A Multiubiquitin Chain Receptors Define a Layer of Substrate Selectivity in the Ubiquitin–Proteasome System

This chapter constitutes a further example of the use of multidimensional protein identification technology for the analysis of moderately complex polypeptide mixtures as resulting from affinity purification of protein complexes, in this case the proteasome from *Saccharomyces cerevisiae*. J. G.'s contribution to this work encompass advice on MudPIT compatible experiment design, MudPIT and data analysis as well as data presentation using `RAYzer` (see section 2.3.9). The copyright for the presented material, published as

> 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(1):99–110.

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A.1 Abstract

Recruitment of ubiquitinated proteins to the 26S proteasome lies at the heart of the ubiquitin–proteasome system (UPS). Genetic studies suggest a role for the multiubiquitin chain binding proteins (MCBPs) Rad23 and Rpn10 in recruitment, but biochemical studies implicate the Rpt5 ATPase. We addressed this issue by analyzing degradation of the ubiquitinated Cdk inhibitor Sic1 (UbSic1) *in vitro*. Mutant

*rpn10*Δ and *rad23*Δ proteasomes failed to bind or degrade UbSic1. Although Rpn10 or Rad23 restored UbSic1 recruitment to either mutant, rescue of degradation by Rad23 uncovered a requirement for the VWA domain of Rpn10. *In vivo* analyses confirmed that Rad23 and the multiubiquitin binding domain of Rpn10 contribute to Sic1 degradation. Turnover studies of multiple UPS substrates uncovered an unexpected degree of specificity in their requirements for MCBPs. We propose that recruitment of substrates to the proteasome by MCBPs provides an additional layer of substrate selectivity in the UPS.

A.2 Introduction

Proteolysis by the UPS is required for the maintenance of cellular homeostasis (Hershko and Ciechanover 1998; and Pickart and Cohen 2004). Proteins destined to be degraded by the proteasome are marked for elimination by the covalent attachment of ubiquitin (Ub). The C terminus of Ub is linked by an isopeptide bond to the α amino group of a lysine residue in the substrate. A multiubiquitin (multiUb) chain is formed by attachment of successive Ubs, primarily to the Lys48 residue of the distal–most Ub tethered to the substrate. Once the multiUb chain contains at least four Ubs, it can bind the proteasome and serve as a signal for degradation (Chau et al. 1989; and Thrower et al. 2000). Following specific binding, the ubiquitinated substrate is unfolded, deubiquitinated, and translocated by the 19S regulatory “cap” of the 26S proteasome into the 20S protease core, where it is proteolyzed to peptide remnants (Hershko and Ciechanover 1998; Verma et al. 2002; and Yao and Cohen 2002).

Recognition of multiUb chains by the proteasome is central to Ub–selective degradation. The receptor(s) that mediates this process has thus been sought intensively. Over the past decade, three different classes of proteins have been advanced as candidate receptors that link Ub conjugates to the proteasome for degradation. Rpn10 was the first protein that was shown to bind selectively to polyubiquitin (polyUb) chains. Because Rpn10 is a bona fide stoichiometric subunit of the 26S proteasome, it was proposed that Rpn10 is the multiUb chain receptor (Deveraux et al. 1994). However, even though proteasomal proteolysis is essential, Rpn10 is dispensable for life in budding yeast (Fu et al. 1998; and Van Nocker et al. 1996). Indeed, only one UPS substrate, Ub–proline–β–galactosidase (Ub–Pro–β–gal, or the related substrate Ub^{V76}–Valine–β–gal), has been shown to be stabilized in *rpn10*Δ cells, and, paradoxically, Ub–Pro–β–gal turnover does not require the Ub binding domain of Rpn10 (Fu et al. 1998). Additionally, Rpn10 assembled into 26S proteasomes does not crosslink to a chemically reactive tetraubiquitin chain (Lam et al. 2002), and recombinant Rpn10 inhibits proteolysis in frog extracts (Deveraux et al. 1995). Taken together, these observations raised doubts as to whether Rpn10 functioned in the context of the 26S proteasome to recruit ubiquitinated substrates for degradation (Pickart and Cohen 2004).

Attention was thus diverted to a second group of proteins exemplified by Rad23 and Dsk2. These proteins each contain a Ub–like domain (UbL) that binds the proteasome (Elsasser et al. 2002; Saeki et al. 2002b; and Schauber et al. 1998) and UBA domains that bind multiUb chains (Rao and Sastry 2002; and Wilkinson et al. 2001). However, the role of Rad23 and Dsk2 in guiding multiUb chain–bearing substrates to the proteasome is equally controversial. Budding and fission yeast *rad23*Δ and *dsk2*Δ mutants accumulate reporter substrates and high molecular

weight Ub conjugates, supporting a positive role for these proteins in the UPS (Chen and Madura 2002; Funakoshi et al. 2002; Rao and Sastry 2002; Saeki et al. 2002a; and Wilkinson et al. 2001). However, *rad23*Δ*rpn10*Δ double mutants are proficient in bulk turnover of short–lived proteins (Lambertson et al. 1999). Additionally, overexpression of Dsk2 or Rad23 in mammalian and yeast cells typically inhibits substrate turnover by the 26S proteasome (Kleijnen et al. 2000; and Ortolan et al. 2000) but can apparently stimulate turnover in some contexts (Funakoshi et al. 2002). Indeed, a key limitation to the argument that Rad23 and Dsk2 serve as substrate receptors is that such a role has never been directly demonstrated. In the only direct test so far of the hypothesis that Rad23 acts as a receptor that links substrates to the proteasome, it was shown that recombinant Rad23 actually inhibits substrate turnover by purified 26S proteasome *in vitro* (Raasi and Pickart 2003). Similar results have been reported for Rpn10 (Deveraux et al. 1995). In light of the lack of conclusive, direct evidence that Rad23 serves as a receptor to guide ubiquitinated substrates to the proteasome, other functions have been sought for this protein. Bioinformatics has revealed that the UBA domain is conserved in a number of enzymes of the UPS, including E2s, E3s, and Ub proteases (Ubps) (Hofmann and Bucher 1996). Some members of the latter class, such as Ubp14, bind polyUb chains and cleave them (Amerik et al. 1997). Although binding of Rad23 to Ub conjugates did not cause cleavage of the Ub chain, it did inhibit Ub chain assembly (Ortolan et al. 2000) as well as disassembly (Hartmann-Petersen et al. 2003; and Raasi and Pickart 2003), suggesting that Rad23 may promote degradation by serving as a shield that retards deubiquitination of substrates that are en route to the proteasome (Pickart and Cohen 2004).

To complicate matters further, a third candidate receptor (S6'/Rpt5) has re-

cently been identified based on UV crosslinking of a tetra–Ub chain to purified 26S proteasomes (Lam et al. 2002). Rpt5 is a member of the AAA ATPase family of enzymes, with an as yet undefined multiUb chain binding domain. A putative receptor function for Rpt5 is appealing based on precedent from other systems. The related AAA ATPases of bacterial compartmentalized proteases contribute to enzyme specificity by directly binding to short peptide degrons within substrates (Flynn et al. 2003), and the mammalian AAA ATPase p97/Cdc48 promotes turnover of IkB by binding directly to multiubiquitin chains (Dai and Li 2001). However, a functional role for S6'/Rpt5 in recruiting ubiquitinated substrates to the proteasome has not been validated yet by either biochemical or genetic studies.

The studies summarized above highlight several key unresolved issues. For example, what is the nature of the primary gateway through which proteins targeted by the numerous cellular ubiquitin ligases are recognized by the proteasome and sent to meet their final fate? Is there a single gateway (e. g., Rpt5) or multiple gateways (e. g., Rad23, Rpn10, and other Ub binding proteins)? If the latter, do the gateways function in parallel or in series? Are all ubiquitinated substrates processed in the same manner, or is there an additional layer of substrate specificity downstream of the ubiquitin ligases? In this work, we employ a combination of *in vitro* reconstitution and *in vivo* turnover assays to address these questions.

