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# Ubiquitin-like domains can target to the proteasome but proteolysis requires a disordered region

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## Abstract

**Ubiquitin and some of its homologues target proteins to the proteasome for degradation. Other ubiquitin-like domains are involved in cellular processes unrelated to the proteasome, and proteins containing these domains remain stable in the cell. We find that the 10 yeast ubiquitin-like domains tested bind to the proteasome, and that all 11 identified domains can target proteins for degradation. Their apparent proteasome affinities are not directly related to their stabilities or functions. That is, ubiquitin-like domains in proteins not part of the ubiquitin proteasome system may bind the proteasome more tightly than domains in proteins that are *bona fide* components. We propose that proteins with ubiquitin-like domains have properties other than proteasome binding that confer stability. We show that one of these properties is the absence of accessible disordered regions that allow the proteasome to initiate degradation. In support of this model, we find that Mdy2 is degraded in yeast when a disordered region in the protein becomes exposed and that the attachment of a disordered region to Ubp6 leads to its degradation.**

**Keywords** degradation; proteasome; ubiquitin-like domain
**Subject Categories** Post-translational Modifications, Proteolysis & Proteomics
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## Introduction

Ubiquitin is a small single-domain protein consisting of 76 amino acids that is conserved in all eukaryotes. It becomes attached to proteins post-translationally, often in long polyubiquitin chains in which the first ubiquitin is modified with additional ubiquitin moieties. The first role discovered for polyubiquitin chains was to target proteins to the proteasome for degradation, but it is now clear that ubiquitin chains are also involved in the regulation of cellular processes via non-proteolytic mechanisms ranging from membrane trafficking to DNA repair (Komander & Rape, 2012). It is not fully understood how cells use ubiquitin chains to specify distinct cellular processes.

Biochemically, ubiquitin chains function as transferable protein–protein interaction tags and are recognized by ubiquitin binding domains, sometimes within the same protein and sometimes in interacting proteins (Husnjak & Dikic, 2012). Individual ubiquitin moieties in polyubiquitin chains are connected to each other through different lysine residues, and chains with different linkage patterns are recognized with different affinities by ubiquitin receptors (Husnjak & Dikic, 2012; Komander & Rape, 2012). However, other properties of the modified proteins also affect their fate and provide additional information to the ubiquitin code (Prakash *et al*, 2004; Takeuchi *et al*, 2007; Zhao *et al*, 2010; Fishbain *et al*, 2011, 2015; Heinen *et al*, 2011; Inobe *et al*, 2011; van der Lee *et al*, 2014).

Cells also encode a number of ubiquitin homologues and in budding yeast (*S. cerevisiae*), at least eleven proteins show homology with ubiquitin. The sequence identity within these ubiquitin-like (UbL) domains ranges between 7 and 53%, and they all share ubiquitin's β-grasp fold (Grabbe & Dikic, 2009) (Fig 1). Although some UbL domains bind to the proteasome, it is thought that none of the proteins that carry UbL domains are degraded by the proteasome.

Ubiquitin-like proteins fall into two classes, ubiquitin-like modifiers (ULMs) and ubiquitin-like domain proteins (UDPs). Ubiquitin-like modifiers are attached to proteins reversibly and post-translationally, like ubiquitin (van der Veen & Ploegh, 2012). In yeast, the ULMs are Atg8, Hub1, Rub1 (Nedd8), Smt3 (SUMO), and Urm1, and these proteins are not thought to bind to the proteasome (Table 1). Rub1 may be an exception in that it can be incorporated into polyubiquitin chains, which in turn can be recognized by the proteasome (Singh *et al*, 2012). Ubiquitin-like domain proteins are larger than ubiquitin and typically contain multiple domains, with only one homologous to ubiquitin. Four of the six UDPs in yeast are associated with the ubiquitin proteasome system (UPS) and interact directly with the proteasome through their UbL domains. Rad23, Dsk2, and Ddi1 contain N-terminal UbLs, which are recognized by the proteasome subunits Rpn1, Rpn10, and Rpn13 (Elsasser *et al*, 2002; Funakoshi *et al*, 2002; Kaplun *et al*, 2005; Husnjak *et al*,

