Cell

Ubiquitin Chains Are Remodeled at the Proteasome by Opposing Ubiquitin Ligase and Deubiquitinating Activities

Bernat Crosas,[1,2] John Hanna,[1] Donald S. Kirkpatrick,[1,3] Dan Phoebe Zhang,[1,3] Yoshiko Tone,[1] Nathaniel A. Hathaway,[1] Christa Buecker,[1] David S. Leggett,[1,4] Marion Schmidt,[1,5] Randall W. King,[1] Steven P. Gygi,[1] and Daniel Finley[1,*]

[1] Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
[2] Institut de Biologia Molecular de Barcelona, CSIC, Jordi Girona 18–26, Barcelona 08034, Spain
[3] These authors contributed equally to this work.
[4] Present address: ArQule Incorporated, 19 Presidential Way, Woburn, MA 01801, USA.
[5] Present address: Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA.
*Contact: daniel_finley@hms.harvard.edu
DOI 10.1016/j.cell.2006.09.051

SUMMARY

The ubiquitin ligase Hul5 was recently identified as a component of the proteasome, a multisubunit protease that degrades ubiquitin-protein conjugates. We report here a proteasome-dependent conjugating activity of Hul5 that endows proteasomes with the capacity to extend ubiquitin chains. *hul5* mutants show reduced degradation of multiple proteasome substrates in vivo, suggesting that the polyubiquitin signal that targets substrates to the proteasome can be productively amplified at the proteasome. However, the products of Hul5 conjugation are subject to disassembly by a proteasome-bound deubiquitinating enzyme, Ubp6. A *hul5* null mutation suppresses a *ubp6* null mutation, suggesting that a balance of chain-extending and chain-trimming activities is required for proper proteasome function. As the association of Hul5 with proteasomes was found to be strongly stabilized by Ubp6, these enzymes may be situated in proximity to one another. We propose that through dynamic remodeling of ubiquitin chains, proteasomes actively regulate substrate commitment to degradation.

INTRODUCTION

The ubiquitin-proteasome system mediates the breakdown of most short-lived regulatory proteins, and in this way it controls many aspects of cell physiology. A central element of this system, the proteasome, is also its most complex, with over 30 distinct integral subunits (Pickart and Cohen, 2004). The proteasome degrades substrates within its core particle (CP or 20S particle), which is a barrel-shaped assembly of 670 kDa whose hollow interior houses multiple proteolytic sites. Protein substrates reach this interior chamber through a gated channel, guided by the regulatory particle (RP, 19S, or PA700), which is a complex of ~1 MDa. The RP recognizes the substrate-bound ubiquitin chain, unfolds the substrate, and directs its translocation into the CP. The need for an unfolding step owes to the narrow cross-section of the channel leading to the CP (Pickart and Cohen, 2004). Critical components of the RP include ubiquitin receptors, deubiquitinating enzymes, and six ATPases that have been implicated in substrate unfolding and translocation.

Recent work has shown that factors associated with the proteasome via loose, often salt-labile interactions play important roles in regulating its function (Schmidt et al., 2005). UBL-UBA proteins, for example, help to deliver specific ubiquitin conjugates to the proteasome (Verma et al., 2004; Elsasser et al., 2004; Madura, 2004). Among the most abundant proteasome-associated proteins of *S. cerevisiae* are two enzymatic factors: Ubp6, a deubiquitinating enzyme, and Hul5, a ubiquitin-protein ligase (Leggett et al., 2002). Ubp6 is one of two deubiquitinating enzymes within the proteasome; in contrast, a much larger number of ubiquitin ligases (nine) have been suggested to interact with the proteasome, including Ubr1, Ufd4, the APC, and the SCF (Xie and Varshavsky, 2000, 2002; Verma et al., 2000; You and Pickart, 2001; Leggett et al., 2002; Seeger et al., 2003; reviewed in Schmidt et al., 2005). However, Hul5 is the only ligase observed on proteasomes in amounts high enough to be visible by conventional protein staining (Leggett et al., 2002). Hul5 is evolutionarily conserved, and its likely mammalian ortholog, KIAA10, also associates with proteasomes (You and Pickart, 2001; Wang and Pickart, 2005).

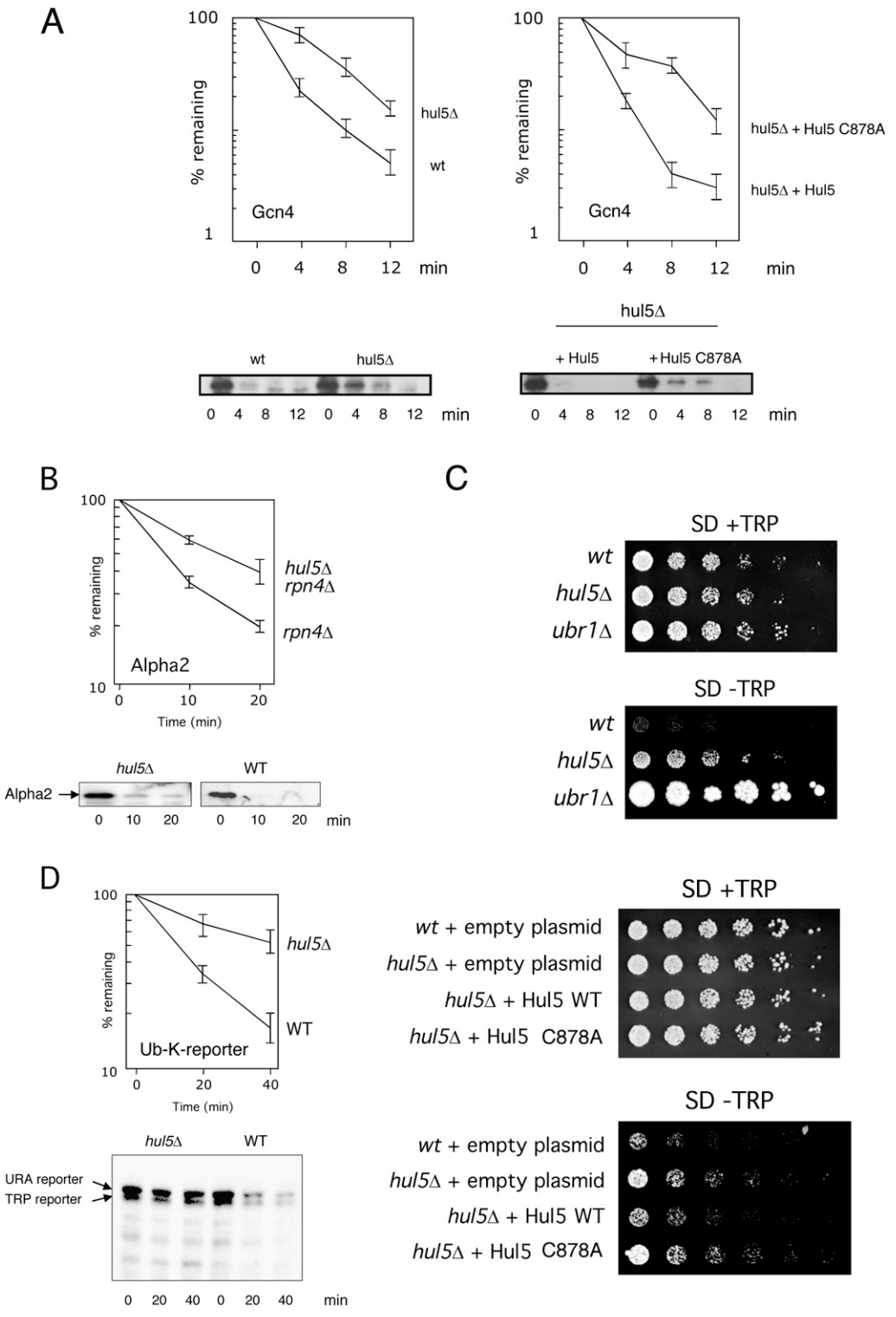


Figure 1. Stabilization of Short-Lived Proteins in *hul5Δ* Mutants

(A) Cycloheximide chase analysis of Gcn4 in wild-type (WT) and *hul5Δ* cells (left panel). The degradation defect was complemented by Hul5 expressed from its own promoter on a *CEN* plasmid but not by the active-site mutant Hul5-C878A (right). *hul5Δ* also stabilized TAP-tagged Met4 (Figure S1). Values represent the average of three independent experiments. Error bars show standard deviation from the mean.

(B) As in (A), but the substrate was α2, and turnover was assessed in *rpn4Δ* and *rpn4Δ hul5Δ* mutants. Values represent the average of three independent experiments. Error bars show standard deviation from the mean.

