the proteasome, a multisubunit protease, which degrades its substrates into polypeptides while releasing ubiquitin for reuse. Yeast proteasomes contain two deubiquitinating enzymes, Ubp6 and Rpn11. Rpn11 promotes protein breakdown through its degradation-coupled activity. In contrast, we show here that Ubp6 has the capacity to delay the degradation of ubiquitinated proteins by the proteasome. However, delay of degradation by Ubp6 does not require its catalytic activity, indicating that Ubp6 has both deubiquitinating activity and proteasome-inhibitory activity. Delay of degradation by Ubp6 appears to provide a time window allowing gradual deubiquitination of the substrate by Ubp6. Rpn11 catalyzes en bloc chain removal, and Ubp6 interferes with degradation at or upstream of this step, so that degradation delay by Ubp6 is accompanied by a switch in the mode of ubiquitin chain processing. We propose that Ubp6 regulates both the nature and magnitude of proteasome activity.

INTRODUCTION

The ubiquitin-proteasome system is the major pathway for intracellular protein degradation in eukaryotes. Substrates of this pathway acquire a covalently linked polyubiquitin chain through the action of a cascade of ubiquitin activating and conjugating enzymes. This ubiquitin chain serves as the recognition motif for a multisubunit protease known as the proteasome, which processively degrades the substrate into polypeptides while releasing ubiquitin for reuse (Pickart and Cohen, 2004).

The proteasome is an approximately 2.5 MDa protein complex consisting of at least 33 distinct subunits in yeast (Pickart and Cohen, 2004). Its proteolytic active sites are housed at the center of the structure in a barrel-shaped subcomplex (Groll et al., 1997) known as the core particle (CP, or 20S complex). At either axial end of the CP, a second subcomplex known as the regulatory particle (RP, or 19S complex or PA700) may bind. The RP comprises two subcomplexes of its own: the base and the lid (Glickman et al., 1998). The base is proximal to the CP and contains, among other proteins, six ATPases that are thought to form a ring structure that abuts the CP.

The proteasome displays a number of characteristics unusual for a protease. First, its proteolytic active sites are sequestered within the hollow cylindrical chamber of the CP. Providing access to this internal chamber are narrow gates at either end of the CP, and gating appears to be modulated by the Rpt2 subunit of the base (Pickart and Cohen, 2004). These features presumably serve to prevent the unregulated destruction of intracellular proteins, and also impose a requirement for substrate unfolding, as most folded proteins are too large to pass through the open translocation channel leading to the CP. Protein unfolding is apparently mediated by the six ATPases of the base. Multiple ubiquitin receptors have been identified, some of which are core proteasomal subunits while others are substoichiometric proteasome-associating factors (Elsasser and Finley, 2005). At some point after recognition, the ubiquitin chain is removed from the substrate to facilitate substrate degradation and to minimize degradation of ubiquitin. This function is carried out by deubiquitinating enzymes that reside in the proteasome.

In budding yeast *S. cerevisiae*, at least two deubiquitinating enzymes are thought to contribute to deubiquitination by the proteasome: Rpn11 and Ubp6 (Leggett et al., 2002; Verma et al., 2002; Yao and Cohen, 2002; Maytal-Kivity et al., 2002; Chernova et al., 2003; Guterman and Glickman, 2004). Rpn11, a metalloprotease, is a core structural component of the lid. Point mutations in its metal-coordinating site impair substrate degradation,

indicating a positive role for Rpn11 in protein degradation (Verma et al., 2002; Yao and Cohen, 2002).

The second proteasomal deubiquitinating enzyme, Ubp6, has been classified as a proteasome-associated protein, largely on the basis of its ready dissociation from the proteasome in the presence of high salt concentrations (Leggett et al., 2002). Ubp6, a cysteine protease, is an abundant component of proteasomes (Verma et al., 2000; Leggett et al., 2002), and in contrast to Rpn11, Ubp6 associates with the base. An N-terminal ubiquitin-like domain (Ubl) of Ubp6 mediates this interaction. Binding of Ubp6 to the proteasome activates Ubp6's catalytic activity over 300-fold (Leggett et al., 2002), indicating an intimate functional relationship between Ubp6 and the proteasome. However, the precise role of Ubp6 in proteasome function has remained uncertain. Some reports have assigned Ubp6 little or no role in proteasome-mediated deubiquitination or degradation, while others have argued that Ubp6, like Rpn11, facilitates proteasome-mediated degradation.

Here, we describe the first detailed analysis of how Ubp6 affects the degradation of a physiological substrate of the proteasome. We report that Ubp6 delays the breakdown of proteins by the proteasome and that a major component of its inhibitory effect is noncatalytic in nature. Negative regulation of degradation by Ubp6 was observed in vivo and in vitro, and on different test substrates. During the degradation delay that Ubp6 imposes, substrate deubiquitination proceeds on proteasomes, but the mode of deubiquitination is altered from that seen in the absence of Ubp6: degradation-linked "en bloc" chain removal by Rpn11 is replaced by progressive deubiquitination by Ubp6. Thus, Ubp6 uses catalytic and noncatalytic mechanisms to modulate proteasome function, and the coordinated activity of multiple proteasomal deubiquitinating enzymes controls substrate fate.

RESULTS

Ubp6 Inhibits the Degradation of Ubiquitinated Cyclin B

To study the potential role of Ubp6 in proteasome function, we developed an in vitro system for proteasome-mediated degradation of a model substrate. Ubiquitination of the short-lived cell cycle regulator cyclin B was achieved via a reaction requiring E1, Ubc4 (E2), immunopurified APC (E3), ubiquitin, and ATP (Kirkpatrick et al., 2006). Upon incubation of ubiquitinated cyclin B with proteasomes purified from *ubp6Δ* mutants, we observed that the rate of cyclin B degradation was much greater than that of wild-type proteasomes (Figure 1A). Significantly, the slower degradation of cyclin B exhibited by wild-type proteasomes was accompanied by progressive removal of ubiquitin groups from the substrate. We verified the absence of Ubp6 in proteasomes purified from the *ubp6Δ* strain (Figure 1B), and that the amounts of proteasome were comparable between the two preparations (Figure 1C).

We next sought to determine whether the observed difference in cyclin B degradation was due specifically to Ubp6. We examined proteasomes by Coomassie blue staining (Figure 1D) and native gel electrophoresis (data not shown) and found no evidence for either an unexpected compositional difference or a gross structural abnormality in mutant proteasomes (see also Guterman and Glickman, 2004). Furthermore, the activity of mutant proteasomes against the peptide substrate suc-LLVY-AMC, which is hydrolyzed in an RP-dependent but ubiquitin-independent manner, was comparable to wild-type (Figure 1E), indicating that delay of cyclin B degradation by Ubp6 was not due to suppression of the core proteolytic activity of the proteasome, nor to closing the gate into the CP. Finally, when bacterially expressed purified Ubp6 was added back to proteasomes lacking Ubp6, we observed a marked delay of cyclin B degradation (Figure 1F), indicating that Ubp6 itself is the inhibitory component of wild-type proteasomes. Inhibition of degradation by recombinant Ubp6 (Figure 1F) was accompanied by progressive deubiquitination, as seen with wild-type proteasomes (Figure 1A).

Rpn11 is known to promote deubiquitination and degradation by the proteasome (Verma et al., 2002; Yao and Cohen, 2002). To verify that Rpn11 was active in our system, we treated *ubp6Δ* proteasomes with the metal chelator o-phenanthroline (o-PA), an inhibitor of Rpn11. o-PA strongly inhibited deubiquitination and degradation of cyclin B (Figure 1G). In contrast, o-PA treatment had no effect on LLVY-hydrolysis by proteasomes (data not shown). Thus, in contrast to Ubp6, Rpn11 promotes cyclin B degradation.