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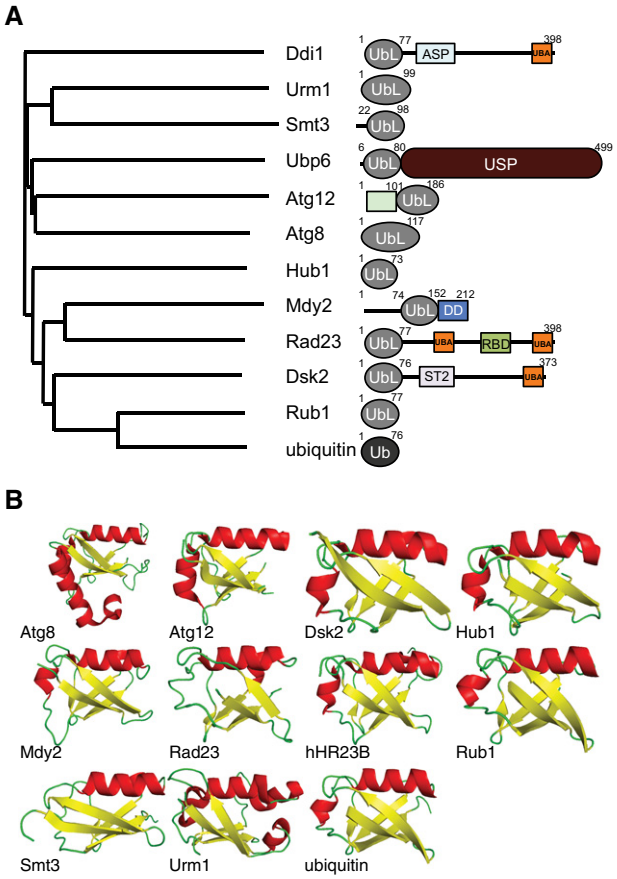


**Figure 1. UbL domains.**

A Phylogenetic relationship of UbL domains in *S. cerevisiae* and schematic representations of the corresponding UbL proteins. Sequences were clustered and mapped onto a N-J tree by ClustalX2 (Larkin *et al*, 2007) and the PHYLIP package (Felsenstein, 1989). ASP, aspartyl protease domain; UBA, ubiquitin-associated domain; USP, ubiquitin-specific protease domain; DD, dimerization domain; RBD, Rad4-binding domain; ST2, Sti1/Sti1 domain pair.

B Structural models of UbL domains generated by PyMOL (The PyMOL Molecular Graphics System, Version 0.99 Schrödinger, LLC). PDB IDs: Atg8 2ZPN, Atg12 3W1S, Dsk2 2BWF, Hub1 1M94, Mdy2 4GOC, Rad23 3M62, hHR23B 1P1A, Rub1 1BT0, Smt3 1EUV, Urm1 2PK0, ubiquitin 1UBQ.

2008; Zhang *et al*, 2009; Gomez *et al*, 2011). Rad23, Dsk2, and Ddi1 also contain one or more ubiquitin-associated (UBA) domains, which bind to polyubiquitin chains and allow the proteins to serve as diffusible proteasome substrate receptors (Saeki *et al*, 2002a; Elsasser *et al*, 2004; Kim *et al*, 2004; Verma *et al*, 2004; Elsasser & Finley, 2005; Kaplun *et al*, 2005; Zhang *et al*, 2009). The Ubp6 protein binds to the proteasome subunit Rpn1 through a UbL domain at its N-terminus and through interactions of its catalytic domain (Leggett *et al*, 2002; Rosenzweig *et al*, 2012; Aufderheide *et al*, 2015; Bashore *et al*, 2015; Shi *et al*, 2016). Ubp6 modulates proteasome activity by trimming ubiquitin chains on proteasome substrates and by modulating proteasome activity allosterically (Crosas *et al*, 2006; Hanna *et al*, 2006; Koulich *et al*, 2008; Peth *et al*, 2009; Lee *et al*, 2010; Bashore *et al*, 2015). Neither the UbL-UBA proteins nor Ubp6 are degraded by the proteasome. The remaining two UDPs are not associated with the UPS. Atg12 contains a C-terminal UbL domain and participates in the autophagy pathway (Mizushima *et al*, 1998), whereas Mdy2 contains a central UbL domain and is involved in the biogenesis of tail-anchored proteins in the endoplasmic reticulum (Wang *et al*, 2010; Chartron *et al*, 2012a).