In this study we report genetic and biochemical characterization of Hul5. *hul5* null mutants are deficient in protein degradation, but, unlike typical ubiquitin ligase mutants, they do not show highly substrate-specific defects in degradation. Rather, the *hul5* phenotype more closely resembles that of a partial proteasome loss-of-function mutant. In vitro, Hul5 endows proteasomes with a potent ubiquitin-ligase activity, which can extend unanchored multiubiquitin chains or ubiquitin chains bound to a proteolytic substrate such as cyclin B. Ubiquitin chain extension by Hul5 is strongly stimulated by proteasomes, suggesting that proteasomes are a principal site of ubiquitination by Hul5. Ubiquitin groups added to proteasome-bound target proteins by Hul5 can be efficiently removed by Ubp6. These and other observations imply that Hul5 and Ubp6 function in opposition with one another. We suggest that by maintaining and extending ubiquitin chains on proteasome-bound substrates, Hul5 stabilizes proteasome-substrate interactions and promotes protein breakdown.

RESULTS

hul5 Mutants Exhibit Reduced Proteolytic Capacity

To test for a role of Hul5 in proteasome function, we examined the effects of deleting the *HUL5* gene on the rates of turnover of several short-lived proteins. Substantial stabilization of the transcriptional activator Gcn4 was observed in *hul5Δ* mutants (Figure 1A). This effect could be complemented by a *HUL5* gene carried on a low copy-number *CEN* plasmid. The HECT-domain ligases, of which Hul5 is a member, form covalent thiolester adducts with ubiquitin as a required step in conjugation. When the adduct-forming cysteine 878 of Hul5 was substituted with alanine, complementation was no longer observed in the Gcn4 degradation assay (Figure 1A).

In contrast to Gcn4, the transcriptional repressor α2 was not noticeably stabilized in the *hul5Δ* mutant (data not shown). However, defects in protein degradation can be compensated for by upregulation of proteasome levels (London et al., 2004; Ju et al., 2004). Rpn4 is a transcription factor that is degraded by the proteasome and that activates transcription of proteasome subunit genes (Xie and Varshavsky, 2001; Leggett et al., 2002; Ju et al., 2004). *rpn4Δ* mutants show reduced proteasome levels and an inability to upregulate proteasome subunit synthesis when the ubiquitin-proteasome system is challenged. In strains lacking Rpn4, we found that turnover of α2 was significantly affected by deletion of *HUL5* (Figure 1B). It is possible that for α2, ubiquitin conjugation is normally rate limiting for degradation; by deleting *RPN4,* proteasome function is attenuated and becomes partially rate limiting, so that underlying defects in proteasome function become more apparent.

Targets of the N-end rule pathway are well-studied model substrates of the proteasome (Varshavsky, 1996). The Trp1 protein was converted into an N-end rule substrate by fusing it to ubiquitin, with a lysine placed adjacent to the ubiquitin C terminus to allow recognition by the ubiquitin ligase Ubr1 (Hanna et al., 2006). A strain bearing this construct grew slowly in the absence of exogenous tryptophan but was Trp^+ when *UBR1* was deleted (Figure 1C). Hul5 appeared to promote degradation of the Ub-K-Trp1 fusion protein, because deletion of the *HUL5* gene conferred a growth advantage to this strain in the absence of tryptophan (Figure 1C). As seen with Gcn4, this function of Hul5 required its active site cysteine (Figure 1C). Cycloheximide chase analysis confirmed that Ub-K-Trp1 and a comparable reporter, Ub-K-Ura3, were stabilized by deletion of *HUL5* (Figure 1D). In summary, the stabilization of a range of substrates in *hul5Δ* mutants implies that Hul5 serves a general function at the proteasome. The defects in protein degradation in *hul5Δ* mutants appear to be more general than expected for a ubiquitin-protein ligase since ubiquitin ligases tend to be highly substrate specific.

Hul5-Dependent Ubiquitination on the Proteasome

The results above, taken together with our earlier finding that Hul5 associates with proteasomes (Leggett et al., 2002), suggested that Hul5 may stimulate the breakdown of multiple proteins by promoting their ubiquitination at the proteasome. To test whether Hul5 can mediate ubiquitination of proteasome-bound proteins, we incubated affinity-purified proteasomes with biotinylated ubiquitin, ubiquitin-activating enzyme (E1), and ATP. Proteasomes converted free ubiquitin into conjugates with surprising efficiency (Figure 2A). When purified from *hul5Δ* mutants, however, proteasomes were virtually devoid of this activity. Thus, although previous studies have indicated that other ligases can bind proteasomes with some affinity, this simple assay for ligase activity is specific for Hul5. Furthermore, the role of Hul5 in the proteasome's ubiquitin ligase activity is direct since proteasomes bearing a catalytically inactive form of Hul5, Hul5-C878A, were comparable to those of *hul5Δ* in their conjugation deficiency (Figure 2A). The selective detection of Hul5 activity may reflect that it is present on proteasomes at higher levels than other ligases in our preparations (Leggett et al., 2002). In addition, we suggest that the assay preferentially detects Hul5 because it is unique among proteasome-associated ligases in the nature or breadth of its substrate specificity.

Proteasomes are associated with deubiquitinating activity as well as ubiquitin ligase activity. An integral subunit

(C) Effect of deleting *HUL5* on growth of a strain expressing a Ub-K-Trp1 fusion protein. Plates were incubated for 3 (+Trp) or 7 (−Trp) days. As in (A), the degradation defect was complemented by plasmid-borne *HUL5* but not by *hul5-C878A*.

(D) As in (A), but the test substrates were Ub-K-Trp1 and Ub-K-Ura3. These two bands were quantitated together. All chase experiments were initiated by the addition of cycloheximide to 100 μg/ml, except in (A) where 20 μg/ml was used. Values represent the average of three independent experiments. Error bars show standard deviation from the mean.

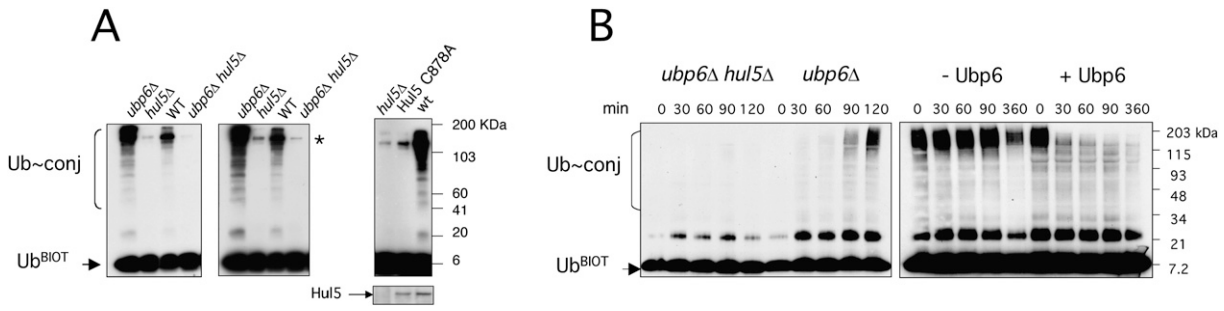


Figure 2. Hul5-Dependent Ubiquitin-Ligase Activity of Purified Proteasomes

(A) Proteasomes from WT and mutant strains were incubated for 1 hr with biotinylated ubiquitin, E1, and ATP. Ub^{BIOT} indicates biotinylated ubiquitin, Ub∼conj indicates ubiquitin-protein conjugates, and * indicates self-ubiquitinated E1 (data not shown). Samples were analyzed by SDS-PAGE. After blotting, ubiquitin conjugates were visualized with HRP-Streptavidin. Left and central panels show different exposures of the same blot.

(B) Ubiquitin-protein conjugation assays were performed with proteasomes purified from *ubp6Δ* and *hul5Δ ubp6Δ* strains as in (A). The leftmost panel shows a time course of the conjugation reaction. The 120 min sample was ATP depleted, then incubated with or without stoichiometric amounts of recombinant Ubp6. Time points were taken, and the samples were analyzed as in (A).

of the proteasome, Rpn11, removes ubiquitin chains en bloc from conjugates, apparently cutting at the substrate-proximal end of the chain (Verma et al., 2002; Yao and Cohen, 2002). A second proteasomal deubiquitinating enzyme is Ubp6, a proteasome-associated protein (Verma et al., 2000; Leggett et al., 2002) that progressively trims chains (Hanna et al., 2006). While cleavage by Rpn11 is coupled to substrate degradation (Verma et al., 2002; Yao and Cohen, 2002), the activity of Ubp6 is not; on the contrary Ubp6 can inhibit degradation (Hanna et al., 2006).

When the conjugate-synthesis assay was carried out using *ubp6Δ* proteasomes, both the yield of conjugates and their average molecular weight increased significantly (Figure 2A), suggesting that conjugates synthesized by Hul5 can be substrates of Ubp6. To test this possibility, we prepared conjugates using *ubp6Δ* proteasomes, then quenched further conjugate synthesis by depleting ATP. Upon addition of purified Ubp6, we observed nearly complete elimination of high molecular weight conjugates by 30 min, while in the absence of Ubp6 these conjugates persisted for at least 90 min (Figure 2B, right panel). Thus, conjugates formed by Hul5 on the proteasome are favorable substrates for Ubp6.