In Vitro Confirmation of Degradation Delay by Ubp6

Chemical inhibitors of the proteasome were used to confirm that the rapid disappearance of cyclin B in the absence of Ubp6 represents cyclin B degradation by proteasomes as opposed to a possible unknown component of our samples. For these experiments, we utilized epoxomicin, an inhibitor of the proteolytic active sites of the CP. Epoxomicin preferentially targets the chymotrypsin-like activity of the CP; at high concentrations, such as used here, epoxomicin can inhibit all three proteolytic sites, although complete proteasome inhibition is generally not observed (Kisselev et al., 2006). The yield of deubiquitinated cyclin B reaction products from *ubp6Δ* proteasomes after a 10 min incubation was greatly increased by epoxomicin, verifying that disappearance of cyclin B involved proteasome-mediated degradation (Figure 2A). A diminished effect of epoxomicin was observed when the same experiment was carried out using proteasomes containing Ubp6, reflecting a reduced rate of cyclin B breakdown in the absence of epoxomicin (Figure 2A). These Ubp6-dependent differences could be recapitulated by endogenous Ubp6 present in wild-type proteasomes (Figure 2B).

The cyclin B used in our in vitro assay may be linked through not only lys-48 (K48) of ubiquitin but also K11

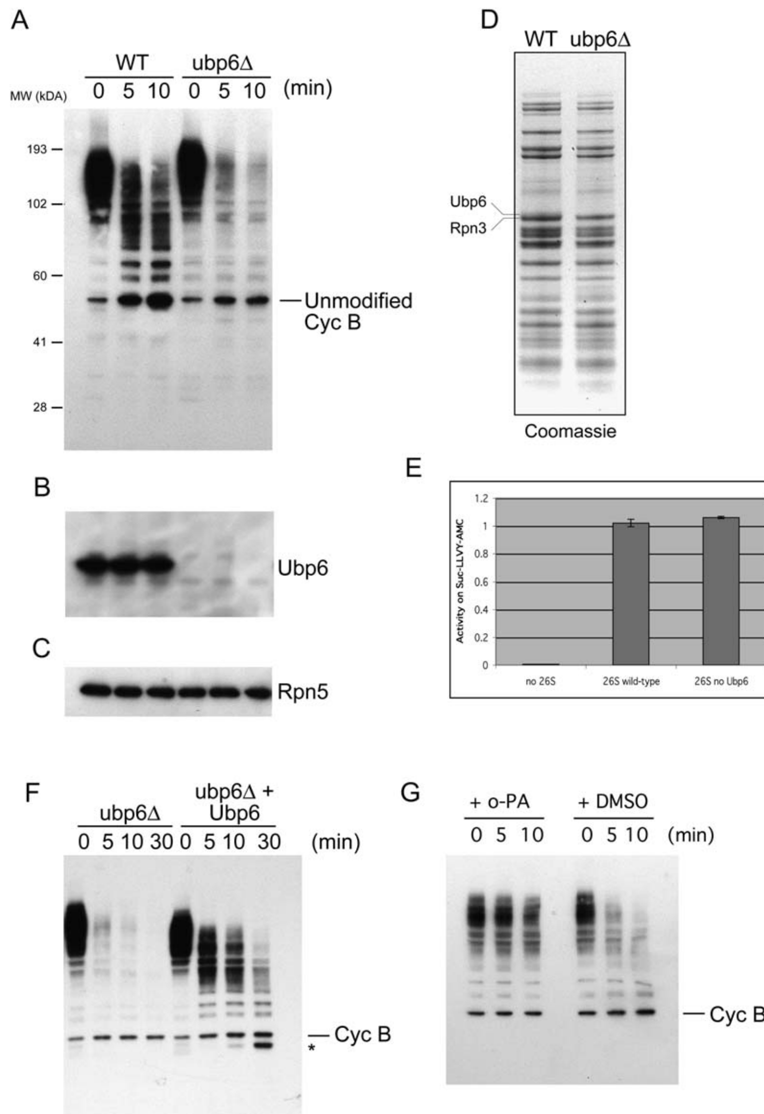


Figure 1. Ubp6 Inhibits Degradation of Ubiquitinated Cyclin B by Purified Yeast Proteasomes

(A–C) Processing of ub-cyclin B in vitro by wild-type or *ubp6Δ* proteasomes, visualized by immunoblot with antibodies to cyclin B1 (A), Ubp6 (B), or proteasome subunit Rpn5 (C).

(D) Comparison of wild-type and *ubp6Δ* proteasomes (10 μg), as determined by SDS-PAGE followed by Coomassie staining.

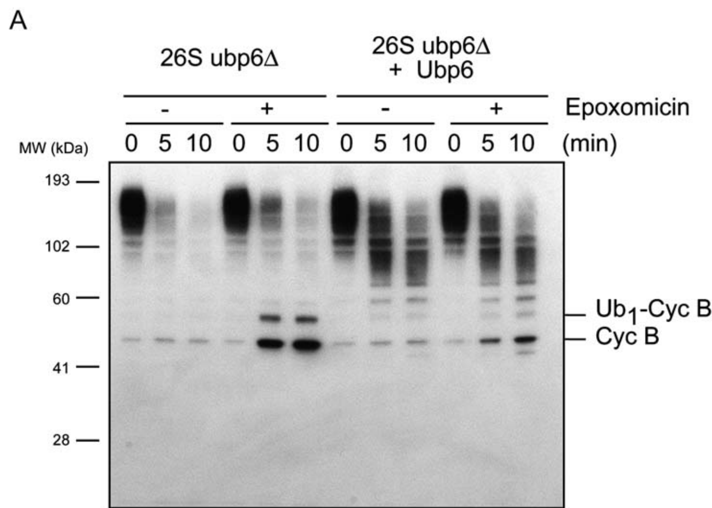
(E) Proteasome-dependent hydrolysis of suc-LLVY-AMC, a ubiquitin-independent fluorogenic proteasome substrate. Error bars reflect standard deviations.

(F) Processing of ub-cyclin B by proteasomes in the presence of purified recombinant Ubp6. Asterisk indicates a cyclin B species generated by residual thrombin activity deriving from recombinant Ubp6 purifications. Comparable Ubp6 add-back results were obtained with *ubp6Δ* proteasomes purified using a CP affinity tag (data not shown).

(G) O-phenanthroline (o-PA) inhibits cyclin B degradation by *ubp6Δ* proteasomes, implicating Rpn11's deubiquitinating activity in this process. o-PA, dissolved in DMSO, was added at 10 mM, 10 min prior to cyclin B.

and K63 (Kirkpatrick et al., 2006). We previously reported that K48 of ubiquitin was not required for in vitro degradation of ub-cyclin B (Kirkpatrick et al., 2006). We extend those results here with a side-by-side comparison of wild-type and *ubp6Δ* proteasomes; in both cases, proteasomes deubiquitinate and degrade cyclin B without regard for the presence of K48 ubiquitin linkages in its attached ubiquitin chains (Figure 2C). Thus, although K48-linked ubiquitin chains may be the dominant chain type promoting degradation in vivo, this linkage is not required for rapid proteasomal degradation in vitro. For many substrates, the requirement for K48 in protein degradation may reflect properties of the E3 enzymes involved in chain synthesis.

Finally, we noticed that for reactions lacking Ubp6, epoxomicin stabilized, in addition to unmodified cyclin B, a second major species, which is apparently monoubiquitinated cyclin B (Figures 2A–2C). These results suggest that in the absence of Ubp6, while a majority of ub-cyclin B is fully deubiquitinated by Rpn11 prior to degradation, a significant fraction is left incompletely deubiquitinated, and the resulting monoubiquitinated species is apparently rapidly degraded. In vivo, ubiquitin undergoes accelerated degradation by the proteasome in the absence of Ubp6 (Leggett et al., 2002; Hanna et al., 2003; Chernova et al., 2003; Figure 5D). We have proposed that when Ubp6 is absent, one or more substrate bound ubiquitin groups are translocated into the CP and degraded along with their substrate (Leggett et al., 2002), but until now there had been no biochemical basis to account for accelerated ubiquitin turnover. We propose that increased turnover of monoubiquitinated degradative intermediates as suggested by Figure 2 may contribute significantly to the rapid degradation of ubiquitin in vivo in the absence of Ubp6.