A.3 Results and Discussion

A.3.1 Intact 26S Proteasomes Can Be Isolated from *rpn10*Δ and *rad23*Δ Mutants

To address the molecular basis for substrate recruitment by the 26S proteasome, we employed a system that recapitulates the selective ubiquitination and degradation of budding yeast S–Cdk inhibitor Sic1 using purified components (Verma et al. 2001). The chromosomal locus that encodes PRE1, a subunit of the 20S core, was tagged with the Flag epitope in wild–type, *rpn10*Δ, and *rad23*Δ mutant cells. 26S proteasomes were purified by single–step affinity chromatography on anti–Flag beads as described (Verma et al. 2000, also see section A.4). The data in Figure A.1A demonstrate that subunit composition, as visualized by SDS–PAGE, was essentially the same for 26S proteasomes purified from wild–type and mutant cells. This result was corroborated by MudPIT mass spec analysis ((Link et al. 1999); see Supplemental Table S2 at http://www.cell.com/cgi/content/full/118/1/99/DC1). Assembly was also normal as determined by Coomassie blue staining (Figure A.1B) and in–gel peptidase assay of purified proteasomes separated on native gels (Figure A.1C). Some decrease in the doubly capped particle (R2C) with concomitant increase in 20S was seen for the mutants, particularly rad23Δ.

A.3.2 *rpn10*Δ and *rad23*Δ 26S Proteasomes Are Defective at Degrading Ubiquitinated Sic1

The protein degradation activity of the wild–type and mutant 26S proteasomes was assessed by incubation with a ubiquitinated maltose binding protein–Sic1 chimera

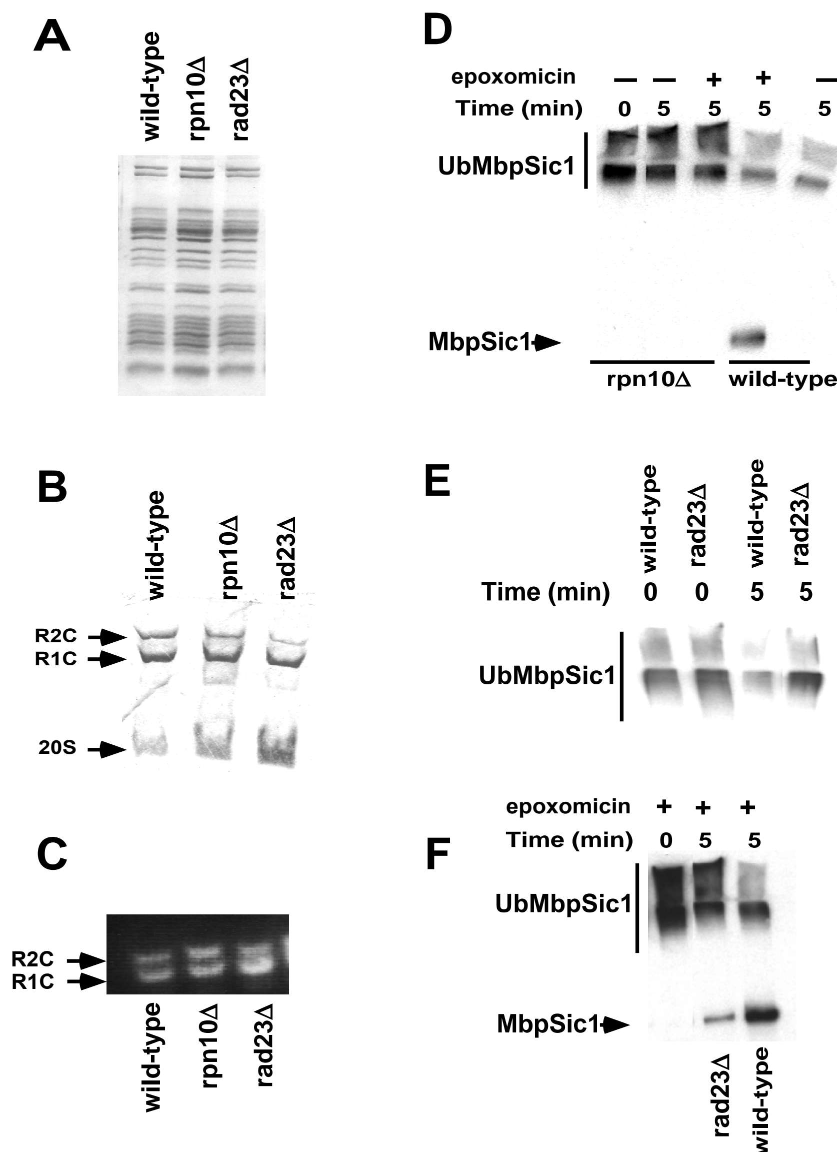

Figure A.1 Structural and Functional Characterization of 26S Proteasomes Isolated from *rpn10*Δ and *rad23*Δ Mutants by Affinity Chromatography. Extracts from wild–type and mutant yeast strains expressing PRE1FH (Supplemental Table S1) were incubated with anti–Flag M2 resin. Bound proteins were eluted with Flag peptide and analyzed by (A) SDS–PAGE and Coomassie blue staining; (B) native gel (nondenaturing) electrophoresis and Coomassie blue staining; or (C) nondenaturing electrophoresis and incubation with a fluorogenic peptide substrate (Verma et al. 2000). (D) *rpn10*Δ 26S are completely defective in the degradation and deubiquitination of UbMbpSic1. UbMbpSic1 was incubated at 30°C with 26S proteasomes isolated from either wild–type or *rpn10*Δ cells. Degradation reactions (lanes 2 and 5) were set up and analyzed by SDS–PAGE followed by immunoblotting with anti–Sic1 polyclonal antibody as described in section A.4. For assessing deubiquitination (lanes 3 and 4), the 26S proteasome preparations were preincubated with 100 μM epoxomicin for 45 min at 30°C before incubation with UbMbpSic1. 26S proteasomes isolated from *rad23*Δ mutants were partially defective in (E) degradation and (F) deubiquitination of UbMbpSic1. Analysis was performed as described for *rpn10*Δ proteasomes in (D).

(UbMbpSic1), which was prepared as described (Seol et al. 1999). Degradation was monitored by loss of high molecular weight Sic1, which typically migrates at the top of a 7.5 % gel and is also observed in the stacker (Verma et al. 2000, 2001). Whereas wild–type 26S proteasomes degraded UbMbpSic1 rapidly, *rpn10*Δ 26S proteasomes were completely defective (compare lanes 2 and 5 with lane 1, Figure A.1D), and *rad23*Δ proteasomes were largely but not completely defective (Figure A.1E). The strength of these defects was surprising given the reported mild phenotype of *rpn10*Δ mutants (Fu et al. 1998; and Van Nocker et al. 1996). To confirm these unexpected results by a different method, we also evaluated whether *rpn10*Δ and *rad23*Δ proteasomes were deficient in Rpn11–dependent substrate deubiquitination (DUB) activity (Verma et al. 2002; and Yao and Cohen 2002). A block in Rpn11 DUB activity leads to a block in degradation. Rpn11 activity is assayed in the presence of the 20S core protease inhibitor epoxomicin, which results in conversion of ubiquitinated substrate to an unmodified protein (MbpSic1; lane 4, Figure A.1D; Verma et al. 2002). We presumed that, concomitant with its deubiquitination by Rpn11, MbpSic1 was translocated into the lumen of the 20S core but was not degraded due to the presence of epoxomicin. This hypothesis is supported by the observation that MbpSic1 formed upon incubation with proteasomes *in vitro*—but not naive MbpSic—was specifically coprecipitated with 20S subunits (see Supplemental Figure S1 at Cell web site). As was observed in the degradation assay, *rpn10*Δ proteasomes were completely deficient in deubiquitination of MbpSic1 (Figure A.1D, lanes 3 and 4), whereas rad23Δ proteasomes were largely but not completely defective (Figure A.1F). Because it is easier to visualize the accumulation of deubiquitinated Sic1 as opposed to the disappearance of ubiquitinated Sic1 to evaluate proteasome function, we sometimes used the DUB assay in lieu of

the degradation assay in subsequent experiments.