For the model substrates shown above to be stabilized in a *hul5Δ* mutant (Figure 1), E3 requirements have previously been defined, so it is unlikely that Hul5 could serve as a major E3 for these proteins (Meimoun et al., 2000; Swanson et al., 2001; Varshavsky, 1996). If not an E3, Hul5 could function as a member of a rarer and more recently recognized group of ubiquitin ligases known as E4 enzymes, which are defined as specialized mediators of ubiquitin chain elongation (Koegl et al., 1999; Richly et al., 2005). Thus, an E4 enzyme should not modify a protein unless it has been previously ubiquitinated through the action of an E3. To test whether Hul5 can elongate preformed ubiquitin chains, we purified Hul5 (Figure S2) and assayed its ubiquitin-ligase activity using tetraubiquitin chains (Ub_4) as substrate. These chains were efficiently elongated in a reaction mixture that included E1, E2, Hul5, and proteasomes, but not when Hul5 was omitted (Figure 3A). Also required for this reaction was the proteasome holoenzyme. These data suggest that Hul5 can serve as an E4, and that its enzymatic activity is stimulated by proteasomes, just as for Ubp6 (Leggett et al., 2002).

The E4 model assumes that tetraubiquitin is being used as a ubiquitin acceptor rather than a donor in these conjugation reactions. This assumption was confirmed through biochemical analysis of the product conjugates. Tetraubiquitin carries a free C-terminal carboxylate group and therefore can, like ubiquitin, be activated by E1 and subsequently be donated to substrates. The "tetraubiquitin donor" model, an alternative to the E4 model, predicts that the conjugates produced by Hul5 lack a ubiquitin-derived C-terminal carboxylate; that is, they are conventional ubiquitin-protein conjugates rather than free chains. However, isopeptidase T, which is specific for free ubiquitin chains, digested the Hul5 reaction products readily (Figure 3B). Thus, tetraubiquitin plays an acceptor role in these conjugation reactions, consistent with the E4 model in which Hul5 extends chains. The predominant donor in these reactions appears to be free ubiquitin, which is supplied in excess (Figure S3).

Proteins are thought to be targeted to the proteasome principally by Lys48-linked ubiquitin chains (Pickart and Cohen, 2004). However, when tested with single-lysine ubiquitin derivatives, Hul5 formed chains more efficiently on Lys63 than on Lys48 (Figure 3C). To confirm the linkage preference of Hul5, we used mass spectrometry to map the linkage site on chains formed with wild-type (WT) ubiquitin. This analysis revealed a strong preference for the formation of Lys63 linkages (Figure 3D). Thus, Hul5 has the capacity to build chains with heterogeneous ubiquitin-ubiquitin linkages. Hul5 can build off Lys48-linked chains, but in vitro it produces mainly Lys63 linkages. Whether this apparent chain specificity represents a functionally significant parameter for Hul5 in vivo remains to be assessed.

The ability of Hul5 to promote protein degradation in vivo suggests that Hul5 should be able to extend ubiquitin chains on defined degradative substrates. We tested this using cyclin B, a highly unstable protein. Cyclin B was expressed in insect cells, complexed to Cdc2, purified, and ubiquitinated by the APC ligase (Kirkpatrick et al., 2006). The resulting conjugates are degraded almost to completion within 5 min by *ubp6Δ* proteasomes (Hanna et al., 2006). Ubp6, when added to such proteasomes, progressively trims the ubiquitin chains of cyclin B. Since the APC modifies cyclin B extensively, the conjugates do not run far into the gel, making it difficult to detect further chain extension. We therefore used Ubp6 to reduce the extent of cyclin B ubiquitination (Figure 3E, compare lanes 2 and 7) while suppressing cyclin B degradation with the proteasome inhibitor PS341 and blocking Rpn11-mediated deubiquitinating activity with o-phenanthroline. After 20 min of Ubp6-dependent processing, Hul5 was added, resulting in efficient addition of ubiquitin groups to the cyclin B conjugate (Figure 3E). Importantly, Hul5 was inactive on cyclin B that had not been ubiquitinated by the APC (Figure 3E, lane 6), indicating that Hul5 does not function as an E3 for cyclin B, but rather as an E4.

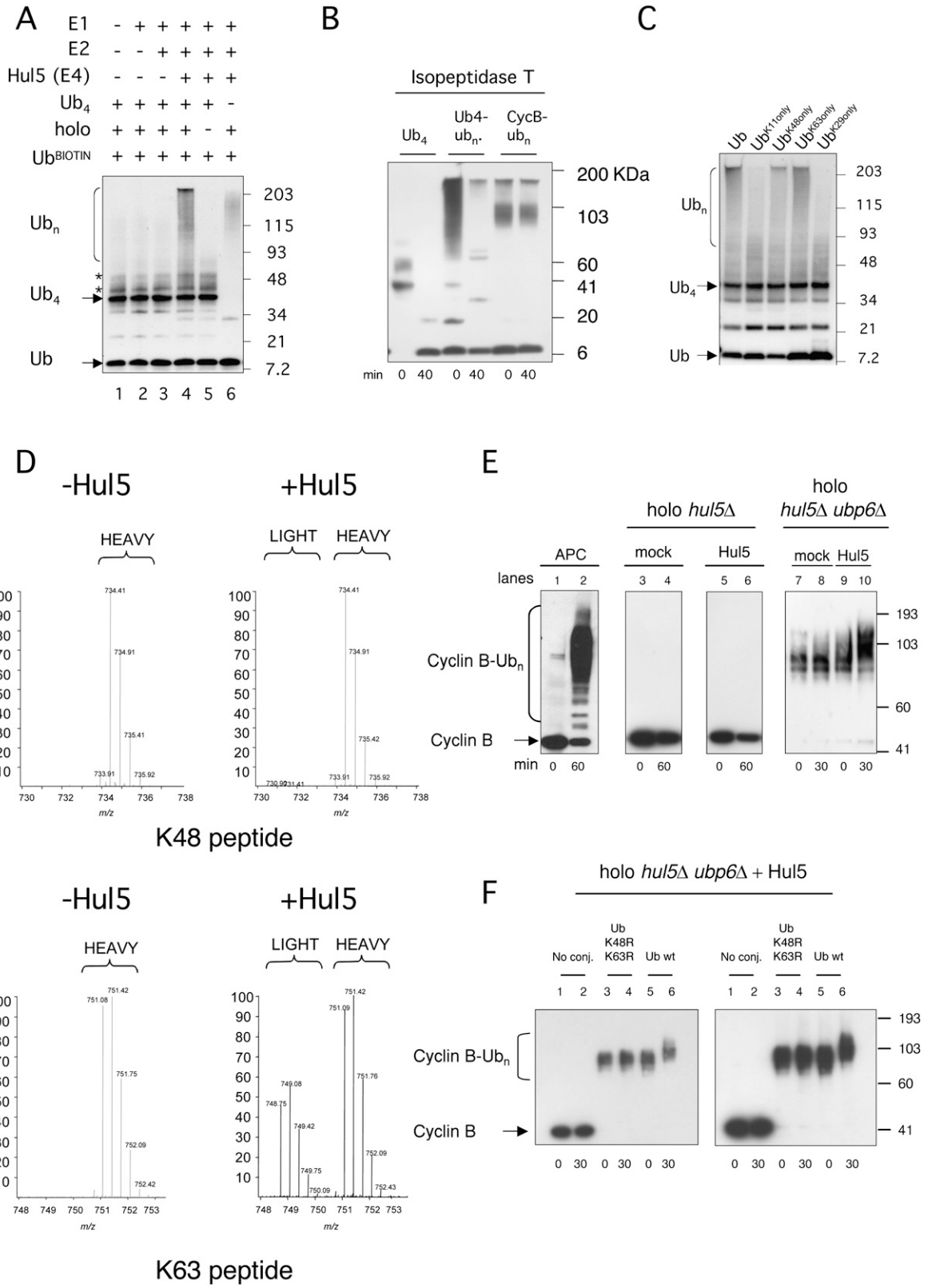
Although Figure 3E shows that Hul5 acts preferentially on previously ubiquitinated cyclin B, it does not show that Hul5 elongates pre-existing ubiquitin chains on cyclin B. Alternatively, Hul5 might conjugate ubiquitin to lysine residues of cyclin B. According to the E4 model, the ubiquitin chains on cyclin B should both target the conjugate to Hul5 and provide the acceptor lysine residues for further ubiquitination. The latter possibility was tested by preparing ubiquitin cyclin B conjugates with mutant ubiquitin lacking Lys48 and Lys63, the two lysines that Hul5 preferentially utilizes for chain extension. Unlike Hul5, the APC can utilize Lys11 in addition to Lys48 and Lys63, and Lys48 is not required for in vitro degradation of ubiquitinated cyclin B by *ubp6Δ* proteasomes (Kirkpatrick et al., 2006). Conjugates lacking Lys48- and Lys63-linked ubiquitin chains were also degraded by purified yeast proteasomes (*ubp6Δ*) with an efficiency close to that of conjugates synthesized with WT ubiquitin (data not shown). However, when Hul5 was challenged with such conjugates in the presence of WT free ubiquitin as a donor, it failed to catalyze significant ubiquitination (Figure 3F). These results imply that Hul5 elongates preformed ubiquitin chains on cyclin B, as expected of an E4 enzyme.