Here, we ask how the UbL domains specify different functions. The simplest model would be that domains in proteins not involved in the UPS do not bind to the proteasome. To test this model, we selected twelve ubiquitin-like domains, eleven encoded by *S. cerevisiae* and one by *H. sapiens*, and examined whether they could target proteins to the yeast proteasome. We found that the yeast proteasome can bind all UbL domains tested, albeit with different affinities. The *in vivo* functions of the domains do not correlate with their apparent proteasome affinities; some UbL domains not involved in the UPS can bind the proteasome more tightly than *bona fide* interactors. We then asked why the proteasome does not degrade UbL proteins. We propose that proteins escape proteasomal degradation when they lack unstructured regions that allow the proteasome to initiate degradation. We tested this hypothesis on two ubiquitin domain proteins, Ubp6 and Mdy2. Ubp6 naturally lacks sites at which the proteasome can initiate degradation, but was depleted rapidly when a disordered region was introduced. The N-terminal domain of Mdy2 is disordered but is buried by its binding partner Get4. Deleting Get4 exposed the disordered region and led to the degradation of Mdy2. We conclude that protein stability is determined not only by proteasome affinity, but also by the presence of sites at which the proteasome can engage its substrates and initiate degradation.

## Results

### UbL domains bind the proteasome

To investigate whether UbL domains bind to the proteasome, we tested whether the purified domains could displace a proteasome substrate and prevent its degradation. We constructed a model substrate with the UbL domain of *S. cerevisiae* Rad23 at its N-terminus followed by a superfolder green fluorescent protein (GFP) domain (Pédelacq *et al*, 2006) and finally a previously characterized C-terminal disordered region of 95 amino acids derived from *S. cerevisiae* cytochrome $b_2$ to allow the proteasome to initiate the degradation (Inobe *et al*, 2011) ($UbL^{Rad23}$-GFP-95; Fig 2A). We then expressed the protein in *E. coli*, isolated it, and presented it to purified yeast proteasome. The protein was degraded efficiently and at a rate comparable to that observed for model substrates targeted to the proteasome by ubiquitin tags under equivalent conditions (Fishbain *et al*, 2011; Kraut & Matouschek, 2012). The degradation followed Michaelis–Menten behavior and the measured $K_M$ value was independent of proteasome concentration, whereas $V_{max}$ values scaled linearly with it (Fig 2C). Thus, it was possible to characterize the degradation process using standard kinetic approaches.

To investigate the binding of different UbL domains to the proteasome, we cloned and expressed the domains in *E. coli* and purified the corresponding proteins. We first characterized the UbL domains from Rad23 and Dsk2 because these two domains are known to bind to the proteasome (Schauber *et al*, 1998; Elsasser *et al*, 2002; Funakoshi *et al*, 2002; Saeki *et al*, 2002b). Adding

**Table 1. Physical and sequence properties of UbL domains analyzed in this study as well as the function of the relevant proteins.**