A.3.3 Restoration of Activity by Recombinant Rpn10 and Rad23

Although *rpn10*Δ and *rad23*Δ proteasomes appeared to be fairly normal by multiple physical and functional criteria (Figure A.1), it remained possible that they were indirectly and/or irreversibly compromised by the absence of either of these proteins. To address this possibility, we performed add–back experiments using recombinant Gst–Rpn10 and Gst–Rad23 purified from *E. coli* (Supplemental Figure S2A). Strikingly, deubiquitination (Figure A.2B) and degradation (Figure A.2A) activities comparable to wild–type levels were obtained upon adding back Gst–Rpn10 to *rpn10*Δ proteasomes. The effect of Gst–Rpn10 was exquisitely dosage sensitive. Very low levels (30–60 nM) were sufficient to rescue *rpn10*Δ proteasomes but had little effect on wild–type proteasomes. However, at a concentration (120 nM) just $\approx$ 1.5– to 2–fold in molar excess over wild–type proteasomes, inhibition was observed, and at $\approx$ 3– to 4–fold molar excess (300 nM), inhibition was complete. Essentially the same effect was seen if Gst–Rpn10 was cleaved with thrombin to remove Gst (data not shown).

The ability of Gst–Rpn10 to rescue *rpn10*Δ proteasomes allowed us to map the domains of Rpn10 required for complementation. Mutational analysis of RPN10 in prior studies has demonstrated that the N–terminal domain of Rpn10 (also called the von Willebrand A or VWA domain, Whittaker and Hynes 2002) is required for conferring resistance to amino acid analogs and Ub–Pro–β–gal degradation (Fu et al. 1998). The C terminus contains the conserved LAMALRL multiUb chain recognition motif that constitutes part of the UIM domain and that is also required

Figure A.2 The Degradation and Deubiquitination Defects of *rpn10*Δ **and** *rad23*Δ **26S Proteasomes Can be Rescued by Recombinant Proteins.** (A–D) Gst–fusion proteins (see Supplemental Figure S2) were isolated from *E. coli* by glutathione sepharose chromatography, and various amounts of purified protein (indicated on top of each figure) were preincubated with wild–type and mutant 26S proteasomes on ice for 15 min. Degradation was initiated by the addition of UbMbpSic1, and reactions were incubated at 30°C for 5 min. DUB assays included a 45 min preincubation of 26S proteasomes with epoxomicin subsequent to addition of recombinant protein. Reactions were analyzed by SDS–PAGE and immunoblotting for Sic1 as in Figure A.1D.

for binding UbMbpSic1 (Supplemental Figure S2C). No phenotype has ever been linked to this domain, even though it constitutes the multiUb chain recognition domain of Rpn10. As shown in Figure A.2D, either point mutation (first five amino acids of the recognition motif mutated; Gst–N5rpn10) or deletion of the UIM domain (Gst–VWARpn10 or UIM^{-}) destroyed Rpn10 activity, underscoring the requirement for the UIM domain of Rpn10 for UbMbpSic1 degradation. To our knowledge, this is the first functional assay in which a direct requirement for the UIM has been demonstrated.

We next investigated the ability of recombinant Rad23 to complement the partial defect in DUB activity observed with *rad23*Δ 26S proteasomes. The results in Figure A.2C demonstrate that bacterially expressed Gst–Rad23 was functional and rescued the DUB defect. As observed for Rpn10, optimal rescue by Gst–Rad23 was highly concentration dependent. Efficient restoration of activity was observed at 40 nM, but high concentrations of Gst–Rad23 actually inhibited the basal activity of *rad23*Δ proteasomes. A recent study using wild–type 26S proteasomes supplemented with a 3–fold molar excess of Rad23 concluded that Rad23 has an inhibitory function in proteolysis (Raasi and Pickart 2003). Likewise, previous reports documented an inhibitory role for Rpn10 *in vitro* (Deveraux et al. 1995). However, our observations indicate that both Rad23 and Rpn10 actually promote protein degradation by the proteasome—at least when the substrate is UbSic1—but that for both proteins it is essential to use mutant proteasome preparations to identify the optimal dose, because these proteins inhibit degradation even when present in only modest stoichiometric excess over the 26S proteasome.

Our results caused us to wonder why Rad23 present in *rpn10*Δ proteasomes and Rpn10 present in *rad23*Δ proteasomes did not provide sufficient activity to sustain

normal rates of UbMbpSic1 turnover. Do these proteins operate in parallel as redundant substrate–targeting factors to sustain a maximal rate of Sic1 turnover, or might they act in series? One simple explanation is that Rad23 is normally present at only substoichiometric levels in 26S proteasome preparations, such that there was not enough to sustain UbMbpSic1 turnover in the absence of Rpn10. This contention is consistent with SDS–PAGE/microsequence analysis of purified yeast proteasomes (Glickman et al. 1998), immunoblot analysis of purified mammalian proteasomes (Raasi and Pickart 2003), and the very low sequence coverage observed for Rad23 in our MudPIT experiments (Supplemental Table S2). Likewise, immunoblotting experiments revealed that Rpn10 was present in *rad23*Δ proteasomes at one–third to one–half the levels observed in wild–type 26S proteasomes (Supplemental Figure S3). Significantly, addition of just 30 nM Rpn10 rescued the defective DUB activity of *rad23*Δ 26S proteasomes (Figure A.2C), arguing that Rpn10 and Rad23 can act redundantly to sustain UbMbpSic1 deubiquitination and turnover, and the action of Rpn10 was not dependent upon Rad23.

A.3.4 Redundant Roles for Rad23 and the UIM Domain of Rpn10 in Sustaining UbSic1 Degradation

Crossrescue of *rad23*Δ 26S proteasomes by Rpn10 encouraged us to investigate if the reverse was true, i. e., could addition of Rad23 restore activity to *rpn10*Δ 26S proteasomes? Surprisingly, although recombinant Gst–Rad23 was fully functional in restoring activity to *rad23*Δ 26S proteasomes (Figure A.2C), it rescued *rpn10*Δ 26S proteasomes weakly (Figure A.2D). Because the requirement for Rpn10 function for *in vivo* turnover of the synthetic reporter substrate Ub–Pro–β–gal mapped to the

N–terminal VWA domain of Rpn10 (Fu et al. 1998), we wondered whether Rad23 would rescue *rpn10*Δ proteasomes in the presence of the VWA domain of Rpn10. Remarkably, although Gst–VWARpn10 (UIM domain deleted) and Gst–N5rpn10 (mutant UIM) by themselves were inactive, the combination of either protein with GstRad23 restored full activity to *rpn10*Δ proteasomes (Figure A.2D). Taken together, these observations support two important conclusions about the functions of Rpn10 and Rad23. First, the Ub binding domains of Rpn10 and Rad23 do not need to act sequentially. Instead, there exists a functional redundancy between Rad23 (see below) and the Rpn10 UIM domain, suggesting that they function in parallel pathways to sustain degradation of Sic1. Second, the VWA domain of Rpn10 was required for Rad23 to promote optimal rates of UbSic1 proteolysis. This was also observed with Dsk2, another UbL–UBA domain protein like Rad23 (Funakoshi et al. 2002). Although rescue was weak, there was clearly an enhancement in activity when the Rpn10 VWA domain and Dsk2 were added together (Figure 2DA.2, lanes 11 and 14). It could be that Dsk2 is less potent than Rad23 because it has only one UBA domain, and Rad23 has two. Indeed, Dsk2 bound less UbMbpSic1 than Rad23 (Supplemental Figure S2C). Since Rpn10 functions to enhance the weak complementation by Rad23 (and Dsk2), we propose the term "facilitator" for Rpn10.

A.3.5 Both the UBA and the UbL Regions of Rad23 Are Required for Function

Rescue of *rad23*Δ 26S proteasomes by recombinant Rad23 allowed us to assess the relative contributions of both its Ub chain binding (UBA) and proteasome binding (UbL) regions. As predicted by prior studies (Schauber et al. 1998; and Wilkinson et al. 2001), a mutant protein (shown in Supplemental Figure S2B) lacking the UbL

but containing both UBA domains bound UbMbpSic1 (Figure A.3A), whereas the reciprocal construct that contains the UbL domain but lacks both UBA domains selectively bound 26S proteasomes (Supplemental Figure S2D). However, neither the UbL nor UBA segments sustained robust rescue of *rad23*Δ (Figure A.3B) or *rpn10*Δ (Figure A.3C) 26S proteasomes.