Hul5 Extends Ubiquitin Chains on Rpn10

To examine how Hul5 might promote proteasome function in vivo, we searched for endogenous ubiquitin conjugates that were hypoubiquitinated in the *hul5Δ* mutant. Ubiquitin was expressed in a 6-histidine-tagged form, and total cellular conjugates were isolated by Ni-NTA-affinity chromatography under nondenaturing conditions and in the presence of proteasome inhibitor. The eluates were subjected to mass spectrometry and to immunoblot analysis with antibodies to various proteins in experiments to be reported more fully elsewhere. Most notably, proteasome subunit Rpn10 was observed via this enrichment procedure to be extensively modified by ubiquitination (Figure 4A). Rpn10 was seen to be modified in both the WT and *hul5Δ* samples, but species bearing more than four ubiquitin groups were much reduced in *hul5Δ* samples (Figure 4A). Thus, Hul5 may act as an E4 for Rpn10 in vivo. The observation of Rpn10 ubiquitination led us to examine its turnover. Surprisingly, Rpn10 is an unstable protein and undergoes Hul5-dependent degradation (Figure 4B). Thus, ubiquitin chain extension by Hul5 appears to promote degradation of this novel proteasome substrate.

We next assayed Hul5 E4 activity on oligo-ubiquitinated Rpn10. We performed conjugation assays with WT and *hul5Δ* proteasomes in the presence of a chromatographic fraction (fraction 8; see Supplemental Data) that was active in forming oligo-ubiquitinated Rpn10. Fraction 8, prepared from a *hul5Δ* strain, catalyzed oligo-ubiquitination of Rpn10 by means of a yet uncharacterized E3. When this reaction was supplemented with *hul5Δ* proteasomes, Rpn10 conjugates did not exceed four ubiquitin moieties (Figure 4C, lane 5), as found for endogenous Rpn10 conjugates recovered from *hul5Δ* cells (Figure 4A, right lane). In contrast, incubations with WT proteasomes produced strong polyubiquitination of Rpn10 (Figure 4C, lane 10). Proteasome inhibitor PS341 dramatically increased the accumulation of high-molecular-weight conjugates (Figure 4C, compare lanes 9 and 10), suggesting that this pool of conjugates includes degradative intermediates of Rpn10. Thus, formation of long conjugates by Hul5 is apparently linked to Rpn10 proteolysis in vitro, as observed in vivo in Figures 4A and 4B.

Affinity-purified proteasomes are associated with ubiquitin conjugates, probably substoichiometrically (Chen and Madura, 2002; Elsasser et al., 2004). The presence of these conjugates could reflect their capture by the proteasome as well as conjugate synthesis at the proteasome. Suggestive of the latter possibility, proteasome-associated conjugates were severely reduced in abundance in *hul5Δ* mutants, and their apparent molecular weights were likewise strongly reduced (Figure 5A). Analysis of proteasome-associated species by two-dimensional IEF/SDS-PAGE (Figure 5A, right panels) and by mass spectrometry (data not shown) indicated that they were heterogeneous. While reduced in *hul5Δ* mutants, the endogenous proteasome-associated conjugates were elevated in *ubp6Δ* mutants (Figure 5B), presumably reflecting that they are substrates of Ubp6 in WT cells. Accordingly, recombinant Ubp6 converted the ubiquitin immunoreactive material in these conjugates into low-molecular-weight species (Figure 5C, lane 5). When the catalytic site of Ubp6 was inactivated by C118A substitution, the conjugates were not processed (Figure 5C, lane 7), indicating that processing is mediated by the deubiquitinating activity of Ubp6. Since Ubp6 is activated by the proteasome (Leggett et al., 2002; Hanna et al., 2006), processing likely occurs on the proteasome. In support of this idea, the Ubl domain of Ubp6, which mediates complex


A
E1
E2
Hul5 (E4)
Ub4
holo
UbBIOTIN
Ubn
Ub4
Ub
203
115
93
48
34
21
7.2
1 2 3 4 5 6
B
Isopeptidase T
Ub4
Ub4-ubn.
CycB-ubn
200 KDa
103
60
41
20
6
min 0 40 0 40 0 40
C
Ub
UbK11only
UbK48only
UbK63only
UbK29only
Ubn
Ub4
Ub
203
115
93
48
34
21
7.2
D
-Hul5
+Hul5
HEAVY
LIGHT HEAVY
Relative Abundance
m/z
K48 peptide
-Hul5
+Hul5
HEAVY
LIGHT HEAVY
Relative Abundance
m/z
K63 peptide
E
holo hul5Δ
holo hul5Δ ubp6Δ
APC
mock
Hul5
mock Hul5
lanes 1 2 3 4 5 6 7 8 9 10
Cyclin B-Ubn
Cyclin B
min 0 60 0 60 0 60 0 30 0 30
193
103
60
41
F
holo hul5Δ ubp6Δ + Hul5
No conj.
Ub K48R K63R
Ub wt
1 2 3 4 5 6
Cyclin B-Ubn
Cyclin B
0 30 0 30 0 30 0 30 0 30 0 30
193
103
60
41

formation with the proteasome, was also required for this reaction (Figure 5C, lane 6).

Interestingly, in cells lacking Hul5, deletion of *UBP6* did not enhance the levels of proteasome-associated ubiquitin conjugates (Figure 5B), suggesting that the disassembly of conjugates synthesized by Hul5 is an important role of Ubp6. We suggest that Hul5 mediates the formation of these proteasome-bound conjugates and that addition of Ubp6 to the isolated complex disassembles the endogenous conjugates as seen with conjugates synthesized by Hul5 in vitro (Figure 2B). Moreover, the endogenous conjugates observed in Figure 5 may serve as the ubiquitin acceptors in the in vitro experiments of Figure 2A. If true, this would imply that Hul5 serves to enhance the recovery on proteasomes of ubiquitin conjugates that it can further ubiquitinate in vitro and that in this assay the products of Hul5 conjugation are themselves the major substrates of Hul5.

Hul5 and Ubp6 Function in Opposition

The analyses of endogenous and in vitro synthesized ubiquitin conjugates in Figures 2–5 suggest that ubiquitin chains bound to the proteasome exist in a dynamic state, with both elongation and attrition of chains occurring. The principal mediators of these reactions appear to be Hul5 and Ubp6. These observations led us to test for functional opposition between these enzymes in vivo. Figure 6A shows that deletion of *HUL5* results in substantial suppression of the previously reported (Amerik et al., 2000; Leggett et al., 2002) canavanine sensitivity of *ubp6Δ* mutants. This suggests that the phenotypic defect in *ubp6Δ* mutants is partly attributable to the presence of Hul5, in that Hul5 activity is unopposed by Ubp6 in the mutant. Another ubiquitin ligase known to bind proteasomes, the E3 enzyme Ufd4 (Xie and Varshavsky, 2000, 2002), was inactive in this suppression assay (Figure 6A). It is currently unclear whether the suppressive effect of *HUL5* deletion is mediated through increased ubiquitin levels or some other mechanism.

Deletion of the gene encoding Ubc4, an E2 enzyme, was found to suppress *ubp6Δ* to approximately the same extent as *HUL5*, possibly because Ubc4 collaborates with Hul5 in ubiquitin conjugation (Figure 6A). Null mutations in genes encoding six other E2 enzymes failed to suppress, indicating that the assay is specific (Figure 6A). Ubc4 is involved in the degradation of short-lived proteins (Seufert and Jentsch, 1990), and significantly it is, like Hul5, found in association with the proteasome (Tongaonkar et al., 2000; Chuang and Madura, 2005). Moreover, our in vitro ubiquitin conjugation assays show that Hul5 and Ubc4 productively interact (Figure 3A). Therefore, Ubc4 is a good candidate for the physiological partner of Hul5. The ability of *hul5Δ* and *ubc4Δ* mutations to enhance growth of *ubp6Δ* mutants in the presence of canavanine is striking, because both *hul5Δ* and *ubc4Δ* mutants exhibit canavanine sensitivity in other contexts (Seufert and Jentsch, 1990; data not shown).