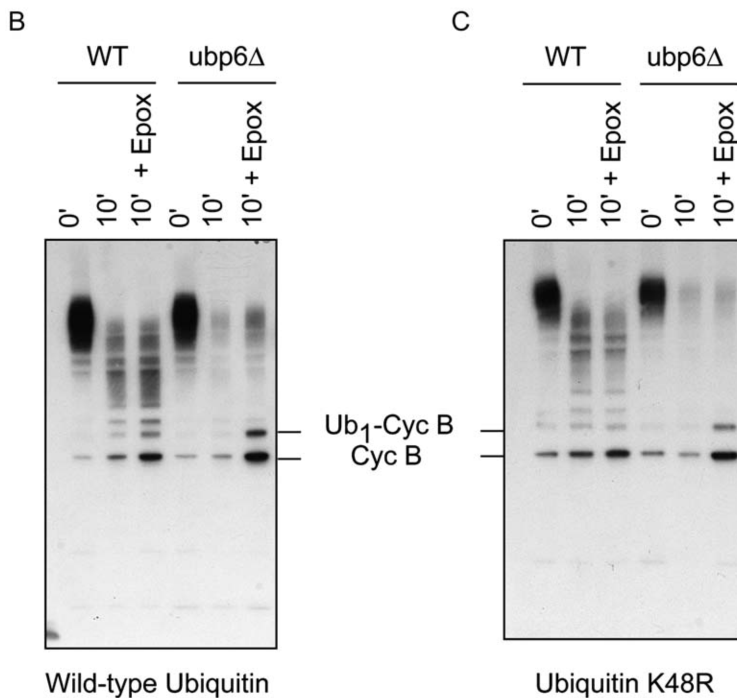


Figure 2. Inhibition of Cyclin B Degradation by Epoxomicin

(A) Effect of purified Ubp6 on proteasome-mediated ub-cyclin B processing in the presence or absence of epoxomicin (100 μM). The assignment of monoubiquitinated cyclin B is consistent with a previous study (Kirkpatrick et al., 2006).

(B and C) Processing of ub-cyclin B by wild-type and *ubp6Δ* proteasomes in the presence or absence of epoxomicin. Ub-cyclin B was synthesized with wild-type or K48R ubiquitin, as indicated.

Ubp6 Reduces Degradation Rates In Vivo

Our in vitro data predict accelerated degradation of at least some proteasomal substrates in vivo in the absence of Ubp6. To test this idea, we used chromosomal integration to render the biosynthetic enzymes Trp1 and Ura3 unstable by appending an N-terminal segment that directs these proteins to the N-end rule pathway for degradation (Varshavsky, 2005). Accordingly, growth of such strains in the absence of the relevant metabolite should reflect the rate of degradation of the respective reporter protein. Strains harboring proteolytic defects should stabilize the reporters, and thus display increased growth relative to wild-type. Ubr1, the E3 of the N-end rule pathway, governs ubiquitination of the reporter proteins. As expected, the *ubr1Δ* mutant displays a strong growth advantage over wild-type when cultured on media lacking tryptophan (Figure 3A) or uracil (data not shown).

Although such assays have typically been used to characterize degradation defects, we reasoned that if significant growth were detectable in wild-type strains, the assay could also be used to identify mutants with increased degradation rates. Indeed, when *ubp6Δ* mutants were tested, they displayed a dramatic growth defect relative to wild-type (Figure 3A), consistent with a proteasome hypermorphic effect. Additionally, a *ubr1Δ ubp6Δ* double mutant retained the robust growth phenotype of the *ubr1Δ* single mutant, ruling out a protein synthesis defect as the cause of the poor growth of the *ubp6Δ* strain (data not shown). Ura3 reporter strains that are wild-type for *UBP6* showed no growth in the absence of uracil,

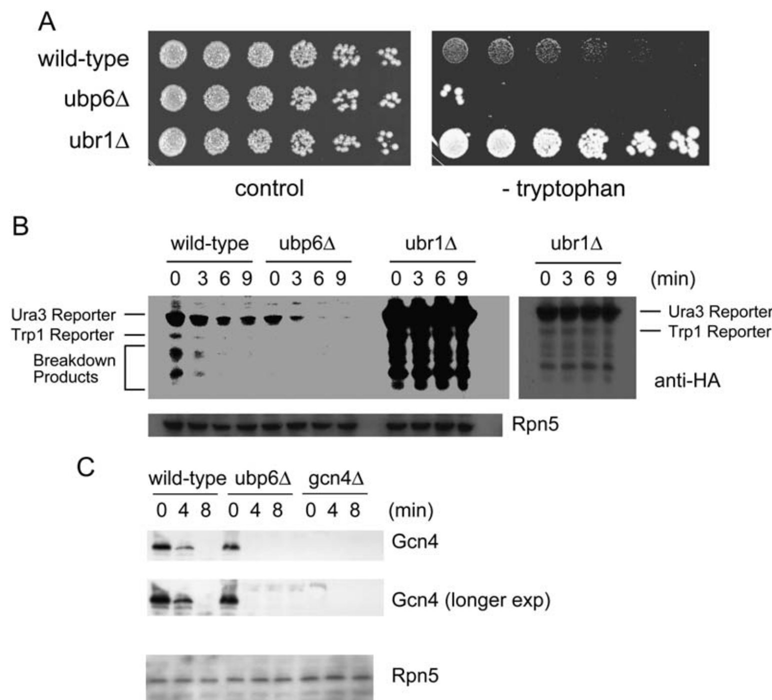


Figure 3. Deletion of the *UBP6* Gene Accelerates Degradation of Proteasome Substrates In Vivo

(A) Growth of wild-type (SYT303), *ubp6Δ* (SJH170), and *ubr1Δ* (SYT304) yeast strains on media containing or lacking tryptophan, as indicated.

(B) Cycloheximide-chase reactions examining turnover of the Ura3 and Trp1 reporter proteins in wild-type (SYT318), *ubp6Δ* (SJH126), and *ubr1Δ* (SYT342) strains. Reactions were visualized with immunoblots using antibodies against the HA-epitope or proteasome subunit, Rpn5, as indicated. Far right, shorter exposure of the *ubr1Δ* lanes.

(C) Cycloheximide chase analysis of Gcn4 turnover in wild-type and *ubp6Δ* strains. Logarithmically growing cultures were treated with cycloheximide (20 μg/ml), and aliquots were taken at the indicated times. Cells were immediately resuspended in 1× SDS-sample buffer (LLB), and endogenous Gcn4 was detected by immunoblot using anti-Gcn4 antibody. Rpn5 (lower panel) served as a loading control.

precluding the corresponding comparison (data not shown).

The results from Figure 3A suggested that the reporter proteins were turned over more rapidly in the *ubp6Δ* strain. To test this idea directly, we conducted cycloheximide-chase analyses in strains harboring both reporter constructs. In the wild-type strain we observed rapid degradation of both reporters, with half-lives on the order of several minutes (Figure 3B). In the *ubr1Δ* mutant, both reporters were highly stabilized (Figure 3B). In contrast, in the *ubp6Δ* mutant, the Ura3 reporter disappeared more rapidly than in wild-type, and showed a decreased steady-state level (Figure 3B). Steady-state levels of the Trp1 reporter were also depressed in the *ubp6Δ* mutant (Figure 3B), falling below the level of detection. The level of a control protein, proteasome subunit Rpn5, remained constant throughout, indicating the specificity of these effects.

In addition to N-end Rule substrates, we examined the short-lived transcription factor Gcn4, expressed from its own promoter in untagged form. Again, we observed accelerated degradation in the *ubp6Δ* mutant, with a concomitant decrease in steady-state levels (Figure 3C). Thus, multiple short-lived proteins are turned over more rapidly in vivo in the absence of Ubp6.