A.3.6 Rad23 and the UIM Domain of Rpn10 Link UbSic1 to the Proteasome

The ability of the UBA domain of Rad23 and the UIM domain of Rpn10 to bind multiUb chains (Figures A.3A and Supplemental S2C) suggested that the redundant function provided by these elements is to target UbSic1 to the proteasome for degradation. To address this hypothesis, the substrate binding capacities of wild–type and *rpn10*Δ 26S proteasomes were investigated by incubating UbMbpSic1 (in the presence of inhibitors of deubiquitination and degradation) with 26S proteasomes immobilized on anti–Flag beads (Figure A.4A). Wild–type 26S proteasomes bound UbMbpSic1 whereas *rpn10*Δ 26S proteasomes displayed little or no binding activity. Gst–Rpn10 efficiently rescued the substrate binding defect of *rpn10*Δ proteasomes (Figure A.4), but Gst–VWARpn10 and Gst–N5rpn10 did not (Figure A.4B), underscoring that this recruitment activity required the UIM domain. Gst–Rad23 bound *rpn10*Δ proteasomes in a UbL–dependent manner (Supplemental Figure S2D) and endowed them with enhanced substrate binding activity (Figure A.4).

A.3.7 Rpn10 VWA Domain Facilitates the Degradation–Promoting Activity of Rad23

Surprisingly, although the VWA domain of Rpn10 was required for optimal proteo-

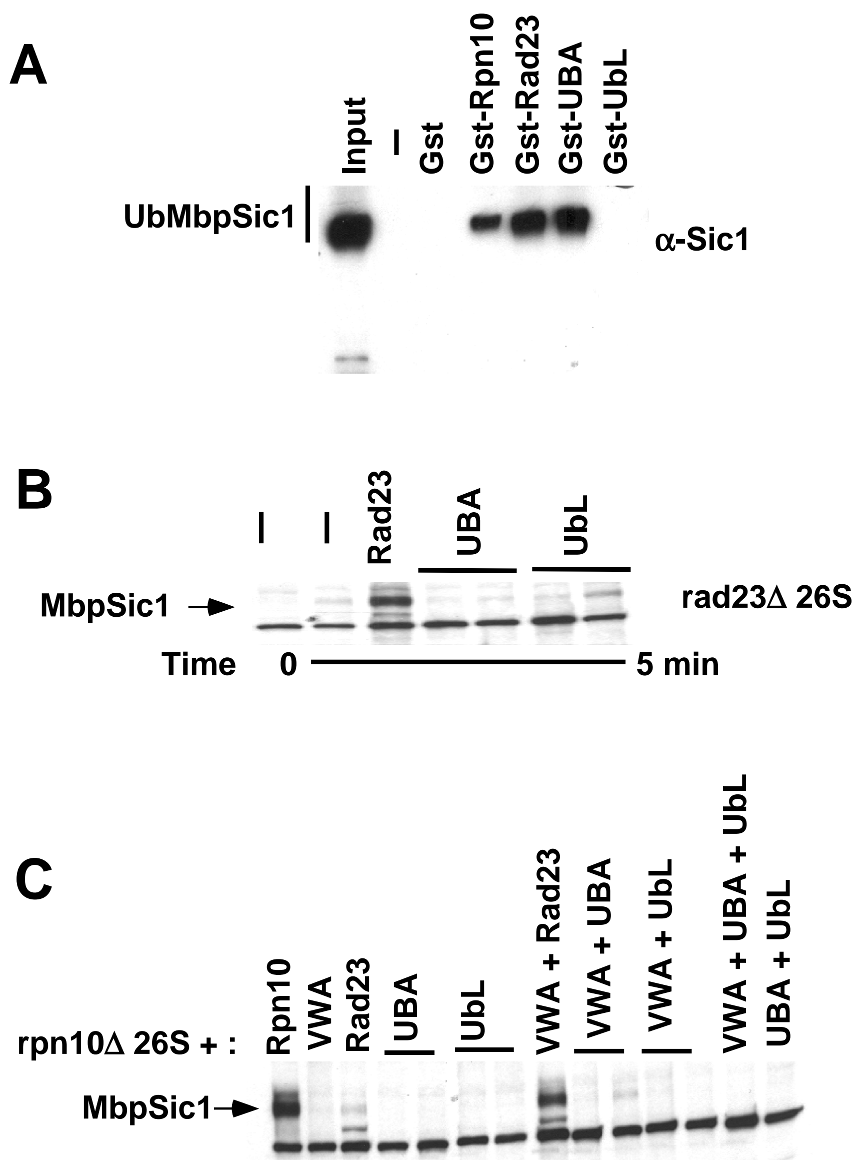

Figure A.3 Complementation of rad23Δ Proteasomes Requires Both the Ub Binding UBA Domains and the Proteasome Binding UbL Domain of Rad23. (A) The UBA domains bind UbMbpSic1. Purified Gst and Gst fusion proteins (1 μg each) bound to glutathione beads were incubated with UbMbpSic1, after which the input (20 % of total) and bound material (33 % of total) were fractionated by SDS–PAGE and visualized by immunoblotting with anti–Sic1 serum. Note that Gst–UBA lacks the UbL domain but contains both UBA domains found in Rad23, whereas Gst–UbL is the reciprocal molecule lacking both UBA domains (Rao and Sastry 2002). (B) Rescue of *rad23*Δ 26S proteasomes by Rad23. Deubiquitination reactions were set up using *rad23*Δ 26S proteasomes and UbMbpSic1 in the presence or absence of Gst–Rad23 (80 nM), Gst–UBA (80 and 40 nM respectively), or Gst–Ubl (80 and 40 nM), respectively, as described in the legend to Figure A.1D.(C) Rescue of *rpn10*Δ 26S DUB defect by full–length Rad23 and Gst–VWA. Deubiquitination reactions were assayed by incubation of UbMbpSic1 with *rpn10*Δ 26S proteasomes in the presence or absence of various Gst–fusion proteins as described above.