| Protein | Class | Full length (aa) | UbL domain (aa) | Identity to Ub (%) | Identity to $UbL^{Rad23}$ (%) | Secondary structure identity to Ub ($\%_{SSE}$) | Function | $K_i$ relative to $UbL^{Rad23}$ (μM) |
|---|---|---|---|---|---|---|---|---|
| Rad23 | UDP | 398 | 1–77 | 22 | – | 83 | DNA excision repair; UPS | 0.45 ± 0.04 |
| hHR23B | UDP | 409 | 1–82 | 30 | 27 | 100 | DNA excision repair; UPS | 2.0 ± 0.2 |
| Dsk2 | UDP | 373 | 1–76 | 29 | 28 | 67 | Spindle pole body duplication; UPS | 3.3 ± 0.3 |
| Atg8 | ULM | 117 | 1–117 | 20 | 14 | 83 | Autophagy | 3.5 ± 0.2 |
| Hub1 | ULM | 73 | 1–73 | 22 | 18 | 83 | Pre-mRNA splicing; morphogenesis | 9.8 ± 0.8 |
| ubiquitin | – | 76 | 1–76 | – | 22 | – | Ubiquitination | 16 ± 1 |
| Ddi1 | UDP | 428 | 1–80 | 20 | 19 | N/A | Mating type switching; UPS | 17 ± 2 |
| Ubp6 | UDP | 499 | 6–80 | 17 | 23 | N/A | Deubiquitination | 24 ± 3 |
| Urm1 | ULM | 99 | 1–99 | 28 | 6 | 83 | Sulfur carrier in tRNA modification | 30 ± 3 |
| Mdy2 | UDP | 212 | 74–152 | 22 | 34 | 83 | Biogenesis of TA proteins | 33 ± 3 |
| Rub1 | ULM | 77 | 1–77 | 53 | 22 | 100 | Cullin proteins neddylation | 34 ± 2 |
| Smt3 | ULM | 101 | 22–98 | 16 | 17 | 50 | Sumoylation | 65 ± 4 |
| Atg12 | UDP | 186 | 101–186 | 7 | 9 | 83 | Autophagy | N/A |

Ub, ubiquitin; UDP, ubiquitin-like domain protein; ULM, ubiquitin-like modifier; UPS, ubiquitin proteasome system; N/A, not available.

increasing amounts of the purified UbL domains inhibited the degradation of $UbL^{Rad23}$-GFP-95 (Figs 2D and E, and EV1). Conversely, increasing amount of substrate overcame the inhibition by the UbL domains (Fig 2E). Thus, substrate and UbL domains competed for binding to the proteasome. The apparent equilibrium constants ($K_i$s) with which the competing UbL domains inhibited substrate degradation reflect the affinity of the domains to the proteasome (Table 1, Fig EV1). The measured $K_i$ values report only on binding to the sites at which the Rad23 UbL domain interacts with the proteasome. It is possible that the other UbL domains also bind to other sites on the proteasome (Gomez *et al*, 2011; Shi *et al*, 2016) but these interactions would not be detected by our assay, causing us to underestimate affinities in these cases.

We then measured the ability of nine additional UbL domains, as well as monoubiquitin, to compete with Rad23's UbL domain for the proteasome (Table 1). The apparent $K_i$s fell into a range from 0.5 to 65 μM. UbL domains that are known to associate with the proteasome were distributed over the entire range of affinities, with monoubiquitin roughly in the middle.

### UbL domains can target artificial substrates for proteasomal degradation *in vitro*

Next we examined whether all UbL domains that bound to the proteasome could also target proteins for degradation. We first addressed this question *in vitro* with model proteasome substrates consisting of a central *E. coli* dihydrofolate reductase (DHFR) domain with the relevant UbL domain fused to its N-terminus and the 95-amino acid tail described above fused to its C-terminus, creating UbL-DHFR-95 (Fig 3A). We expressed these proteins by coupled *in vitro* transcription and translation and presented them to purified yeast proteasome. The UbL domain of Rad23 mediated efficient degradation. However, DHFR-95 without the UbL domain and $UbL^{Rad23}$-DHFR without the tail were stable (Fig 3A). Treatment with the proteasome inhibitor MG132 stabilized the $UbL^{Rad23}$-DHFR-95 protein, indicating that degradation was proteasome dependent (Fig 3A). Strikingly, all of the other UbL domains tested also mediated degradation (Fig 3B). As before, degradation depended on the presence of a disordered tail and was repressed by the proteasome inhibitor MG132. The rates and extent of degradation in the *in vitro* assays varied somewhat for the different UbL domains, but the variation did not seem to depend on whether the domains were naturally involved in proteasome-dependent processes, nor did they correlate with the apparent inhibition constants ($K_i$s). For example, the yeast SUMO homolog Smt3 targeted DHFR for degradation as effectively as the Rad23 UbL domain but bound to the proteasome more weakly (Fig 3A and B, Table 1).