The Deubiquitinating Activity of Ubp6 Is Not Required for Degradation Delay

To determine which functional elements of Ubp6 are required for degradation delay, we generated and tested a number of Ubp6 mutants (Figure 4A). An N-terminal ubiquitin-like domain (Ubl) is necessary and sufficient to mediate binding to the proteasome, but is not required for the catalytic activity of purified Ubp6 (Leggett et al., 2002). Deletion of the Ubl domain rendered Ubp6 noninhibitory in the cyclin B degradation assay (Figure 4B), suggesting that Ubp6 must be bound to the proteasome to inhibit cyclin B degradation. The Ubl domain by itself also had no effect on cyclin B degradation (Figure 4E). Thus, the ability of Ubp6 to delay degradation of cyclin B jointly requires the Ubl and C-terminal domains of the protein.

Although efficient degradation of ubiquitin conjugates by the proteasome is thought to require removal of ubiquitin groups prior to translocation of the substrate into the CP, premature removal of ubiquitin chains could result in dissociation of the substrate and antagonize its degradation (Lam et al., 1997). Such a scenario could provide a straightforward means by which deubiquitinating activity at the proteasome could inhibit protein breakdown. The joint requirements of the Ubl and C-terminal domains of Ubp6 for inhibiting cyclin B degradation are consistent with this view. However, a catalytically inactive point mutant of Ubp6 (Ubp6-C118A) proved competent for degradation delay (Figure 4B). Thus, Ubp6 does not prevent cyclin B degradation simply through premature deubiquitination. To substantiate this conclusion, we verified that Ubp6-C118A is completely devoid of deubiquitinating activity against the model substrate Ub-AMC (Figure S1 in the Supplemental Data available with this article online), but retains wild-type binding affinities for the proteasome (data not shown; Chernova et al., 2003).

As an alternate approach to assay noncatalytic inhibition, we utilized ubiquitin-vinyl sulfone (Ub-VS), an irreversible active site inhibitor of Ubp6. Although it completely eliminated catalytic activity of wild-type Ubp6 as measured by Ub-AMC hydrolysis, inhibition of cyclin B

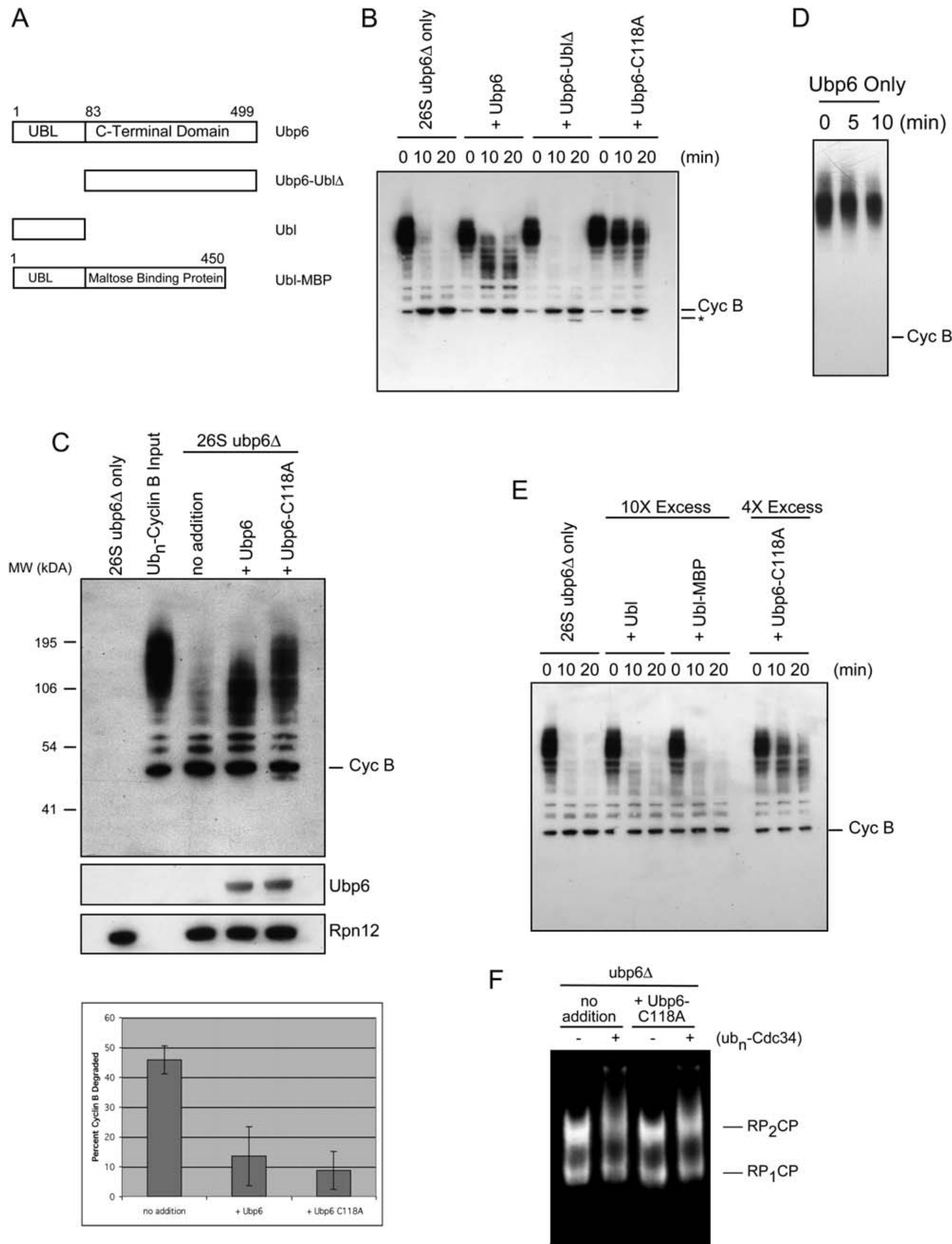


Figure 4. A Noncatalytic Function of Ubp6 in Proteasome Inhibition

(A) Schematic of the various Ubp6 mutants. MBP, maltose binding protein. (B and E) Ub-cyclin B processing by *ubp6Δ* proteasomes, in the presence or absence of the indicated Ubp6 species. Inhibition of degradation by Ubp6-C118A was also observed using proteasomes purified via a CP affinity tag (data not shown). (C) A comparison of the extent of proteasome inhibition by wild-type Ubp6 and Ubp6-C118A by quantitative mass spectrometry. Upper panels, immunoblots using anti-cyclin B, -Ubp6, and -Rpn12 antibodies as indicated. Lower panel, quantitation results. Error bars represent the standard deviation of two independent quantitations. (D) Ubp6 fails to deubiquitinate cyclin B when proteasomes are absent. (F) Electrophoretic mobility shift of proteasome bound to ubiquitinated Cdc34. Proteasomes were visualized using the fluorogenic substrate suc-LLVY-AMC.

degradation by Ubp6 persisted (Figure S2). This finding also indicates that degradation delay by Ubp6-C118A cannot be attributed to nonproductive ubiquitin chain binding at the active site of the mutant enzyme. Indeed the large excess of substrate over Ubp6 (see Experimental Procedures) is inconsistent with a model in which Ubp6 delays degradation by sequestering substrate. Taken together, these data show that Ubp6 has two distinct functions, one catalytic and one noncatalytic. Although these functions are distinct, each requires both the Ubl domain and the C-terminal domain. We suggest that the Ubl domain serves in both cases to target Ubp6 to the

proteasome, and that both functions are obligatorily executed on the proteasome.

Although Ubp6-C118A was, like wild-type Ubp6, competent to delay cyclin B degradation, the fate of cyclin B over the time course of the incubation was affected by the loss of deubiquitinating activity in the mutant enzyme. When wild-type Ubp6 was used for reconstitution, a progressive reduction in electrophoretic mobility of cyclin B immunoreactive material was seen, whereas these mobility shifts were not observed with Ubp6-C118A (Figure 4B). These data imply that the mobility shifts represent trimming of ubiquitin or ubiquitin chains bound to cyclin B, and that the trimming reaction is mediated predominantly or exclusively by the deubiquitinating activity of Ubp6.