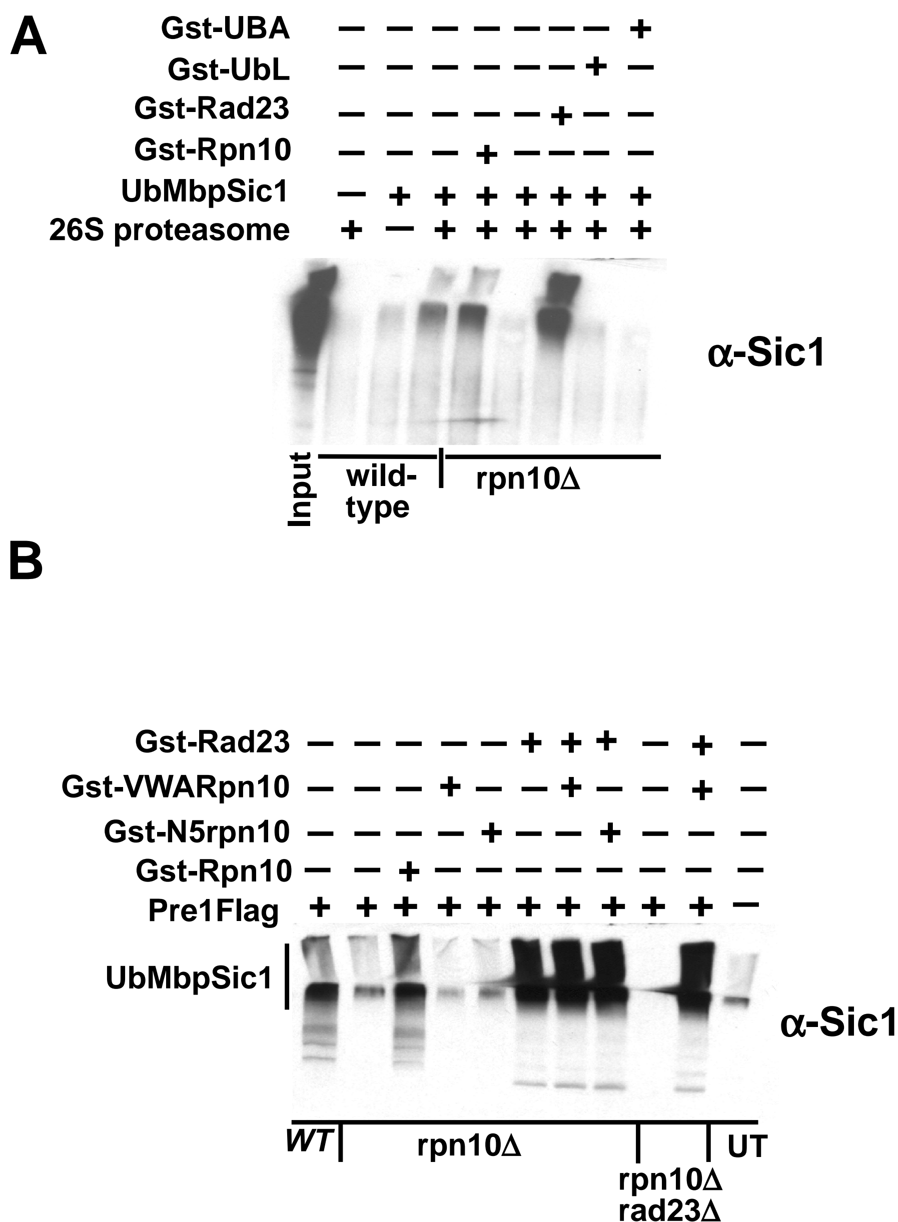

Figure A.4 26S Proteasomes from *rpn10*Δ Are Defective in Binding UbMbpSic1. (A and B) The binding defect of *rpn10*Δ 26S proteasomes can be rescued by either recombinant Rpn10 or Rad23. Extracts from wild–type (WT), *rpn10*Δ, and *rpn10*Δ*rad23*Δ cells expressing PRE1FH (Supplemental Table S1) or untagged PRE1 (UT) were bound to anti–Flag M2 resin in the presence of ATP and washed with buffer containing ATP as described for 26S purification (section A.4). Resin–immobilized 26S proteasomes were then incubated with 1 mM phenanthroline, 2.5 μM Ub aldehyde, 100 μM MG132, 1 mM ATP, and 5 mM $MgCl_2$ in the absence or presence of the various Gst–fusion proteins on ice for 60 min. UbMbpSic1 was then added, and, after 90 min incubation at 4°C, the bound fraction was washed and analyzed by SDS–PAGE and immunoblotting for Sic1. In (A), 5 % of input and 25 % of the bound fractions were loaded.

lysis–promoting activity of Rad23 (Figure A.2D), it was not required for Rad23–dependent tethering of UbMbpSic1 to the proteasome (Figure A.4). Thus, binding is not a reliable surrogate assay for degradation. We conclude that the VWA domain acts downstream of Rad23 and enables proteasome bound, ubiqutinated substrate to engage productively with the degradation machinery. Owing to its additional facilitator function encoded within the VWA domain, we suggest that the term facilitator be applied to Rpn10 to distinguish it from substrate receptors such as Rad23. A widespread role for Rpn10 as a substrate receptor facilitator is suggested by the findings that deletion of RPN10 in *Drosophila* results in pupal lethality (Szlanka et al. 2003), and its downregulation by RNAi causes G2/M phase arrest in *Trypanosoma brucei* (Li and Wang 2002). Given that yeast *rpn10*Δ mutants are viable, we surmise that either Rad23, Dsk2, or other substrate receptors retain sufficient function to sustain life (note the weak albeit detectable activity of Rad23 in the absence of Rpn10VWA; Figure A.2D, lane 10), or other proteins provide a facilitator function *in vivo* that is redundant with that of Rpn10's VWA domain.

A.3.8 Both RPN10 and RAD23 Contribute to Sic1 Turnover *In Vivo*

The *in vitro* assays indicate important roles for Rpn10 and Rad23 in Sic1 turnover. To date, all studies on these mutants *in vivo* have relied either on artificial substrates (Van Nocker et al. 1996); indirect readouts for degradation, such as steady state analysis (Wilkinson et al. 2001); or a substrate (Clb2) whose degradation is subject to indirect regulation via cell cycle checkpoints (Lambertson et al. 1999). Thus, to monitor Sic1 degradation *in vivo*, we evaluated turnover during the appropriate cell cycle phase. Wild–type and mutant cells were arrested in G1 with α factor and then

released synchronously into the cell cycle (Figure A.5). Both GAL1–expressed and endogenous Sic1 are normally degraded at the G1/S boundary (Verma et al. 1997). As shown in Figure A.5, both GAL1–expressed and endogenous Sic1 tapered off by 45 min as cells entered S phase. Based on our reconstitution experiments, we reasoned that Sic1 might be targeted for degradation *in vivo* by either Rad23 or the UIM domain of Rpn10. Indeed, whereas Sic1 was degraded with normal kinetics in *rad23*Δ and in a mutant lacking the UIM domain of Rpn10 (*rpn10*VWA+), significant stabilization was observed in an *rpn10*VWA+ *rad23*Δ double mutant. As expected from the facilitator role played by the VWA domain in the operation of other receptor pathways *in vitro*, Sic1 was significantly more stable in *rpn10*Δ than in *rpn10*VWA+ cells. Additionally, failure to promptly degrade Sic1 correlated with a reduced rate of entry into S phase, as shown for the *rpn10*Δ *rad23*Δ mutant (Figure A.5), which remained in G1 phase 75 min after release from α factor. Degradation of Sic1 is essential for entry into S phase (Verma et al. 1997). Delayed entry into S phase and residual turnover of Sic1 in *rpn10*Δ *rad23*Δ cells indicate that there must exist a third receptor pathway (possibly Dsk2, Figure A.2D) by which Sic1 can engage the proteasome and be degraded, albeit at a greatly reduced rate.

Since the *rpn10*Δ*rad23*Δ double mutant displayed unexpectedly strong stabilization of Sic1, the growth phenotype of this mutant was reassessed. It has been reported that these mutants are cold sensitive at 13°C (Lambertson et al. 1999). However, we observed a severe growth defect even at 25°C (Supplemental Figure S4), which was exacerbated in synthetic medium. Consistent with the *in vitro* and *in vivo* data presented here and elsewhere (Fu et al. 1998), the slow growth phenotypes of the double mutant were linked to the absence of the VWA domain of