### UbL domains can target proteins for proteasome degradation *in vivo*

We next investigated whether UbL domains could mediate degradation by the proteasome in yeast. We created model substrates based on imidazoleglycerol-phosphate dehydratase (His3) and yellow fluorescent protein (YFP). The abundance of His3 protein was estimated by its ability to support growth, and the abundance of yellow fluorescent protein (YFP) by measuring cellular fluorescence.

His3 catalyzes an essential step in histidine production in yeast, so that *his3* mutant strains cannot grow in medium lacking histidine unless the mutation is complemented by a plasmid-borne wild-type *HIS3* gene (Alifano *et al*, 1996). However, if the His3 protein expressed from the plasmid is degraded, then the complementation will fail. Proteasomal degradation of His3 protein can therefore modulate the yeast growth rate. We tested whether UbL domains could target His3 protein for degradation in yeast by attaching them to the N-terminus of His3. The fusion proteins were expressed from a *GAL1* promoter on a 2-micron (multicopy) plasmid in a *his3* mutant yeast strain (Fleming *et al*, 2002) (Appendix Table S1).

 

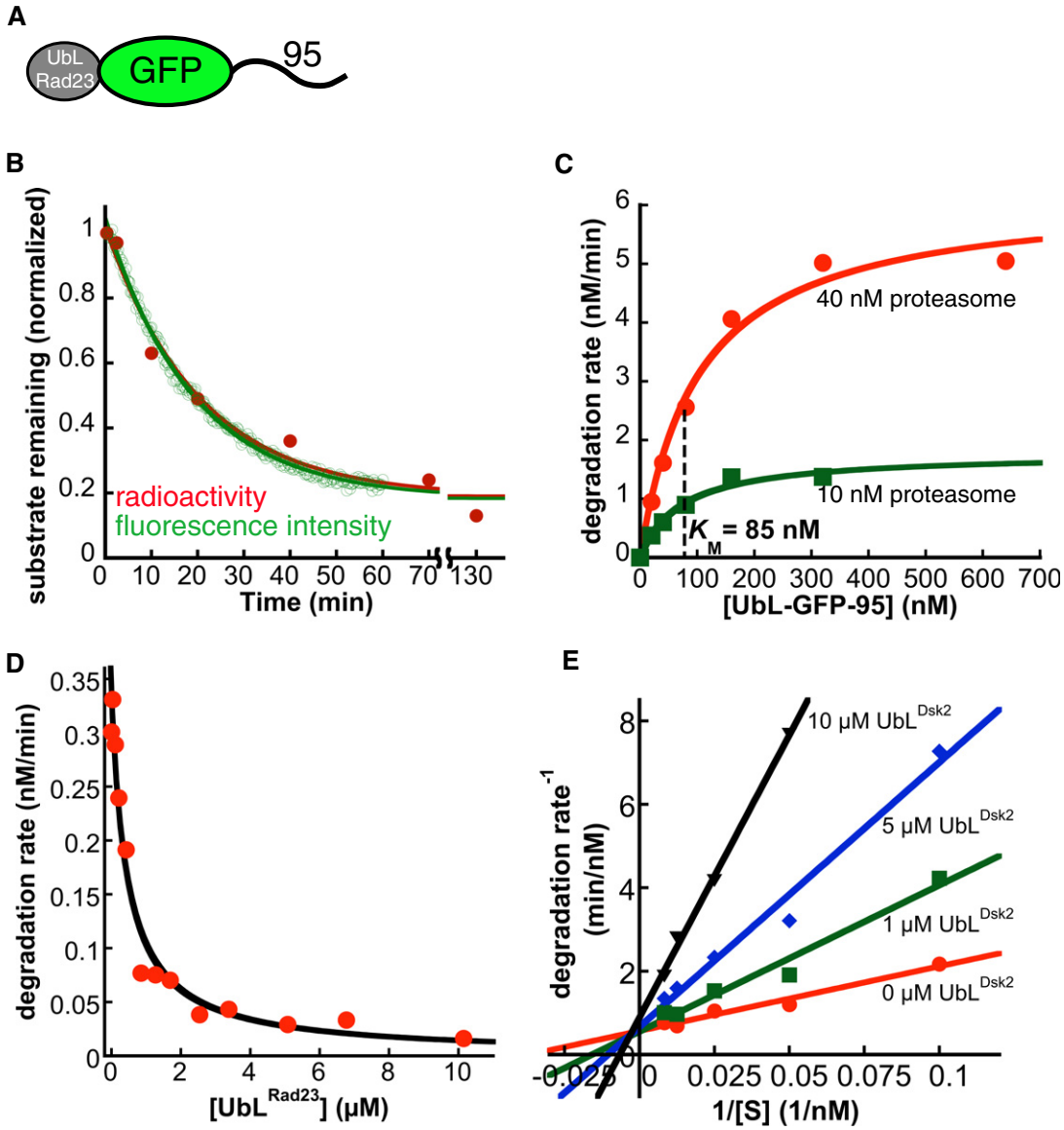


**Figure 2. UbL domains bind with proteasome.**

A Sketch of $UbL^{Rad23}$-GFP-95 consisting of the UbL domain of *S. cerevisiae* Rad23, followed by superfolder GFP and a 95-amino acid-long tail derived from *S. cerevisiae* cytochrome $b_2$.

B *In vitro* degradation of $UbL^{Rad23}$-GFP-95 by purified *S. cerevisiae* proteasome. The graph plots the amount of substrate over time, estimated by fluorescence intensity monitored by plate reader (green) or electronic autoradiography of SDS–PAGE gel bands (red), normalized to the initial substrate amount as described in Materials and Methods.

C Michaelis–Menten analysis of $UbL^{Rad23}$-GFP-95 degradation by different concentrations of purified *S. cerevisiae* proteasome (green, 10 nM; red, 40 nM).

D $UbL^{Rad23}$ inhibits $UbL^{Rad23}$-GFP-95 degradation. The initial degradation rates of $UbL^{Rad23}$-GFP-95 at different concentrations of purified $UbL^{Rad23}$ domain are plotted and fitted to the equation describing competitive inhibition.