To quantify the extent of degradation delay by Ubp6, we utilized a recently developed method of quantitative mass spectrometry (Kirkpatrick et al., 2006). After a 20 min incubation, approximately half of the total cyclin B was degraded by *ubp6Δ* proteasomes (Figure 4C). High molecular weight ubiquitin-cyclin B conjugates were almost completely eliminated by this time, but degradation would not be expected to go to completion because a fraction of the input cyclin B had not been ubiquitinated. Addition of purified Ubp6 or Ubp6-C118A resulted in approximately 70% inhibition of degradation at this time point (Figure 4C), which may underestimate the Ubp6 inhibitory effect, since the course of degradation seemed to be nearing completion in the control sample.

Ubp6 Prevents Rpn11-Dependent Ubiquitin Chain Removal

The linkage of Rpn11 activity to substrate degradation can be abrogated by proteasome inhibitors, presumably because they act downstream of Rpn11 (Verma et al., 2002; Yao and Cohen, 2002). Thus, to visualize the activity of Rpn11 on cyclin B, we used proteasome inhibitor treated *ubp6Δ* proteasomes, as in Figure 2. This figure showed, as described above, that the effect of Ubp6 on cyclin B disappearance is mediated by an altered rate of proteasome-mediated degradation. However, since the results of Figure 4 indicate that the deubiquitination seen during degradation delay is mediated by Ubp6, and not by Rpn11, it appears that Ubp6 inhibits the proteasome at a point in the reaction pathway such that Rpn11-mediated chain removal is prevented.

In the presence of epoxomicin, Rpn11 can be seen to cleave substrate-linked chains at or near their base, resulting in the production of a prominent band of unmodified substrate protein (Verma et al., 2002; Yao and Cohen, 2002; Figure 2). When we compared the reaction products from wild-type and *ubp6Δ* proteasomes pretreated with epoxomicin, we observed in *ubp6Δ* reactions a greater amount of unmodified cyclin B, reflecting substrate deubiquitinated but not degraded by the proteasome (Figures 2A–2C). In the presence of Ubp6, a lesser amount of unmodified cyclin B was stabilized by the inhibitor; instead, the majority of the cyclin B remained as higher molecular weight species, most likely reflecting partial deubiquitination. The increased yield of unmodified cyclin B in the presence of *ubp6Δ* proteasomes as compared to wild-type indicates that Rpn11-dependent chain removal from substrate is suppressed in the presence of Ubp6. However, it remains unclear whether Ubp6 directly inhibits Rpn11 or some other activity that may function upstream of Rpn11.

Evidence that Interference with Substrate-Proteasome Interaction Cannot Explain Proteasome Inhibition by Ubp6

One potential explanation for the degradation-inhibitory effect of Ubp6 is that binding of Ubp6 to the proteasome is competitive with that of substrate. Several lines of evidence indicate that Ubp6 does not inhibit the proteasome by this mechanism. As seen in Figure 4B, the deubiquitination of cyclin B that occurs in the complete reaction mixture is mediated by Ubp6. However, when Ubp6 is incubated in the presence of cyclin B, but without proteasomes, no deubiquitination of cyclin B is observed (Figure 4D). Thus, Ubp6 requires the proteasome for its deubiquitinating activity on cyclin B. These data suggest that both Ubp6 and cyclin B are bound to proteasomes so long as cyclin B deubiquitination proceeds, which can be an extended period, since the deubiquitination reaction is progressive over the time courses shown in Figure 4.

Ubp6 could conceivably inhibit cyclin B degradation via a substrate-titration mechanism. However, we were unable to observe direct interaction between Ubp6 and ub-cyclin B using a GST pull down assay that sensitively detects binding of ub-cyclin B to ubiquitin receptors such as Rpn10, Rad23, and Dsk2 (Figure S3 and Kirkpatrick et al., 2006). These findings are consistent with a previous report that the K_M of Ubp6 for ubiquitin ethyl ester is so high as to be unmeasurable (Chernova et al., 2003).

Ubp6 is large enough, at 57 kDa, that its presence on the proteasome could conceivably impede binding of substrates or substrate receptors. Also, the pathway of substrate translocation through the proteasome might be blocked by nonspecific steric interference. To address this possibility we constructed a fusion protein in which the Ubl of Ubp6 was fused N-terminally to the maltose binding protein of *E. coli* (Figure 4A), resulting in a fusion protein of nearly the same size as Ubp6. Despite binding the proteasome (data not shown), this construct, as for the Ubl alone, failed to delay cyclin B degradation (Figure 4E).

Finally, we directly evaluated the effect of Ubp6 on conjugate binding to the proteasome by using autoubiquitinated Cdc34, a substrate that binds proteasomes but is not degraded (Elsasser et al., 2004). Using an activity-based electrophoretic mobility shift assay (Elsasser et al., 2004), we observed comparable conjugate binding in the presence and absence of Ubp6-C118A (Figure 4F). Conjugate binding was observed in the presence of wild-type Ubp6, but was also accompanied by deubiquitination, as

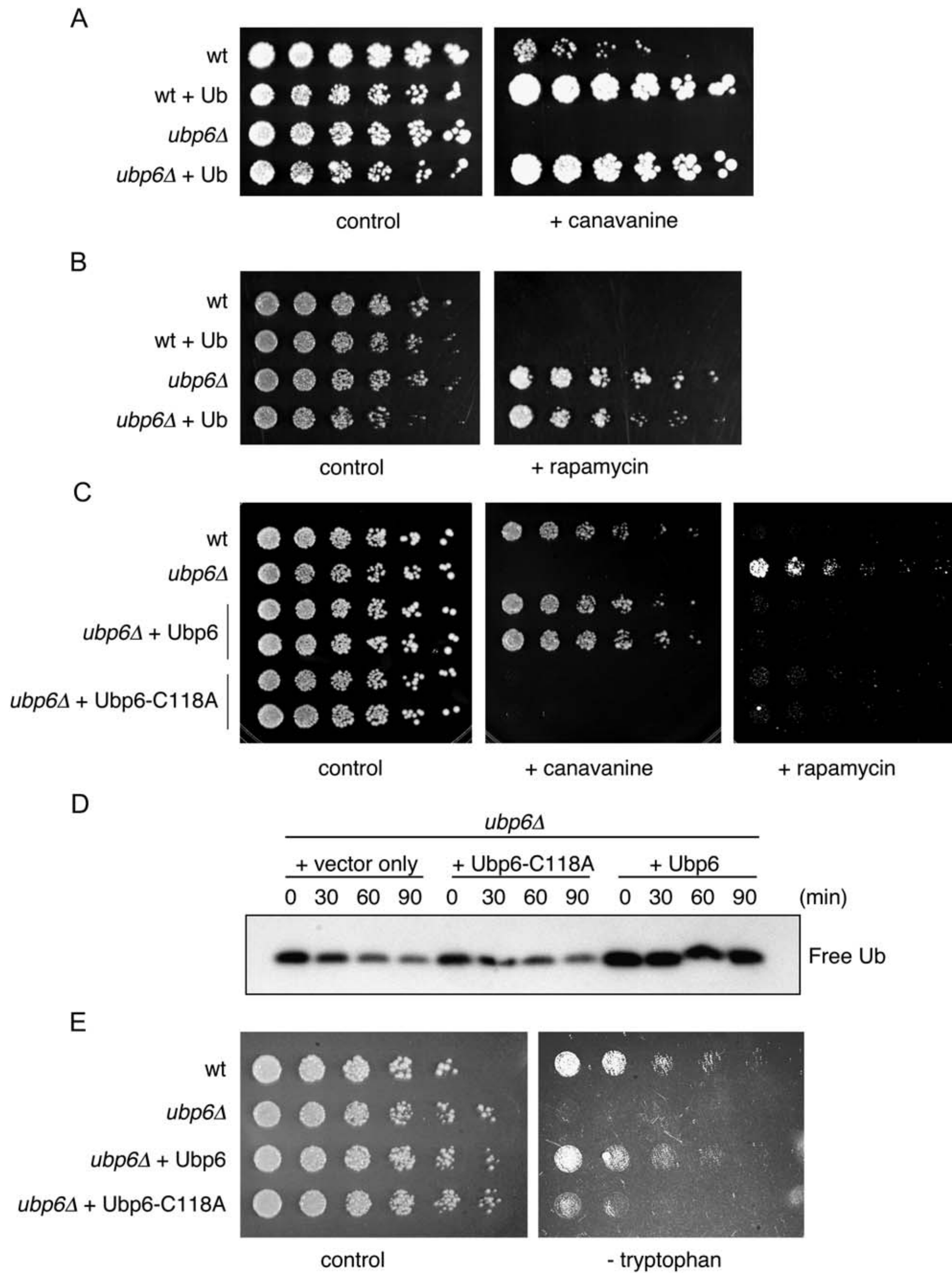


Figure 5. In Vivo Differentiation of *ubp6Δ* and *ubp6-C118A* Mutants

(A and B) Growth of wild-type (SJH30), wild-type overexpressing ubiquitin (SJH34), *ubp6Δ* (SJH31), and *ubp6Δ* overexpressing ubiquitin (SJH35) yeast strains on selective plates containing copper sulfate (100 μM) in the presence or absence of canavanine (1.5 μg/ml) or rapamycin (200 ng/ml), and grown at 30°C for 3–7 days.