The specific relationship between Hul5 and Ubp6 was also apparent from compositional analysis of proteasomes isolated from the corresponding null mutants. Under relatively stringent conditions of proteasome preparation, the recovery of Hul5 on *ubp6Δ* proteasomes was significantly reduced (Figure 6B). However, it was unclear how *hul5Δ* could suppress *ubp6Δ* if Hul5 were not bound to proteasomes in vivo in the *ubp6Δ* mutant. We therefore isolated proteasomes again from *ubp6Δ* mutants, but in the absence of salt. These proteasomes were immobilized on beads, and the column was step-eluted with NaCl in 25 mM increments. Hul5 dissociated from WT proteasome primarily at 175–200 mM NaCl but from the *ubp6Δ* proteasome at 50–75 mM (Figure 6C). Substitution of the active site cysteine of Ubp6 had little or no effect on the salt dependence of the Hul5-proteasome association (Figure 6C). Thus, the presence of Ubp6, whether in an active

Figure 3. Ubiquitin Chain-Extending Activity of Hul5

(A) Hul5 can extend unanchored multiubiquitin chains in a proteasome-dependent reaction. Ubiquitin-conjugation assays were performed in reactions supplied with ubiquitin, E1, E2 (UbcH5), Hul5, proteasome holoenzyme (holo), and tetraubiquitin (Ub_4) as shown. Reaction products were analyzed by SDS-PAGE and antiubiquitin immunoblotting. Proteasomes were derived from a *hul5Δ ubp6Δ* strain and had been previously incubated with Ubp6 to reduce antiubiquitin immunoreactive material (see Supplemental Data). The two asterisked bands just above Ub_4 are minor contaminants present in the input Ub_4, presumably Ub_5 and Ub_6 (data not shown).

(B) Tetraubiquitin (1 μg), tetra-Ub (1 μg) extended by Hul5 as in (A), and APC-generated cyclin B conjugates were incubated with isopeptidase T (0.1 μg) for 40 min at 30°C. Cyclin B-ubiquitin conjugates, a negative control, are not expected to be digested with isopeptidase T.

(C) As in (A), but the added free ubiquitin contained in each case only a single lysine residue, as indicated, to support chain formation.

(D) Full MS scan spectra corresponding to either the K48 and K63 -GG signature peptides as measured in high-molecular-mass gel regions from complete reactions with or without addition of Hul5 (lanes 3–4 from [A]). The m/z range shown includes both the digested native peptide as well as its corresponding isotopically labeled internal standard peptide. The spectra shown represent an average of all MS spectra collected during peak elution.

(E) E4 activity of Hul5 on cyclin B conjugates. Cyclin B conjugates were formed by the APC in 1 hr incubations (lanes 1 and 2). Conjugation assays performed with Hul5 and proteasomes but without prior APC treatment did not show detectable ubiquitination (lanes 3–6). Cyclin B conjugates were partially trimmed by adding purified Ubp6 then incubating for 20 min. Ubp6 was then inhibited by adding ubiquitin-aldehyde. Hul5-dependent ubiquitination was observed on cyclin B conjugates partially trimmed by Ubp6 (lanes 7–10). Proteasomes were incubated with 10 μM PS341 and 10 mM o-phenanthroline prior to reactions, and conjugation reactions were supplied with E1 and Ubc4. Mock reactions were supplemented with BSA instead of Hul5. Here and in (F), conjugates were visualized with antibody to cyclin B (Kirkpatrick et al., 2006).

(F) Cyclin B (lanes 1 and 2) and cyclin B conjugates generated by the APC using either ubiquitin carrying K48R and K63R mutations (lanes 3 and 4) or WT ubiquitin (lanes 5 and 6) were washed and subsequently incubated with proteasomes, Hul5, and WT ubiquitin as in (E). Different exposures of the same gel are shown.

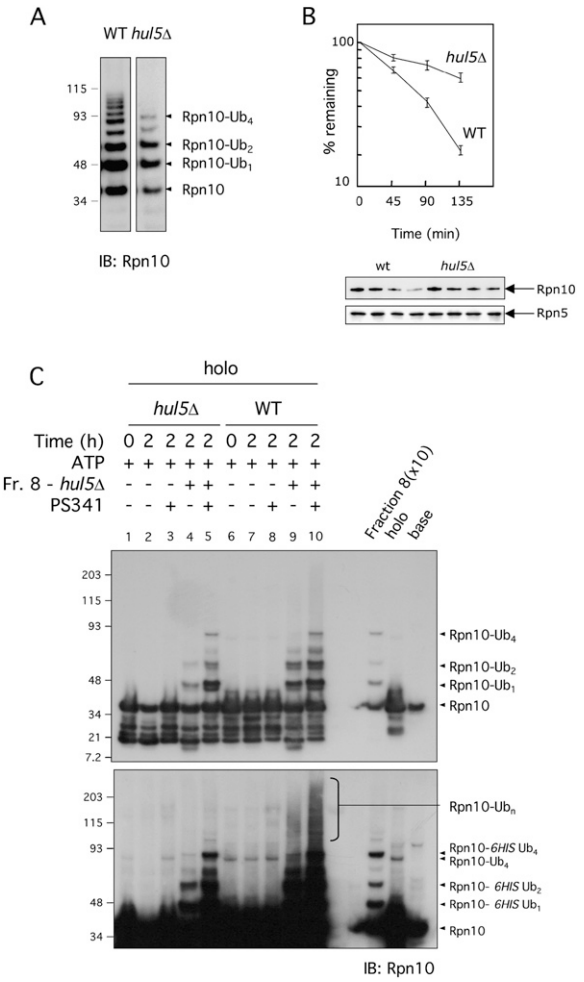


Figure 4. Hul5 Promotes Rpn10 Ubiquitination and Turnover

(A) Ubiquitination of endogenous Rpn10. Purification of whole-cell 6HIS-ubiquitin-protein conjugates under nondenaturing conditions, from SJR125 (WT) and SBC9 (*hul5Δ*) strains (see Supplemental Data for details). Conjugates were eluted with imidazole and analyzed by SDS-PAGE and anti-Rpn10 immunoblotting.

(B) Cycloheximide chase analysis of Rpn10 in WT and *hul5Δ* cells. Rpn5 was used as a loading control. Values represent the average of three independent experiments. Error bars show standard deviation from the mean.

(C) In vitro analysis of Hul5-dependent Rpn10 ubiquitination. WT and *hul5Δ* proteasomes were incubated with ATP, 6HIS-ubiquitin, PS-341, and *hul5Δ* extract (fraction 8) containing Rpn10 oligo-ubiquitinating activity. The lower panel shows a longer exposure.

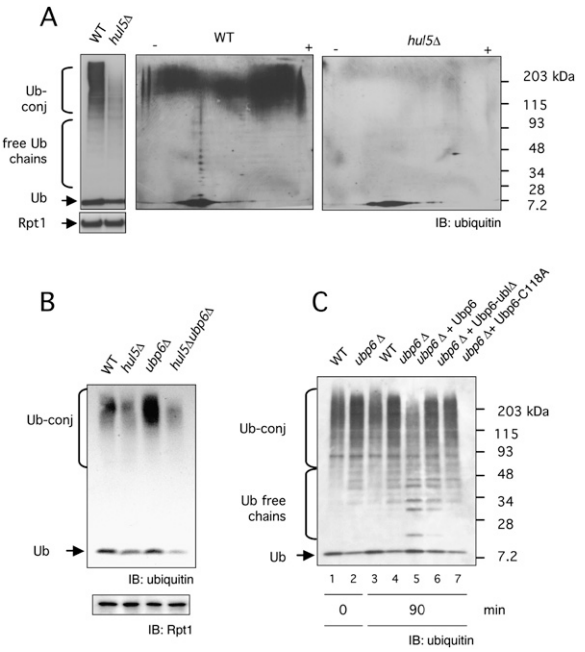


Figure 5. Antagonistic Effects of Hul5 and Ubp6 on the Recovery of Endogenous Conjugates with Affinity-Purified Proteasomes

(A) Proteasomes (2 μg) from WT and *hul5Δ* strains were analyzed by SDS-PAGE (left panel) or by two-dimensional isoelectric focusing/SDS-PAGE followed by antiubiquitin immunoblotting (center and right panels). Rpt1 was used as a loading control. Ub∼conj indicates ubiquitin-protein conjugates.

(B) Proteasomes from WT and mutant strains were analyzed by SDS-PAGE and antiubiquitin immunoblotting.

(C) WT or *ubp6Δ* proteasomes (12 nM) were incubated with ATP (1 mM) and a 4-fold molar excess of the indicated recombinant Ubp6 construct. Reactions were stopped by the addition of 5× SDS-loading buffer and analyzed by SDS-PAGE and immunoblotting. Note that a second deubiquitinating enzyme, Rpn11, appears to be largely inactive against the ubiquitin immunoreactive material.

or an inactive form, stabilizes the association of Hul5 with proteasomes. A possible consequence of this would be that Hul5 partitions predominantly onto Ubp6-containing proteasomes in vivo. However, Ubp6 is not essential for recognition of Hul5 by proteasomes. Therefore, to better understand the association of Hul5 with proteasomes, we attempted to identify the primary Hul5 receptor.