E The $UbL^{Dsk2}$ domain is a competitive inhibitor of $UbL^{Rad23}$-GFP-95 degradation. Lineweaver–Burk plot of $UbL^{Rad23}$-GFP-95 degradation with different concentrations of purified $UbL^{Dsk2}$ domain.

Data information: (B–E) Proteasomal degradation of $UbL^{Rad23}$-GFP-95 monitored by fluorescence intensity in a Tecan plate reader as described in Materials and Methods.

We added 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the His3 enzyme, to make the growth assay more sensitive (Hawkes *et al*, 1995). To allow the proteasome to initiate the degradation of any bound His3 fusion protein, we also fused a 51-amino acid disordered tail (derived from subunit 9 of the $F_o$ component of the *N. crassa* ATPase, abbreviation Su9) to the C-terminus of His3 (Fig 4A). Expression of His3 with an N-terminal DHFR domain and the Su9 tail complemented *his3* mutant cells, indicating that N- and C-terminal modifications alone did not impede His3 activity (Fig 4B). However, fusing the Rad23 UbL domain to the N-terminus of His3 with a Su9 tail prevented the complementation and inhibited cell growth (Fig 4B). His3 fusion protein could not be detected by

Western blotting in cell lysate, unless the proteasome was inhibited by the proteasome inhibitor bortezomib (Fig EV2A), suggesting that the growth defect was caused by the depletion of UbL-His3-tail protein by the proteasome. Indeed, most of the yeast UbL domains we investigated prevented His3 from complementing the *his3* mutation to some extent. The exceptions were the UbL domains of Ddi1 and Urm1, which affected growth similarly under selective and nonselective conditions, indicating that $UbL^{Ddi1}$ and $UbL^{Urm1}$ failed to target His3 fusion proteins to degradation effectively. None of the UbL domains themselves disrupted His3 function as UbL-His3 fusion proteins lacking the C-terminal proteasome initiation tails were stable (Fig EV2B) and supported growth (Fig 2B). Expressing either UbL-His3-tail or UbL-His3 constructs when histidine was present in the medium did not affect yeast growth. The simplest interpretation of our observations is that the different UbL domains target His3 to the proteasome. Furthermore, degradation by the proteasome required a disordered region at which the proteasome could initiate the degradation.