(C) Growth of wild-type (SJH152), *ubp6Δ* (SJH153), *ubp6Δ* expressing wild-type Ubp6 (SJH154), and *ubp6Δ* expressing Ubp6-C118A (SJH155) yeast strains on selective plates containing no drug, canavanine (1.5 μg/ml), or rapamycin (200 ng/ml) as indicated, and grown at 30°C for 3-7 days. For SJH154 and SJH155, duplicates represent two independent transformants.

(D) Cycloheximide-chase analyses of free ubiquitin turnover were conducted in wild-type (SJH120), *ubp6Δ* overexpressing wild-type Ubp6 (SJH20), and *ubp6Δ* overexpressing Ubp6-C118A (SJH22) strains, as indicated.

(E) Growth of wild-type (SJH171), *ubp6Δ* (SJH172), *ubp6Δ* expressing wild-type Ubp6 (SJH173), and *ubp6Δ* expressing Ubp6-C118A (SJH174) yeast strains on selective media containing or lacking tryptophan, as indicated.

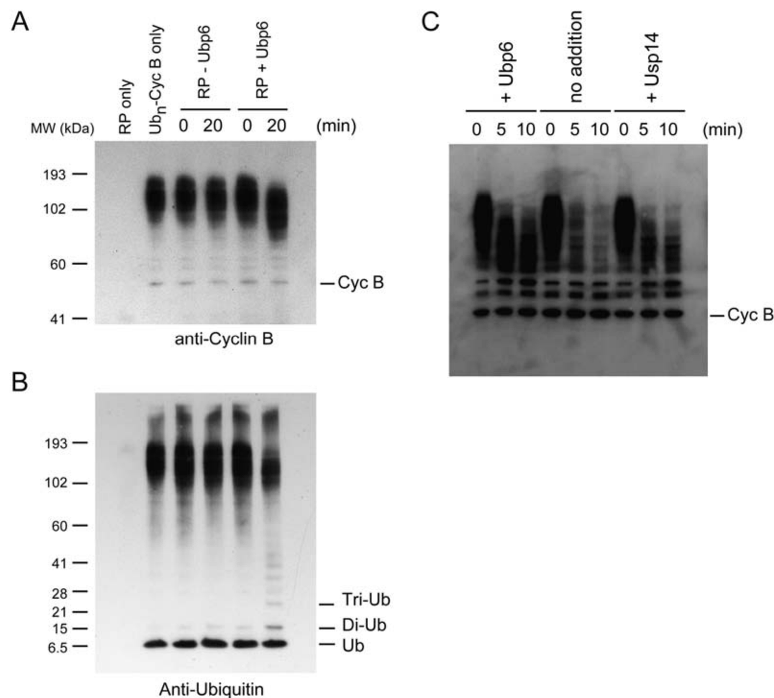


Figure 6. Degradation Independence and Evolutionary Conservation of Ubp6 Function

(A and B) Processing of ub-cyclin B by RP (*ubp6Δ*) in the presence or absence of purified Ubp6. Reactions were visualized by immunoblotting with anti-cyclin B antibody or anti-ubiquitin antibody, as indicated.

(C) Effect of Ubp6 or its human homolog, Usp14, on the processing of ub-cyclin B by yeast *ubp6Δ* proteasomes.

determined by immunoblot (S.E., J.H., and D.F., unpublished data). Taken together, the data of Figures 4E and 4F indicate that noncatalytic proteasome inhibition by Ubp6 is unlikely to be achieved through simple sterically based mechanisms but instead may involve specific functionalities within the C-terminal domain of Ubp6.

Noncatalytic Effects of Ubp6 In Vivo

We next sought in vivo evidence for the nonequivalence of the null and catalytic point mutants of Ubp6. A number of *ubp6Δ* phenotypes that can be suppressed by ubiquitin overexpression have been described (Chernova et al., 2003; Hanna et al., 2003), including hypersensitivity to the amino acid analog canavanine (Figure 5A) and to several translational inhibitors. Intriguingly, we have found that not all *ubp6Δ* phenotypes are ubiquitin dependent. For instance, we observed a strong resistance of *ubp6Δ* to rapamycin, an inhibitor of the TOR pathway (Wullschleger et al., 2006), which globally regulates the state of the yeast cell accord to nutritional conditions. The rapamycin resistance of *ubp6Δ* was not suppressed by ubiquitin overexpression (Figure 5B). This result suggests functions of Ubp6 outside of ubiquitin regeneration, possibly reflecting noncatalytic functions of Ubp6. To test this hypothesis, we expressed wild-type Ubp6 and Ubp6-C118A from its endogenous promoter in a *ubp6Δ* strain. Wild-type Ubp6 complemented both the canavanine and rapamycin phenotypes (Figure 5C). As expected, expression of Ubp6-C118A failed to rescue canavanine hypersensitivity (Figure 5C; Leggett et al., 2002) but in contrast largely complemented rapamycin resistance (Figure 5C). Ubp6-ublΔ recapitulated the null phenotype, suggesting that the rapamycin-related noncatalytic functions of Ubp6 occur on the proteasome (Figure S4). These results provide in vivo evidence that the *ubp6Δ* and *ubp6-C118A* mutants are not equivalent and support the hypothesis that Ubp6 performs noncatalytic functions, as observed in vitro.

In the absence of Ubp6, free ubiquitin is rapidly turned over (Figure 5D; Leggett et al., 2002). The failure of Ubp6-C118A to complement ubiquitin-dependent phenotypes such as canavanine hypersensitivity suggested aberrant ubiquitin turnover in *ubp6-C118A* mutants. Overexpression of wild-type Ubp6, but not Ubp6-C118A, restored ubiquitin stability (Figure 5D), indicating that the ubiquitin recycling function of Ubp6 is catalytic in nature. These results provide a mechanistic basis for understanding why the Ubp6-C118A mutation affects ubiquitin-dependent but not ubiquitin-independent phenotypes, and indicate that the noncatalytic function of Ubp6 is distinct from its function in ubiquitin homeostasis.

Finally, we utilized the Ub-K-Trp1 substrate described in Figure 3 to test whether noncatalytic inhibition of degradation by Ubp6 was also operational in vivo. As shown in Figure 5E, expression of Ubp6-C118A restored growth on media lacking tryptophan, indicating stabilization of the Ub-K-Trp1 protein. Importantly, wild-type Ubp6 was more effective in stabilizing Ub-K-Trp1 than was Ubp6-C118A. Thus both catalytic and noncatalytic features of Ubp6 appear to mediate proteasome inhibition in vivo (see Discussion).

In summary, the results of Figure 5 show that Ubp6 has a noncatalytic inhibitory effect on protein breakdown in vivo and that the biology of the cell is sensitive to a noncatalytic function of Ubp6 as shown for example by the strongly rapamycin resistant phenotype of the mutant.

Protein turnover effects were observed without overexpression of Ubp6, indicating that significant proteasome inhibition is in effect under normal growth conditions in wild-type cells.