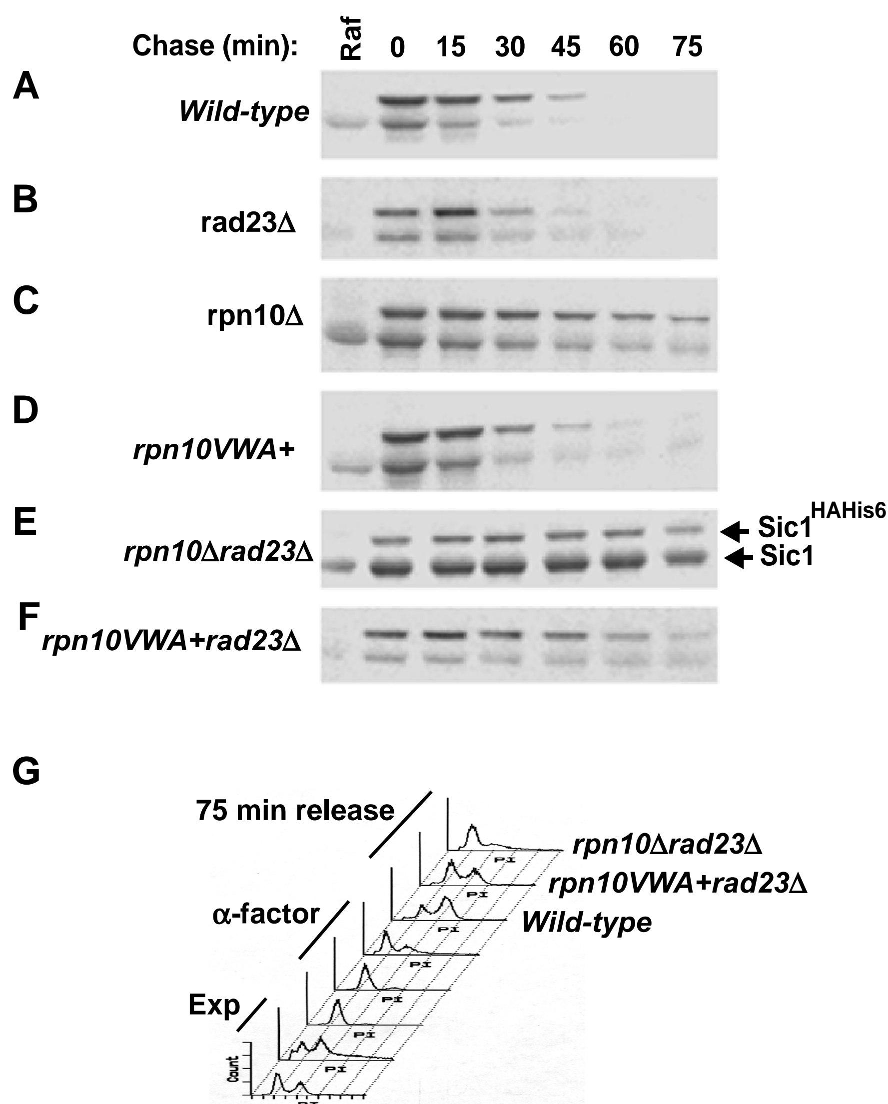

Figure A.5 Rpn10 UIM Domain and Rad23 Serve Redundant Roles in Sic1 Turnover ***In Vivo*****.** (A–F) Wild–type and mutant cells (Supplemental Table S1) expressing a GAL1–driven, epitope–tagged (HaHis6) allele of SIC1 in addition to endogenous untagged SIC1 were arrested with α factor and released synchronously into the cell cycle at 25°C (except *rpn10*Δ*rad23*Δ, which were released at 30°C because they grew poorly at 25°C). Extracts were prepared at the indicated time points and analyzed by SDS–PAGE followed by immunoblotting with anti–Sic1 serum that detects both the endogenous and the epitope–tagged versions of Sic1. (G) Wild–type, *rpn10*VWA *rad23*Δ, and *rpn10*Δ *rad23*Δ cells collected at the indicated time points were evaluated for cell cycle distribution by flow cytometry.

RPN10 (Supplemental Figure S4).

A.3.9 Specificity in the Requirement for Different MCBPs for *In Vivo* Turnover of UPS Substrates

To address the generality of our observations, we next tested whether the relative contributions of Rad23 and Rpn10 to Sic1 degradation would hold true for another physiological substrate of the UPS—the G1 cyclin Cln2 (Deshaies et al. 1995). HA–tagged Cln2 expressed from the GAL1 promoter was rapidly degraded in G1 phase cells and unlike Sic1 was not stabilized in *rpn10*Δ, *rad23*Δ, or *rpn10*Δ*rad23*Δ mutants. This prompted us to look at its turnover in additional MCBP mutants. As shown by the data in Figure A.6A, mutations in the genes encoding the UBA domain–containing putative targeting factors Ddi1, Dsk2 (Saeki et al. 2002a), and the UT3 domain–containing Ufd1 (Ye et al. 2003) had no effect on Cln2 turnover. From this analysis, we conclude that an as yet unknown receptor or set of receptors, possibly including Rpt5, functions to link ubiquitinated Cln2 to the proteasome.

Whereas Sic1 is a substrate of the E3 Ub ligase SCF^{Cdc4} (Seol et al. 1999), Cln2 is an SCF^{Grr1} substrate (Seol et al. 1999; and Skowyra et al. 1999). To determine if the identity of the ubiquitin ligase influenced the different receptor dependencies exhibited by Sic1 and Cln2, we examined the turnover of the SCF^{Cdc4} substrate Far1 (Henchoz et al. 1997) and the SCF^{Grr1} substrate Gic2 (Jaquenoud et al. 1998). Far1 is a G1 cyclin–Cdk inhibitor, and Gic2 is an effector of the Cdc42 cell polarity regulator. In both cases, turnover of the endogenous protein was examined during G1 phase, when Far1 and Gic2 are normally degraded (Jaquenoud et al. 1998; also

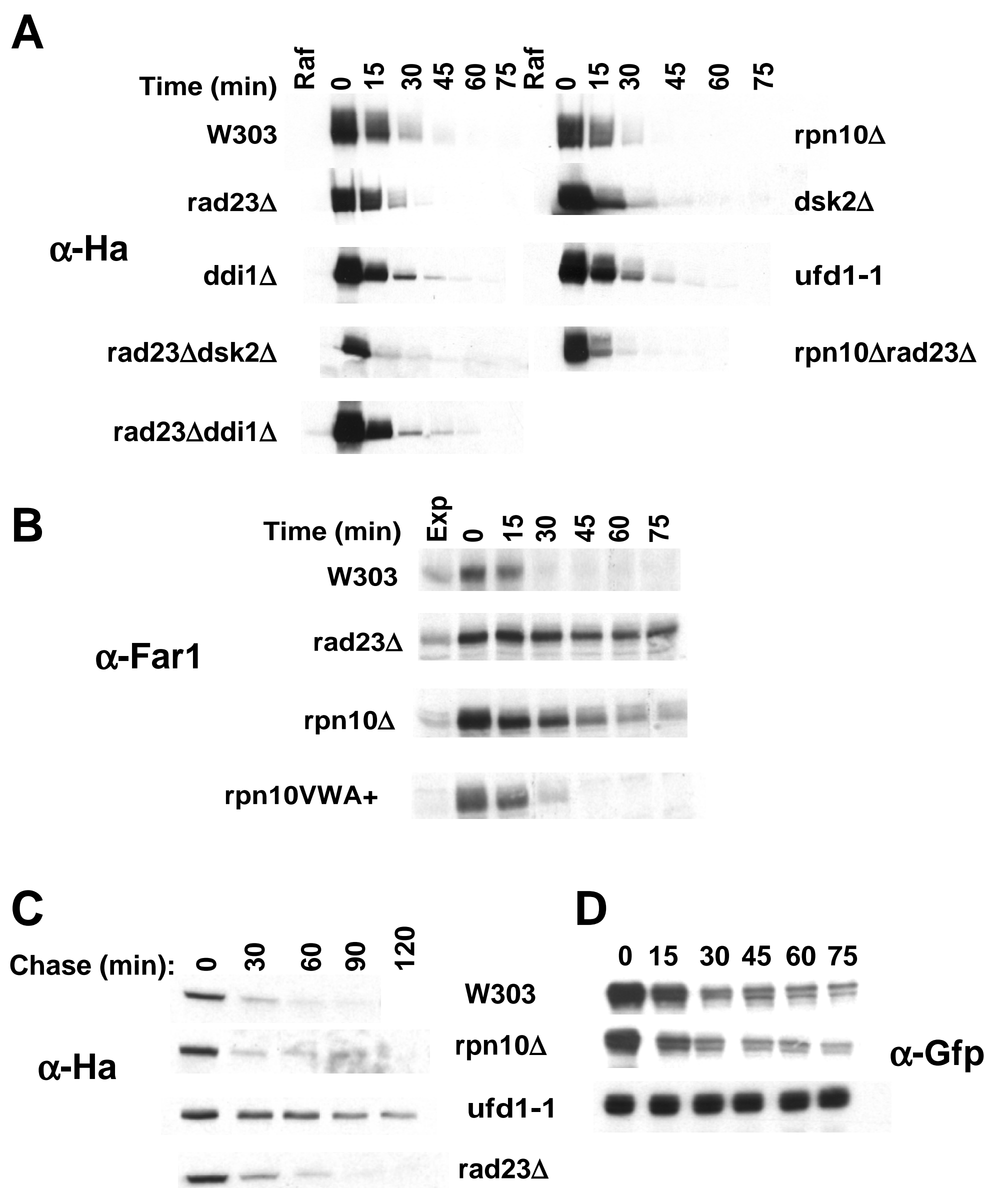

Figure A.6 UPS Substrates Have Differential Requirements for Multiubiquitin Chain Receptors *In Vivo*. For experiments shown in panels (A)–(D), aliquots of cells of the indicated genotypes were withdrawn at various times after initiation of chase (min), and whole cell lysates were fractionated by SDS–PAGE and immunoblotted with the indicated antibodies. (A) Wild–type and mutant cells expressing Ha epitope–tagged Cln2 from the GAL1 promoter were grown in YP raffinose at 30°C, and expression of Cln2–Ha was induced with 2 % galactose at 25°C for 90 min. Induction was terminated and chase was initiated by transfer of cells to YP–2 % dextrose. (B) To monitor turnover of Far1, wild–type and mutant cells were arrested with α factor for 3 h at 25°C, and the chase period was initiated by release into fresh medium in the absence of α factor, which results in rapid downregulation of Far1 message (see http://www.yeastgenome.org/ for expression analysis) (C) The stability of CPY*HA was monitored upon initiating a chase period by adding 100 μg/ml cycloheximide to wild–type and mutant cultures at 25°C. (D) Cycloheximide chase was done as described in (C) to monitor turnover of Deg1–Gfp.