We first examined subassemblies of the proteasome, which are stable and can be purified in large amounts. These consisted of the 28-subunit CP, the 19-subunit RP, and the two complexes that make up the RP: the nine-subunit base, which is proximal to the CP, and the nine-subunit lid, which is distal to the CP (Glickman et al., 1998; Schmidt et al., 2005). Hul5 was recognized by the RP and its constituent, the base, but it was not recognized by the CP or the lid (Figure 7A). We also tested eight proteasome subunits individually for Hul5 binding, and among these Rpn2 and Rpn10 were positive (Figure 7B). However, Rpn2 bound Hul5 in an approximately stoichiometric complex, while Rpn10 captured Hul5 inefficiently, according to Coomassie blue staining intensities (Figure 7C). Rpn10 may not be a physiologically significant mediator of Hul5 binding because proteasomes isolated from *rpn10Δ* strains have WT levels of Hul5 (Figure 7D), whereas the assignment of Rpn2 as the physiological Hul5-binding subunit is supported by Hul5′s binding to the base, which contains Rpn2. In summary, these data suggest that Rpn2 is the principal proteasomal receptor for Hul5. Rpn2 and the related

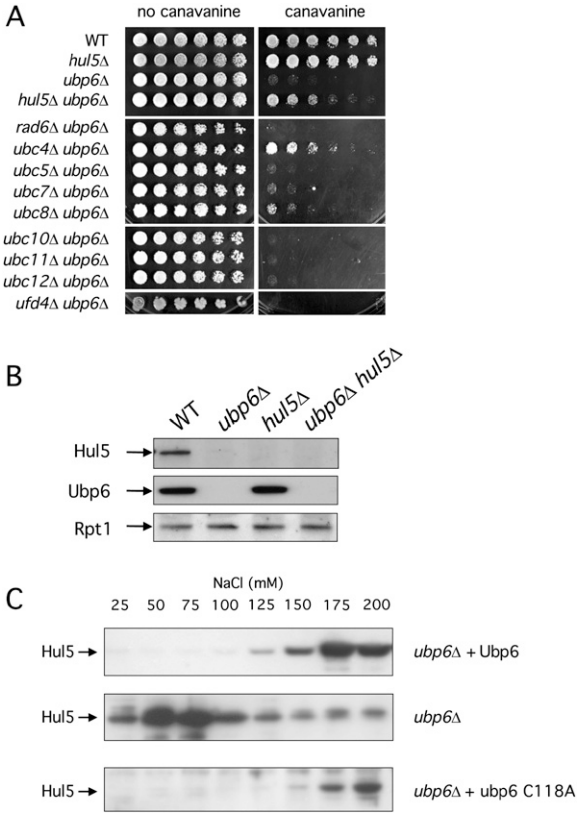


Figure 6. Genetic and Biochemical Interactions between Hul5 and Ubp6

(A) Cells were spotted onto selective plates with or without 0.8 μg/ml canavanine sulfate and grown at 30°C for 3 days (above). *ubc* (E2) and *ufd4* (E3) mutants were also screened for suppression of *ubp6Δ*-associated canavanine sensitivity.

(B) Proteasomes from WT and mutant strains were extensively washed (500 bed vol.) prior to elution. Equal protein amounts were analyzed by SDS-PAGE and immunoblotting.

(C) Proteasomes from WT, *ubp6Δ,* and *ubp6–C118A* mutants were bound to resin and washed with a NaCl gradient. Washes were collected and analyzed by SDS-PAGE and immunoblotting.

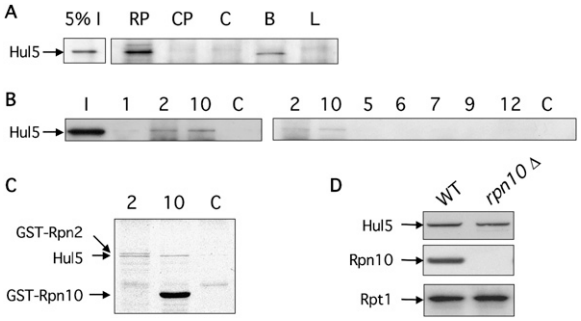


Figure 7. Proteasome Subunit Rpn2 Binds Hul5

(A) Immobilized RP, CP, base (B), and lid (L) were prepared as described (Leggett et al., 2002). A control sample (C) was from a congenic, untagged strain. Hul5 was bound to resin, and, after washing, protein remaining bound to the column was analyzed by SDS-PAGE and Hul5 immunoblotting. The leftmost lane represents 5% of input Hul5.

(B) GST fusions of proteasome subunits and a GST control (C) were bound to glutathione Sepharose and tested for Hul5 binding. Numerical lane designations indicate the identity of the Rpn subunit tested: Rpn1, Rpn2, Rpn10, and so forth. Beads were treated as in (A). Equal amounts of beads were analyzed by SDS-PAGE and immunoblotting.

(C) GST-Rpn2 and GST-Rpn10 beads were loaded with Hul5. Proteins were then eluted with glutathione and visualized by Coomassie staining after SDS-PAGE.

(D) Equal amounts of ProA-TEV-Rpt1-tagged proteasomes, affinity-purified from WT and *rpn10Δ* mutants, were analyzed by SDS-PAGE and immunoblotting.

proteasome subunit Rpn1, which is apparently the major proteasomal binding site for Ubp6 (Leggett et al., 2002; Stone et al., 2004), may together provide a scaffold that brings Hul5 and Ubp6 into proximity.

DISCUSSION

A diverse set of reversibly bound proteins participates in and regulates the function of the proteasome. Affinity purification, mass spectrometry, and other methods have allowed for the identification of hundreds of proteasome-associated proteins (see for example Verma et al., 2000; Cagney et al., 2001; Leggett et al., 2002; Guerrero et al., 2006). These proteins remain poorly characterized on the whole and probably represent a mixture of proteasome cofactors, ubiquitin-protein conjugates, and non-specifically associated proteins. However, only a few proteins are associated with proteasomes in high amounts (Verma et al., 2000; Leggett et al., 2002), and each of these tested so far modulates proteasome function and associates with proteasomes in both yeast and mammals (reviewed by Schmidt et al., 2005).

Among the abundant proteasome-associated proteins is a ubiquitin ligase, Hul5. The ligase activity of Hul5 has thus far not shown discriminatory capacity for the acceptor protein of the ubiquitin conjugate that it takes as a substrate. Hul5 is active on bulk protein associated with the proteasome, on synthetic tetraubiquitin chains, on ubiquitin-Rpn10 conjugates, and on ubiquitin-cyclin B conjugates. The relaxed in vitro specificity of Hul5 parallels its effects on protein turnover in vivo, where it was seen to stabilize a variety of substrates. Since all substrates examined are ubiquitinated by known E3 enzymes, it is difficult to envisage Hul5 functioning as an E3 for these degradative events. In contrast to E3 enzymes, E4 enzymes are specialized in the elongation of ubiquitin chains and may characteristically lack the capacity to recognize specific determinants in the target protein (Koegl et al., 1999; Richly et al., 2005). Instead, our data and those of Richly et al. (2005) suggest that their specificity of action may be determined principally by the E4's association with a particular protein complex. The E4-like activity of Hul5 is indicated not only by its specificity for ubiquitinated forms of cyclin B but also by its ability to elongate tetraubiquitin chains. These engineered chains are not attached

to a target protein, so an E3 mode of conjugate recognition is not possible in this case.

A Hul5 substrate of particular interest is Rpn10, a proteasomal ubiquitin receptor. Rpn10 is the first proteasome subunit to be identified as a proteasome substrate and as a major target of ubiquitination, although it should be noted that a nonproteasomal pool of Rpn10 exists (van Nocker et al., 1996). Hul5 appears to function as an E4 for Rpn10 because its presence is not required for the generation of mono- or diubiquitinated forms of Rpn10 but is required to form extensively modified species of Rpn10 in vivo and in vitro. Many ubiquitin receptors are ubiquitinated (Kumar et al., 1999; Shih et al., 2003; Di Fiore et al., 2003; Hoeller et al., 2006), and these modifications are thought to be regulatory in nature. Conjugated ubiquitin often blocks receptor function, apparently by occupying the ubiquitin-binding site of the receptor. In contrast, chain extension by Hul5 appears to be critical for efficient Rpn10 degradation in vivo and in vitro. An interesting question is why ubiquitination targets Rpn10 for degradation, given that Rpn10 can associate with the proteasome in the absence of this modification.

We have thus far not found Hul5 to fully stabilize any substrate in vivo under standard growth conditions. These observations seem consistent with our in vitro data; since Hul5 does not seem to function efficiently unless the conjugate is already bound to the proteasome, Hul5 may be inherently unable to promote the initial recognition of a substrate by the proteasome. These properties of Hul5 would predict a facilitative rather than obligatory role in degradation. If Hul5 is, as we propose, irrelevant to the initial recognition event, it could promote substrate turnover by slowing dissociation of the substrate from the proteasome, thus increasing the probability of degradation as the outcome of a particular substrate-proteasome-binding event.