Deubiquitination of Cyclin B by Ubp6

Rpn11 has been reported to function in the context of the 26S proteasome, but not in the context of the RP or the lid (Verma et al., 2002), although there have been differing reports (Yao and Cohen, 2002; Guterman and Glickman, 2004). In our assay, RP purified from a *ubp6Δ* mutant produced no detectable deubiquitination of cyclin B conjugates (Figure 6A). In contrast, addition of purified Ubp6 to RP resulted in progressive deubiquitination, although to a lesser extent than with 26S proteasomes (Figure 6A). Addition of Ubp6-C118A to RP had no effect on substrate processing (Figure S5), further verifying that deubiquitination seen with wild-type Ubp6 reflects the catalytic activity of Ubp6 itself. The immunoreactivity at the position of unmodified cyclin B before and after Ubp6 processing was of approximately equal intensity. Thus, although it can progressively remove ubiquitin groups from cyclin B, Ubp6 produces unmodified cyclin B less readily than Rpn11.

We examined the fate of ubiquitin in the presence of RP-bound Ubp6 by anti-ubiquitin immunoblots. We found a decrease in the overall size and intensity of the high molecular weight material, consistent with the data from Figure 6A, but also observed reaction products comigrating with diubiquitin, triubiquitin, and larger ubiquitin polymers (Figure 6B). Presumably, monoubiquitin was also generated, although this was difficult to discern because of the high levels of free ubiquitin present in the reactions. In reactions utilizing lower amounts of substrate, Ubp6-dependent generation of free ubiquitin was readily observed (Figure S5). These results suggest a preference of Ubp6 for the distal end of ubiquitin chains (see also Hu et al., 2005); alternatively, Ubp6 may cleave proximally to remove monoubiquitin and small ubiquitin chains. A third deubiquitinating enzyme, Uch37, which is absent from budding yeast, has been reported to possess a distal-end-directed activity on the proteasome (Lam et al., 1997). However, whereas Uch37 appears to remove ubiquitins one at a time, progressive deubiquitination by Ubp6 is not limited to the trimming of single ubiquitins.

The In Vitro Functions of Ubp6 are Evolutionarily Conserved

Ubp6 and its human homolog Usp14 share 32% sequence identity (Chernova et al., 2003). Several features of Ubp6, including proteasome binding and activation by proteasome binding, are shared by Usp14 (Borodovsky et al., 2001). Indeed, overexpressed Usp14 complements several in vivo phenotypes of *ubp6Δ* in yeast (Chernova et al., 2003). We therefore tested whether purified recombinant Usp14 could substitute for Ubp6 in the cyclin B degradation assay, using *ubp6Δ* proteasomes from yeast. Usp14 largely recapitulated the effects of Ubp6 (Figure 6C): ub-cyclin B was not only stabilized against degradation by Usp14, but progressively deubiquitinated to lower molecular weight forms. Usp14 is slightly less efficient than Ubp6 in this assay, perhaps reflecting a decreased affinity of Usp14 for yeast proteasomes compared to Ubp6. These results, in combination with the reported in vivo complementation of *ubp6Δ* by Usp14, indicate that the observed effects of Ubp6 in our reconstituted system are likely to represent major functions of Ubp6 in vivo.

DISCUSSION

A Proteasome Component that Delays Proteasome Action

Proteasomes regenerate ubiquitin by separating it from substrate prior to substrate degradation. Interestingly, the proteasomes of most eukaryotes are associated with three deubiquitinating enzymes. Indeed, the mammalian proteasome has as many distinct active sites for deubiquitination as it has proteolytic active sites for substrate hydrolysis. It is unclear why so many activities are required for deubiquitination at the proteasome, how they are related to one another, how their function is coupled to substrate degradation, and to what extent deubiquitination may antagonize degradation by removal of the substrate's targeting signal.

The activity of one proteasomal deubiquitinating enzyme, Rpn11, is coupled to and facilitates degradation (Verma et al., 2002; Yao and Cohen, 2002). Here, we report that another proteasomal deubiquitinating enzyme, Ubp6, has the opposing capacity to delay degradation. Studies with Uch37 have shown that a proteasomal deubiquitinating enzyme can potentially antagonize degradation by premature deubiquitination (Lam et al., 1997). However, Ubp6 antagonizes degradation by a novel mechanism that does not require deubiquitination of the degradative substrate: it appears to inhibit the proteasome directly. Proteasomes operate on ub-cyclin B in vitro with substantially reduced efficiency when they are bound to Ubp6, and Ubp6 shows a comparable in vivo effect in reducing flux of at least some specific substrates through the proteasome. These negative effects are seen under standard growth conditions, where Ubp6 is a major proteasome component.

Substrate Deubiquitination by Rpn11 is Negatively Controlled by Ubp6

One aspect of Ubp6's inhibitory action is to prevent en bloc deubiquitination of the substrate by Rpn11 (Figure 2). During the degradation delay imposed by Ubp6, its own deubiquitinating activity is operational, as can be seen in a gradual reduction in the number of substrate bound ubiquitin groups (Figures 4 and 6). Thus, Ubp6 appears to delay the degradation of and progressively deubiquitinate the same substrate. In this way, the mode of substrate deubiquitination is significantly altered from that seen in the absence of Ubp6. These results show that the proteasome has distinct modes of deubiquitination,

and that the en bloc mode is subject to negative control, principally by a noncatalytic function of Ubp6.

If Ubp6 were to inhibit degradation downstream of Rpn11, the substrate would lack ubiquitin modification during degradation delay. In this scenario, release from Ubp6-mediated degradation delay might be unproductive, as substrates lacking a ubiquitin modification would presumably dissociate from the proteasome rather than be degraded. Thus, inhibition of the proteasome by Ubp6 appears to delay the decision of whether the substrate will be degraded, and during the delay, attrition of substrate bound ubiquitin on the substrate proceeds gradually through the activity of Ubp6. The length of time allowed for delay of degradation may be an important parameter. During this time period, shortening of ubiquitin chains beyond a critical length may lead to dissociation of the substrate from the proteasome. This model implies that the presence of two distinct activities in Ubp6, catalytic and noncatalytic in nature, is unlikely to be fortuitous.

An interesting possibility is that the catalytic activity of Ubp6 should also inhibit degradation, and moreover it may do so in a manner that is strongly dependent on the duration of noncatalytic inhibition. Our in vivo data (Figure 5E) provide initial support for this model, although further work will be required. We have so far not observed catalytically based inhibition in vitro. However, it may be that the extent of cyclin B ubiquitination achieved in our in vitro reaction is high enough that limited deubiquitination does not compromise binding of the conjugate to the proteasome, while substrates bearing shorter chains, or exposed to longer time courses, would display such an inhibitory capacity of Ubp6 more clearly.

The Mechanism of Degradation Delay by Ubp6

This study shows that a deubiquitinating enzyme can utilize noncatalytic functions to modulate the activity of another protein—in this case, the proteasome. As there seems to be no interference with substrate binding on the part of Ubp6, an interaction between Ubp6 and the proteasome most likely mediates degradation delay. Ubp6 is tethered to the proteasome through its Ubl domain, which binds subunit Rpn1 of the base (Leggett et al., 2002; Stone et al., 2004). However, proteasome binding by the Ubl domain itself has no inhibitory effect (Figure 4E). Indeed, both the Ubl and C-terminal domains are required for inhibition. A simple model is that the Ubl domain serves only a proteasome-tethering role and that the C-terminal domain contains the specific sequence information for inhibition. Crystal structures of Ubp6 and its mammalian homolog, USP14, indicate several regions within the C-terminal domain of Ubp6, including a notable surface-exposed alpha helix, that are not conserved with the unrelated deubiquitinating enzyme HAUSP, and are unlikely to contribute to enzymatic activity (Hu et al., 2005). In contrast, these regions are highly conserved among Ubp6 homologs (J.H. and D.F., unpublished data), and it may be that one of these regions mediates the noncatalytic activity of the protein.