see http://www.yeastgenome.org/). In contrast to Sic1, Far1 degradation was impeded more in *rad23*Δ than in *rpn10*Δ mutants (Figure A.6B). Meanwhile, Gic2 mimicked Sic1 and not Cln2 in that it was strongly stabilized in *rpn10*Δ cells (Supplemental Figure S5A). Additionally, Clb2, an APC substrate (Harper et al. 2002), also mimicked Sic1 (Supplemental Figure S5). Thus, no simple rule could be formulated that relates a ubiquitinated substrate's dependency upon a targeting receptor to the identity of its E3.

In addition to proteolysis of regulatory proteins, the UPS is also required for the degradation of misfolded proteins. Secretory pathway proteins that fail to fold properly in the ER are retrotranslocated into the cytosol and degraded by the 26S proteasome in a process called ER–associated degradation (ERAD, Tsai et al. 2002). The Cdc48/Ufd1/Npl4 complex is required for ERAD and recognizes membrane–associated Ub conjugates via the UT3 domains of Ufd1/Cdc48 (Ye et al. 2003). The ERAD substrate CPY* is stabilized in mutants defective in individual subunits of the Cdc48/Ufd1/Npl4 complex (Jarosch et al. 2002; Figure A.6C). To determine if ERAD substrates are "handed off" to proteasomal receptors following their extraction from the membrane by Cdc48/Ufd1/Npl4 (Flierman et al. 2003), we evaluated the turnover of CPY* in *rpn10*Δ and *rad23*Δ mutants. Surprisingly, no stabilization was observed (Figure A.6C). These data suggest that Cdc48/Ufd1/Npl4 may shepherd the extracted CPY* directly to the proteasome or deliver it to Rpt5 or an as yet unknown receptor.

The Cdc48/Ufd1 complex binds specifically to K48–linked polyUb chains via the UT3 domain (Ye et al. 2003) and also participates in degradation of non–ERAD substrates such as cytosolic Ub^{V76}–V–β–galactosidase (Johnson et al. 1995) and spindle disassembly factors Cdc5 and Ase1 (Cao et al. 2003). We monitored the

turnover of the cytoplasmic Deg1–Gfp, which contains the degradation signal from the transcriptional repressor MATα2. This fusion substrate is interesting because, although it is soluble, it is ubiquitinated by enzymes resident in the ER membrane (Swanson et al. 2001). As shown in Figure A.6D, Deg1–Gfp was stabilized in *ufd1–1*. However, like the ERAD substrate CPY*, Deg1–Gfp was not stabilized in *rpn10*Δ mutants.

A.3.10 Natural versus Synthetic Substrates of the UPS

An important principle emerges from considering the targeting requirements observed for physiological versus synthetic substrates. Reporter substrates such as Ub–Pro–β–gal, Ub^{V76}–V–β–gal, and Ub^{V76}–V–DHFR exhibit simultaneous dependence on multiple putative receptor pathways, including Rpn10, Rad23, and Cdc48/Ufd1 (Johnson et al. 1995; Rao and Sastry 2002; and Xie and Varshavsky 2002; see also Supplemental Figure S5D). This simultaneous dependence suggests that these factors typically serve nonredundant, possibly even sequential (Chen and Madura 2002) roles in degradation. By contrast, none of the physiological substrates examined in this study (including Far1, Sic1, Gic2, Cln2, CPY*, and Clb2) exhibited an equivalently broad dependence on multiple putative receptor pathways. Thus, although synthetic substrates have proved very useful for defining components of the UPS system, we caution that their turnover may not be reflective of typical physiologic mechanisms, and, thus, general conclusions about the mechanism/specificity of the UPS should be rooted in the study of physiological substrates.

A.3.11 One Universal Targeting Signal with Multiple Receptors

It is commonly thought that specificity in substrate turnover by the UPS lies at the level of ubiquitin chain assembly controlled by E2, E3, and isopeptidase enzymes. Our findings, however, lead to the unexpected conclusion that proteasome–targeting pathways downstream of the ubiquitin ligases exhibit a surprising degree of substrate specificity. A scheme that graphically summarizes our key proposals is depicted in Figure A.7. Rpn10, Rad23, Dsk2, and possibly Ufd1/Cdc48 and Rpt5 are envisioned to comprise distinct receptor pathways that link ubiquitinated substrates to the proteasome. It is important to note that there are no functional data indicating that either Ufd1/Cdc48 or Rpt5 recruits ubiquitinated substrates to the proteasome. However, others have suggested a receptor function for Rpt5 based on crosslinking data (Lam et al. 2002), and we suggest a receptor activity for Ufd1/Cdc48 as a working hypothesis in light of data reported here and elsewhere (Flierman et al. 2003; and Ye et al. 2003).

Some substrates, like Sic1 and Clb2, are recruited to the proteasome and degraded in a manner that depends strongly on the receptor and/or facilitator (FA) functions of the proteasome subunit Rpn10, whereas others, such as Far1, show a weaker dependence on Rpn10 and a correspondingly stronger dependence on Rad23. Yet other substrates such as CPY* and Deg1–Gfp appear to bypass Rpn10 entirely but depend on a complex containing Ufd1 and Cdc48. (It has been reported that Far1 degradation also depends upon Cdc48 using a novel G1–specific td allele (Fu et al. 2003), but we have not observed a defect in Far1 turnover in cdc48–3 or *ufd1–1* mutants; data not shown). Finally, at least one substrate, Cln2, does not depend upon any known receptor pathway. However, our data on Sic1 underscore that it is