Opposing Activities of Hul5 and Ubp6

Our data suggest that the chain-elongating activity of Hul5 functions specifically in opposition to deubiquitination by Ubp6. That is, Hul5 serves not simply to elongate chains but to preserve chains against attrition by Ubp6. Although opposing pairs of ligases and deubiquitinating enzymes are probably rare, several have been described (Wertz et al., 2004; Rumpf and Jentsch, 2006; Kee et al., 2005; and references therein). Hul5 and Ubp6 appear to provide the first example of an E4 and a deubiquitinating enzyme that reside simultaneously on the same protein complex.

We recently found that Ubp6 can, for at least some substrates, act noncatalytically to inhibit the proteasome (Hanna et al., 2006). The degradation delay resulting from this activity of Ubp6 may be significant for the function of Hul5 because it provides a window of time necessary for refashioning of ubiquitin chains by Hul5 and Ubp6. When Ubp6 inhibits the proteasome, it also prevents chain removal by Rpn11, directly or indirectly. Thus, Ubp6 opposes Rpn11 via its noncatalytic activity and Hul5 via its catalytic activity.

The chain-extending activity of Hul5 may promote degradation of a protein by producing a longer chain that binds the proteasome more tightly. The greater effectiveness of long chains in targeting degradation (Thrower et al., 2000) may reflect their ability to engage multiple proteasomal ubiquitin receptors. A second possible view is that Hul5′s chain-extending activity rarely affects net chain growth but serves to prevent Ubp6 from shortening the chain to such an extent that it can no longer tether the substrate to the proteasome. In either scenario, the combined effect of Hul5 and Ubp6 in regulating the lengths of chains on proteasome-bound conjugates may function principally to modulate the dissociation rate of substrate from the proteasome (Varshavsky, 1996) and, as a consequence, the probability of the substrate's degradation.

ATP-dependent proteases from eukaryotes and prokaryotes share many properties but differ in that only eukaryotic systems show ubiquitin dependence. An important aspect of this difference that, to our knowledge, has not been commented upon is that the reversibility of the ubiquitin targeting signal allows eukaryotic proteasomes in particular to play an active role in modulating the strength of the targeting signal. Our studies suggest that Hul5 and Ubp6 are key factors that provide this capacity.

A Scaffold on the Proteasome May Organize Factors that Act on Ubiquitin Chains

Many of the proteins that mediate the dynamics of ubiquitin chains on the proteasome are not true proteasome subunits but are weakly associated factors. This applies to Hul5, Ubp6, and Ubl-Uba proteins, such as Rad23, that are major mediators of ubiquitin conjugate delivery to the proteasome. Thus, chain delivery, elongation, and attrition are all mediated at least in part through proteasome-associated proteins. Interestingly, proteasome subunits Rpn1 and Rpn2 appear to serve together as a scaffold that organizes these factors. Rpn1 is a receptor for both Rad23 and Ubp6 (Elsasser et al., 2002; Leggett et al., 2002; Saeki et al., 2002; Seeger et al., 2003; Elsasser et al., 2004; Stone et al., 2004), whereas Rpn2 binds Hul5 avidly (Figure 7). In addition, both Rpn1 and Rpn2 crosslink to Rad23 (Saeki et al., 2002), suggesting that Rpn1 and Rpn2 are close neighbors. Rpn1 and Rpn2 are both components of the base, have similar structural motifs and size (Kajava, 2002), may bind one another (Gorbea et al., 2000), and are thought to have evolved from a common ancestor. Thus we propose that Rpn1 and Rpn2 impose a close alignment of Rad23, Ubp6, and Hul5 on the proteasome. The stabilization of Hul5 binding to the proteasome by Ubp6 supports this view. The detailed organization of these factors may be significant since both Hul5 and Ubp6 appear to work efficiently only in the context of the proteasome (Leggett et al., 2002; Hanna et al., 2006; this work). This may be due in part to the proteasome playing a role in the presentation of ubiquitin chains to these enzymes.

Convergence of Cdc48 and the Proteasome

Cdc48, also known as p97 and VCP, is a homohexameric ATPase ring complex. It falls into the AAA family of ATPases, which also includes the Rpt subunits of the proteasome. Interestingly, many AAA proteins exhibit protein-unfolding activity. Recent studies have revealed that Cdc48 is an intricate assembly for the processing of ubiquitin-protein conjugates (Richly et al., 2005; Rumpf and Jentsch, 2006; reviewed in Elsasser and Finley, 2005). Some proteins that are ubiquitinated and destined for degradation are shuttled first to Cdc48, where their ubiquitin chains are extended to a length required for association with the proteasome (Richly et al., 2005). These remarkable activities of Cdc48 are not intrinsic to its ATPase rings but reflect the properties of associated cofactors: ubiquitin receptors such as Ubx-Uba proteins, an E4 enzyme known as Ufd2, and the Otu1 deubiquitinating enzyme (Rumpf and Jentsch, 2006). Based in part on the present results, it can be seen that the ensemble of Cdc48 cofactors is deeply similar to that of the proteasome. Thus, Ubx-Uba proteins are cognates of Ubl-Uba proteins, Otu1 is a cognate of Ubp6, and Ufd2 is a cognate of Hul5. This represents a dramatic evolutionary convergence, especially because for the most part, these factors cannot have been produced by a recent evolutionary bifurcation. For example, while Hul5 is a HECT-domain ubiquitin ligase, Ufd2 is a member of the U-box family of ligases. Similarly, Ubp6 falls into the DUB family of ligases, and Otu1 falls into the OTU family. Even Cdc48 apparently diverged from its sister subunits in the proteasome far before the eukaryotic radiation and thus prior to the evolution of ubiquitination.

Despite these similarities, key properties of these complexes differ, and in particular Hul5 differs functionally from Ufd2. For example, we have found that the binding of Hul5 to proteasomes is promoted by Ubp6. In contrast, the binding of Ufd2 and Otu1 to the same Cdc48 complex is thought to be disfavored (Rumpf and Jentsch, 2006), so that Ufd2 and Otu1 presumably act on distinct conjugates while positioned on different complexes. The Ufd2 reaction, as presently understood, stereotypically takes substrates with chains of one or two ubiquitin groups and extends them to approximately six groups (Richly et al., 2005). The Hul5 reaction seems to embrace a greater range of chain lengths and to be more dynamic, in that it involves a competition with chain attrition by Ubp6.

Although it is the final arbiter of protein degradation, the proteasome has traditionally been viewed as having a passive role. It has been assumed that the fate of individual substrates has effectively been determined before their engagement by the proteasome, primarily through the activity of E3 enzymes. The discovery that Hul5 and Ubp6 mediate powerful ubiquitin-chain-remodeling activities on the proteasome suggests that this view may be too simplistic. In a multistep pathway such as the ubiquitin-proteasome system, the capacity to control the final and only irreversible step in protein breakdown should be advantageous. Our data suggest a new discriminatory step in protein breakdown, in which substrates are actively scrutinized by the proteasome.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

Media are described in the Supplemental Data. Strain genotypes are given in Table S1.

Purification of Hul5, Proteasomes, and Proteasome Subcomplexes

Hul5 was purified using Hul5-TEV-ProA (SBC8) following the procedure described for proteasome purification (Leggett et al., 2002), except that binding and washing buffers were 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 300 mM NaCl. Procedures for proteasome purification are given in the Supplemental Data.

Assays of Ubiquitin-Protein Ligase Activity

Incubations were performed in 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM EDTA, 5 mM $MgCl_2$, and 5 mM ATP buffer in 100 μl reactions at 30°C supplied with 2 μg of holoenzyme, 0.1 μg of E1, and 1 μg of either biotinylated, 6-histidine-tagged ubiquitin or mutated ubiquitins. Assays with WT ubiquitin contained 21 μg of this molecule per reaction. Depending on the reaction, additional reagents included 0.1 μg Hul5, 0.1 μg E2 (Ubc4 or UbcH5), 10 μM PS341, 10 mM o-phenanthroline, 0.1 μg tetraubiquitin, 0.5 μg ubiquitin chains, or 1 μg ubiquitin-aldehyde. When required, ATP was depleted by adding hexokinase (0.3 units/μl) and glucose (10 mM) for 15 min at 30°C. A description of preincubations of holoenzyme and conjugate substrates is in the Supplemental Data.

Protein Turnover Analysis

Cells were grown in YPD media. Logarithmically growing cells were treated with 100 μg/ml cycloheximide, and time points were taken. Samples were analyzed by immunoblot, using HRP-conjugated secondary antibodies and ECL detection or ^{125}I-protein A followed by quantitation with a PhosphorImager.

Mass Spectrometry

Gel slices were incubated with trypsin. Digest products were mixed with isotope-labeled internal standard peptides for both K48- and K63-linked polyubiquitin chains. A fraction of the sample corresponding to 0.2 pmol of each internal standard peptide was analyzed by LC-MS/MS using an LTQ-FT mass spectrometer. The MS spectra shown were obtained as an average across the eluted peak.

Supplemental Data

Supplemental Data include three figures, two tables, Experimental Procedures, and References and can be found with this article online at http://www.cell.com/cgi/content/full/127/7/1401/DC1/.