We have largely ruled out roles for Ubp6 in substrate targeting (Figures 4E and 4F), gate opening (Figure 1E), modulation of CP active sites (Figure 1E), as well as modulation of overall proteasomal ATPase activity (data not shown). Interestingly, our previous studies suggested that the C-terminal domain of Ubp6 has lid binding activity (Leggett et al., 2002); this interaction might mediate degradation delay. This speculative model, if confirmed, would be particularly interesting since the lid is required for full activation of Ubp6 upon complex formation with the proteasome (Leggett et al., 2002). Thus, the same Ubp6-lid binding event could conceivably inhibit the proteasome and assist in the activation of Ubp6. Since Rpn11 is a component of the lid, the lid interaction model also suggests that Ubp6 may delay degradation by directly inhibiting Rpn11 rather than an unknown step upstream of Rpn11.

Proteasome inhibition by Ubp6 is not complete. Although this is not surprising—complete inhibition of the proteasome would be detrimental—the mechanism by which inhibition is relieved is unclear. It is also unclear whether release of the proteasome from Ubp6-mediated degradation delay is itself a regulated step. Since Ubp6 is not an integral subunit of the proteasome, the fraction of proteasomes associated with Ubp6 can in principle be freely varied, so the overall extent of proteasome inhibition could be regulated through simple means such as altering cellular Ubp6 levels, or alternatively via posttranslational modulation of Ubp6 activity.

Ubp6 and the Selectivity of the Proteasome

An outstanding question concerns the generality of the degradation-inhibitory effect of Ubp6. Interestingly, the inhibitory effects are not restricted to poor degradative substrates. To determine whether degradation delay by Ubp6 is to any extent substrate-specific, it will be of interest to determine the effect of *ubp6* mutations on the rates of degradation of a wider variety of substrates. If substrates are affected to very different degrees by Ubp6, it would suggest that Ubp6 may slow degradation rates to enhance the selectivity of protein degradation by the proteasome. Studies of nucleotide polymerases (Joyce and Benkovic, 2004) as well as protein synthesis (Cochella and Green, 2005) have shown that that speed and fidelity cannot be optimized simultaneously, but are competing functional criteria. The definition of fidelity for a protease cannot be as clear as for a polymerase, but fidelity may be more easily defined from the standpoint of the fate of ubiquitin groups than of substrates. Ubp6 clearly enhances ubiquitin sparing by the proteasome both in vitro and in vivo, and thus enhances a significant aspect of its fidelity.

Several previous studies have reported Ubp6 to play positive roles in the degradation of specific proteins (Leggett et al., 2002; Miura and Abe, 2004), in contrast to the present work. These observations do not appear to reflect limited scope of the proteasome-inhibitory effect described here, but rather the critical role of Ubp6 in maintaining cellular ubiquitin levels, and the importance of ubiquitin levels in determining the degradation rates of these

particular proteins. For example, yeast Tat2 can be degraded in a *UBP6*-dependent manner, but Tat2 is a membrane protein and its degradation appears to proceed through the vacuole rather than the proteasome. In addition, Tat2 degradation is sensitive to mutations in other genes that affect cellular ubiquitin levels (Miura and Abe, 2004). The other known example is ubiquitin-proline-β-galactosidase (Leggett et al., 2002), whose degradation is highly sensitive to ubiquitin levels (Johnson et al., 1995).

Implications for Protein Turnover in Mammals

The substrate-stabilizing effects of ubiquitin deficiency could potentially obscure the destabilizing effects of release from proteasome inhibition in the *ubp6Δ* mutant. It will be particularly interesting to uncouple these opposing effects in *ataxia* mice, whose biochemical basis is loss-of-function mutation in Ubp6/Usp14. These mice display a severe neurologic phenotype culminating in widespread paralysis and premature death (Wilson et al., 2002). Whether these phenotypes are a result of ubiquitin depletion or enhanced proteasomal degradation, or both, and whether either of these cellular phenomena may have relevance for human neurologic or neurodegenerative diseases remains an intriguing open question.

In recent years, pharmacologic inhibition of the proteasome's proteolytic active sites has emerged as an effective anti-cancer treatment in such clinical contexts as multiple myeloma (Adams, 2004). The efficacy of such drugs may reflect an increased requirement for proteasome function in some cancer cells. On the other hand, there may exist clinical scenarios in which a deficit of proteasome function contributes significantly to pathophysiology. Deficient proteasome function has been suggested, for example, to underlie various proteinopathies and neurodegenerative diseases (Goldberg, 2003). For diseases characterized by proteasome deficiency, drugs that inhibit a broadly acting proteasome inhibitor such as Ubp6 may provide an effective therapy.

EXPERIMENTAL PROCEDURES

Yeast Strains

Yeast strains and plasmids are given in Table S1. General methods for yeast are described in the supplement.

Recombinant Proteins

See Table S1. Recombinant proteins were expressed as GST-fusion proteins, purified by glutathione-Sepharose affinity chromatography, and eluted by thrombin cleavage, as described (Leggett et al., 2002). Eluates were treated with benzamidine or benzamidine-Sepharose to inactivate thrombin.

Ubp6-C118A mutants were generated by site-directed mutagenesis using the Quikchange system (Stratagene) and verified by DNA sequencing. The Ubl-MBP construct was generated by fusing the first 83 codons from *UBP6* to the 5′ end of the complete coding sequence of the maltose binding protein from *E. coli.*

Proteasome Purification

A previously described procedure for purification of the proteasome (Leggett et al., 2002) was modified by adding 1 mM ATP and 5 mM $MgCl_2$ to all purification buffers and by washing resins with 100 bed volume of buffer containing 50 mM NaCl, as opposed to 50 bed volume of buffer containing 100 mM NaCl. RP was purified in the presence of ATP and $MgCl_2$ as described (Leggett et al., 2002).

In Vitro Deubiquitination/Degradation Assays

Proteasomes (135 nM) were incubated with ub-cyclin B in the presence of 1 mM ATP and buffer (50 mM Tris [pH 7.5], 1 mM EDTA, 5 mM $MgCl_2$) for the indicated times. Reactions were terminated by the addition of 5× SDS Laemmli loading buffer (Leggett et al., 2002) and boiled for 5 min. Where indicated, recombinant, purified Ubp6 or Usp14 species (540 nM) were preincubated with proteasomes for 5 min prior to the reaction. Ubl and Ubl-MBP species were used at 1.35 μM to compensate for lower affinity of these species for proteasome binding compared to full-length Ubp6 species (Leggett et al., 2002; data not shown). Epoxomicin (100 μM) was used to inhibit proteolytic activity of proteasomes. Unless otherwise noted, immunoblots were visualized with anti-cyclin B1 polyclonal antibodies. Polyclonal antibodies recognizing Ubp6, Rpn5, Rpn12, and ubiquitin were used as indicated. Ub-cyclin B was added in molar excess over proteasome, generally on the order of 25-fold, except for Figures 4C, 6A, and 6B, where the substrate: enzyme ratio was increased by a factor of five.

In the experiments of Figures 1, 4 (except 4C), and 6C, time points were sampled after the preparation of a single reaction mixture. The zero time points therefore reflect a lag which represents the amount of time required to sample the first time point after having prepared the reaction mixture. This time lag was consistent between samples, and less than 20 seconds. However, the activity of Ubp6 is rapid enough to reduce cyclin B ubiquitination by time zero. In the experiments of Figures 2, 4C, and 6 (except 6C), each time point was carried out as an independent reaction. The zero time points were prepared in the presence of 5× LLB, and accordingly, no processing could occur before time zero.

In Vivo Degradation Assays

For plating assays, 3-fold serial dilutions of yeast cultures grown in YPD were spotted onto plates containing or lacking tryptophan. For cycloheximide chase assays, exponential cultures in YPD supplemented with 100 μM copper sulfate (to further induce expression of the reporter proteins) were normalized to an equivalent cell density and cycloheximide (50 μg/mL unless otherwise noted) was added. At the indicated time points, an aliquot of each culture was removed, and cells were pelleted and resuspended in 1× LLB. Samples were immediately frozen on dry ice for the remainder of the time course, and subsequently boiled for 5 min. Visualization was by immunoblotting with anti-HA antibody (12CA5) or anti-Rpn5 antibody, as indicated.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one table, and five figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/1/99/DC1/.

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