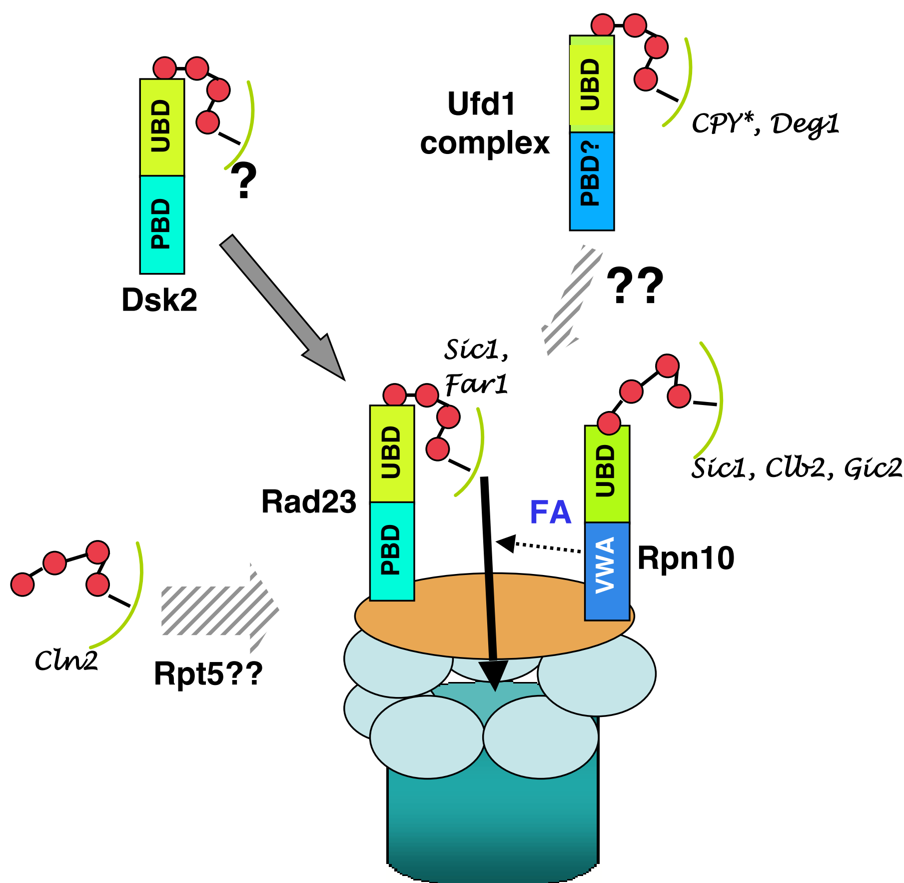

Figure A.7 Hypothetical Model for Physiological Targeting Pathways that Deliver Ubiquitinated Substrates to the 26S Proteasome. The schematic shows the 20S proteolytic core capped by the base, which comprises a hexameric ring of the AAA ATPases (Rpt1–Rpt6, depicted as light blue ovals) and the PC repeat containing proteins Rpn1 and Rpn2 (collectively depicted as a beige oval). Rad23 and Rpn10 associate with the proteasome via the Rpn1/Rpn2 subunits to deliver substrates tethered to their Ub binding domains (UBD), including Far1, Sic1, Gic2, and Clb2. Deubiquitination and degradation of substrates delivered by Rad23 requires a facilitator activity (FA) encoded within the VWA domain of Rpn10. Dsk2, a UBA domain containing protein like Rad23, is postulated to also deliver substrates to the same entry port used by Rad23, but the identity of these substrates remains unknown. Ufd1–containing complexes that contain Cdc48 are proposed to deliver ERAD and non–ERAD substrates such as CPY*, Deg1, and Cdc5 to the proteasome, but the putative proteasome binding domain (PBD) and docking site employed by this complex remain unknown. Ubiquitinated Cln2 is targeted for degradation by a pathway that remains unknown but does not require the activity of Rpn10, Rad23, Dsk2, or Ufd1. It is possible that Cln2 gains access to the proteasome via the putative Rpt5 gateway or an unknown receptor or utilizes multiple receptor pathways in a highly redundant manner.

important to distinguish “dependency” from “involvement.” Rad23 can be involved in Sic1 turnover (as evidenced by the fact that Sic1 was unstable in *rpn10*$^{\mathrm{VWA}}$ but

was stabilized in *rpn10*$^{\mathrm{VWA}}$*rad23*Δ), even though Sic1 turnover does not normally depend upon Rad23 (as evidenced by rapid Sic1 turnover in a *rad23*Δ mutant). Thus, Cln2 may not depend upon the known receptors, because it can be targeted by multiple receptors in a highly redundant manner, or because it arrives at the proteasome by a distinct route involving Rpt5 or an unknown receptor. Yet other targeting strategies are likely to exist, given that ubiquitin ligases such as Parkin, Ufd4, and Hul5 can bind directly to the proteasome (Demand et al. 2001; Sakata et al. 2003; Xie and Varshavsky 2002; and Leggett et al. 2002). Interesting challenges for the future will be to determine how many receptor pathways exist, to sort out the mechanism underlying the allocation of substrates to different receptor pathways, and to determine whether individual receptor pathways are differentially regulated to modulate the repertoire of proteins degraded by the UPS in response to specific signals.

Our data indicate that a putative receptor activity intrinsic to Rpt5 (Lam et al. 2002) by itself is insufficient to target UbSic1 for degradation in a defined *in vitro* system. Moreover, our *in vivo* analysis implies that an Rpt5–mediated targeting mechanism would appear to be insufficient to sustain normal rates of degradation *in vivo* for seven of eight UPS substrates characterized in this study. What, then, is the role of Rpt5 in substrate targeting? It is possible that Rpt5 serves as the primary conduit by which a subset of unstable proteins poorly represented in this study (but possibly including Cln2) gains access to the proteasome. On the other hand, we favor the notion that Rpt5 serves as a central conduit that gathers together substrates delivered by different receptor pathways (Rpn10, Rad23, and Cdc48/Ufd1) and positions them for subsequent unfolding, deubiquitination, and translocation. This latter possibility calls to mind translocation of secretory precursors cross the

ER membrane, where the primary signal peptide–mediated targeting step is carried out by upstream receptors such as Signal Recognition Particle, following which the signal peptide is transferred to the Sec61 channel to enable precursor translocation across the membrane. An analogous two–step recognition system may operate in *E. coli*, where the SspB protein functions as a specificity factor for the AAA ATPase ClpX, enhancing degradation of ssrA–tagged substrates (Levchenko et al. 2000).

A.3.12 Note Added in Proof

While this manuscript was under review, Elsasser et al. (2004) reported that Rad23 and Rpn10 can tether autoubiquitinated Cdc34 to 26S proteasome. In a second publication, Medicherla et al. (2004) reported that *rad23*Δ*dsk2*Δ mutants are defective in CPY* turnover. Medicherla et al. (2004) also reported that Deg1–GFP is degraded normally in *ufd1–1*, a result that conflicts with our Figure A.6D. We do not know the reason for this discrepancy.

A.3.13 Acknowledgements

We thank H. Rao, H. Yokosawa, H. Fu, M. Funakoshi, K. Madura, R. Vierstra, R. Hampton, E. Johnson, M. Peter, S. Reed, Y. Zheng, and D. Kellogg for yeast strains, expression plasmids, and antibodies. We thank the members of the Deshaies lab for helpful discussions; and A. Varshavsky and T. Mayor for critically reading the manuscript. We gratefully acknowledge the Caltech Flow Cytometry Cell Sorting Facility and R. Diamond for performing flow cytometry; and the Caltech Protein

Expression Facility and P. Snow for expression of the G1 Cdk and SCF complexes in insect cells. This work was supported by the Howard Hughes Medical Institute.

A.4 Experimental Procedures

A.4.1 Yeast Strains and Extract Preparation

Yeast strains used in this study are listed in Supplemental Table S1.

For turnover analysis of UPS substrates, wild–type and mutant cells grown to an $OD_{600\ nm}$ of 0.2–0.3 were processed as described in Figure A.4 and Figure A.5. Cells were harvested by centrifugation and drop frozen in liquid nitrogen. They were thawed and washed in a buffer containing 50 mM Tris (pH 7.5); 10 mM EDTA; 20 mM NaF; 0.05 % azide; 5 mM NEM; 1 mM PMSF; 0.5 mM AEBSF; and 1× protease inhibitor cocktail containing pepstatin, chymostatin, aprotinin, and leupeptin at 5 μg/ml. Glass beads (Sigma, 425–600 μm, acid washed) equal in volume to the cell pellet were added, and the cell pellets were plunged into boiling water for 3 min after brief vortexing. Cells were then resuspended at uniform concentration (26.7 OD units/ml) in 1× SDS sample buffer and vortexed in a ThermoSavant Fast-Prep at 4°C for 45 s at the maximum speed setting (6.5). Vortexed cell pellets were boiled again for 3 min and aliquots resolved by SDS–PAGE. Ponceau S staining was done after transfer to nitrocellulose membrane to assess uniformity of protein levels across the gel. The blot was incubated with the appropriate antibody and processed using ECL.

A.4.2 Degradation and Deubiquitination Assays

Ubiquitinated MbpSic1 substrate (Seol et al. 1999) and affinity–purified 26S proteasomes (Verma et al. 2000) were prepared essentially as described. Degradation and deubiquitination assays ($\approx$ 300 nM substrate, $\approx$ 100 nM proteasome, incubated at 30°C for 5 min) were conducted as described previously (Verma et al. 2002).

A.4.3 Preparation of Gst–Fusion Proteins

Gst–fusion proteins were expressed in BL21/pLysS according to standard procedures. Proteins were eluted from glutathione resin with 50 mM Tris (pH 8.8), 50 mM NaCl, 5 mM DTT, 1 mM EDTA, and 40 mM glutathione at 4°C for 3 h and then dialyzed against buffer containing 25 mM Tris (pH 7.5), 100 mM NaCl, and 15 % glycerol. Aliquots were drop frozen in liquid N_2 and stored at −70°C.

A.4.4 FACS Analysis

Yeast cells were processed for flow cytometry as described (Verma et al. 1997).

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