ACKNOWLEDGMENTS

This work was supported by NIH grants GM65592 (D.F.), GM67945 (S.P.G.), and GM66492 (R.W.K.); Spanish Government (MEC) grant RyC3582 (B.C.); and a JSPS Postdoctoral Fellowship for Research Abroad (Y.T.). We thank Millennium Pharmaceuticals for the gift of PS341 and R. Baker, M. Hochstrasser, R. Vierstra, D. Kornitzer, R. Li, and C. Mann for antibodies. We also thank S. Elsasser and J. Roelofs for yeast strains.

Received: March 8, 2006
Revised: August 25, 2006
Accepted: September 29, 2006
Published: December 28, 2006

REFERENCES

Amerik, A.Y., Nowak, J., Swaminathan, S., and Hochstrasser, M. (2000). The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. Mol. Biol. Cell *11*, 3365–3380.

Cagney, G., Uetz, P., and Fields, S. (2001). Two-hybrid analysis of the Saccharomyces cerevisiae 26S proteasome. Physiol. Genomics *7*, 27–34.

Chen, L., and Madura, K. (2002). Rad23 promotes the targeting of proteolytic substrates to the proteasome. Mol. Cell. Biol. *22*, 4902–4913.

Chuang, S.M., and Madura, K. (2005). Saccharomyces cerevisiae Ub-conjugating enzyme Ubc4 binds the proteasome in the presence of translationally damaged proteins. Genetics *171*, 1477–1484.

Di Fiore, P.P., Polo, S., and Hofmann, K. (2003). When ubiquitin meets ubiquitin receptors: a signalling connection. Nat. Rev. Mol. Cell Biol. *4*, 491–497.

Elsasser, S., and Finley, D. (2005). Delivery of ubiquitinated substrates to protein-unfolding machines. Nat. Cell Biol. *7*, 742–749.

Elsasser, S., Gali, R.R., Schwickart, M., Larsen, C.N., Leggett, D.S., Müller, B., Feng, M.T., Tübing, F., Dittmar, G.A.G., and Finley, D. (2002). Proteasome subunit Rpn1 binds ubiquitin-like protein domains. Nat. Cell Biol. *4*, 725–730.

Elsasser, S., Chandler-Militello, D., Mueller, B., Hanna, J., and Finley, D. (2004). Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. J. Biol. Chem. *279*, 26817–26822.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell *94*, 615–623.

Gorbea, C., Taillandier, D., and Rechsteiner, M. (2000). Mapping subunit contacts in the regulatory complex of the 26S proteasome. J. Biol. Chem. *275*, 875–882.

Guerrero, C., Tagwerker, C., Kaiser, P., and Huang, L. (2006). An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. Mol. Cell. Proteomics *5*, 366–378.

Hanna, J., Hathaway, N.A., Tone, Y., Crosas, B., Elsasser, S., Kirkpatrick, D.S., Leggett, D.S., Gygi, S.P., King, R.W., and Finley, D. (2006). Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. Cell *127*, 99–111.

Hoeller, D., Crosetto, N., Blagoev, B., Raiborg, C., Tikkanen, R., Wagner, S., Kowanetz, K., Breitling, R., Mann, M., Stenmark, H., and Dikic, I. (2006). Regulation of ubiquitin-binding proteins by monoubiquitination. Nat. Cell Biol. *8*, 163–169.

Ju, D., Wang, L., Mao, X., and Xie, Y. (2004). Homeostatic regulation of the proteasome via an Rpn4-dependent feedback circuit. Biochem. Biophys. Res. Commun. *321*, 51–57.

Kajava, A.V. (2002). What curves alpha-solenoids? Evidence for an alpha-helical toroid structure of Rpn1 and Rpn2 proteins of the 26 S proteasome. J. Biol. Chem. *277*, 49791–49798.

Kee, Y., Lyon, N., and Huibregtse, J.M. (2005). The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. EMBO J. *24*, 2414–2424.

Kirkpatrick, D.S., Hathaway, N.A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R.W., and Gygi, S.P. (2006). Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nat. Cell Biol. *8*, 700–710.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635–644.

Kumar, S., Talis, A., and Howley, P. (1999). Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. J. Biol. Chem. *274*, 18785–18792.

Leggett, D.S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R.T., Walz, T., Ploegh, H., and Finley, D. (2002). Multiple associated proteins regulate proteasome structure and function. Mol. Cell *10*, 495–507.

London, M.K., Keck, B.I., Ramos, P.C., and Dohmen, J.R. (2004). Regulatory mechanisms controlling biogenesis of ubiquitin and the proteasome. FEBS Lett. *567*, 259–264.

Madura, K. (2004). Rad23 and Rpn10: perennial wallflowers join the melee. Trends Biochem. Sci. *29*, 637–640.

Meimoun, A., Holtzman, T., Weissman, Z., McBride, H.J., Stillman, D.J., Fink, G.R., and Kornitzer, D. (2000). Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. Mol. Biol. Cell *11*, 915–927.

Pickart, C.M., and Cohen, R.E. (2004). Proteasomes and their kin: proteases in the machine age. Nat. Rev. Mol. Cell Biol. *5*, 177–187.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell *120*, 73–84.

Rumpf, S., and Jentsch, S. (2006). Functional division of substrate processing cofactors of the ubiquitin-selective cdc48 chaperone. Mol. Cell *21*, 261–269.

Saeki, Y., Sone, T., Toh-e, A., and Yokosawa, H. (2002). Identification of ubiquitin-like protein-binding subunits of the 26S proteasome. Biochem. Biophys. Res. Commun. *296*, 813–819.

Schmidt, M., Hanna, J., Elsasser, S., and Finley, D. (2005). Proteasome-associated proteins: regulation of a proteolytic machine. Biol. Chem. *386*, 725–737.

Seeger, M., Hartmann-Petersen, R., Wilkinson, C.R., Wallace, M., Samejima, I., Taylor, M.S., and Gordon, C. (2003). Interaction of the anaphase-promoting complex/cyclosome and proteasome protein complexes with multiubiquitin chain-binding proteins. J. Biol. Chem. *278*, 16791–16796.

Seufert, W., and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. EMBO J. *9*, 543–550.

Shih, S.C., Prag, G., Francis, S.A., Sutanto, M.A., Hurley, J.H., and Hicke, L. (2003). A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. EMBO J. *22*, 1273–1281.

Stone, M., Hartmann-Petersen, R., Seeger, M., Bech-Otschir, D., Wallace, M., and Gordon, C. (2004). Uch2/Uch37 is the major deubiquitinating enzyme associated with the 26S proteasome in fission yeast. J. Mol. Biol. *344*, 697–706.

Swanson, R., Locher, M., and Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matα2 repressor degradation. Genes Dev. *15*, 2660–2674.

Thrower, J.S., Hoffman, L., Rechsteiner, M., and Pickart, C.M. (2000). Recognition of the polyubquitin proteolytic signal. EMBO J. *19*, 94–102.

Tongaonkar, P., Chen, L., Lambertson, D., Ko, B., and Madura, K. (2000). Evidence for an interaction between ubiquitin-conjugating enzymes and the 26S proteasome. Mol. Cell. Biol. *20*, 4691–4698.

van Nocker, S., Sadis, S., Rubin, D.M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R.D. (1996). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in Saccharomyces cerevisiae and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. *16*, 6020–6028.

Varshavsky, A. (1996). The N-end rule: functions, mysteries, uses. Proc. Natl. Acad. Sci. USA *93*, 12142–12149.

Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R.J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol. Biol. Cell *11*, 3425–3439.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R., 3rd, Koonin, E.V., and Deshaies, R.J. (2002). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science *298*, 611–615.

Verma, R., Oania, R., Graumann, J., and Deshaies, R.J. (2004). Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. Cell *118*, 99–110.

Wang, M., and Pickart, C.M. (2005). Different HECT domain ubiquitin ligases employ distinct mechanism of polyubiquitin chain synthesis. EMBO J. *24*, 4324–4333.

Wertz, I.E., O'Rourke, K.M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D.L., et al. (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-KB signaling. Nature *430*, 694–699.

Xie, Y., and Varshavsky, A. (2000). Physical association of ubiquitin ligases and the 26S proteasome. Proc. Natl. Acad. Sci. USA *97*, 2497–2502.

Xie, Y., and Varshavsky, A. (2001). RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc. Natl. Acad. Sci. USA *98*, 3056–3061.

Xie, Y., and Varshavsky, A. (2002). UFD4 lacking the proteasome-binding region catalyses ubiquitination but is impaired in proteolysis. Nat. Cell Biol. *4*, 1003–1007.

Yao, T., and Cohen, R.E. (2002). A cryptic protease couples deubiquitination and degradation by the proteasome. Nature *419*, 403–407.

You, J., and Pickart, C.M. (2001). A HECT domain E3 enzyme assembles novel polyubiquitin chains. J. Biol. Chem. *276*, 19871–19878.