The *his3* complementation experiments report on cellular protein abundance indirectly through the His3 protein's enzymatic activity and its effect on cell metabolism and growth. The abundance of fluorescent proteins can be estimated directly from the total cell fluorescence measured by flow cytometry (Yen *et al*, 2008; Sharon *et al*, 2012). Therefore, we repeated the degradation experiments with yellow fluorescent protein (YFP) as the reporter protein. We fused either one of the eleven UbL domains or a DHFR domain to the N-terminus of YFP and a Su9 tail to its C-terminus, and then expressed the fusion proteins (UbL-YFP-tail) from the constitutive promoter p*ACT1* on a *CEN* plasmid derived from pYCplac33 (Gietz & Sugino, 1988). The same plasmid also expressed the red fluorescent protein dsRed Express2 (Strack *et al*, 2008) from a second p*ACT1* promoter as a normalization control for variation in plasmid copy number, global protein expression levels, and cell size. We again first investigated the yeast Rad23 UbL domain, measuring the fluorescence of yeast cells expressing the fusion protein $UbL^{Rad23}$-YFP-tail together with dsRed (Fig 5A). Replacing the UbL domain with DHFR increased the yellow fluorescence roughly 9-fold, while the red fluorescence remained unchanged (Fig 5A). Inhibiting the proteasome with bortezomib increased yellow fluorescence without changing red fluorescence (Figs 5A and EV3B). Degradation required a C-terminal tail; removing the tail increased YFP fluorescence 9-fold and bortezomib did not affect the fluorescence of cells expressing $UbL^{Rad23}$-YPF protein without the tail (Fig EV3B and C).

We then investigated the 10 other yeast UbL domains. Here, we compared the fluorescence of cells expressing the UbL-YFP fusion with and without the proteasome initiation tail to minimize the effects of the N-terminal UbL domain on protein expression (Fig 5B). The tail itself had little effect on YFP fluorescence as cells expressing DHFR-YFP fusions with or without the tail showed similar fluorescence (Fig 5A and B). All UbL domains reduced cellular YFP fluorescence at least twofold (Fig 5B), and inhibiting the proteasome with bortezomib increased YFP fluorescence (Fig EV3B), suggesting that all the UbL domains targeted YFP for degradation by the proteasome. The proteasome inhibitor affected neither the fluorescence of cells expressing UbL-YFP fusions without proteasome initiation tails nor the fluorescence of cells expressing DHFR-YFP fusions (Fig EV3B). Therefore, both the N-terminal UbL domain and the C-terminal tail were required for degradation by the proteasome.

Degradation of the His3 and YFP fusion proteins depended on the UbL domain but was not due to ubiquitination. *S. cerevisiae* encodes a single ubiquitin-activating enzyme (McGrath *et al*, 1991), Uba1, and its temperature-sensitive allele *uba1-204* makes it possible to reduce protein ubiquitination effectively by shifting cells to the restrictive temperature (Ghaboosi & Deshaies, 2007). When expressed in *uba1-204* cells, the steady-state levels of all the UbL fusion proteins with or without proteasome initiation tail were similar at the restrictive and permissive temperatures, while a protein containing a classic N-end rule degron was stabilized at the restrictive temperature (Figs EV2 and EV3C). Thus, the UbL domains did not serve as ubiquitination signals but rather targeted proteins to the proteasome directly.

The UbL domains share the β-grasp fold of ubiquitin (Fig 1). The proteasome recognizes ubiquitin through a hydrophobic patch on its surface centered around the residues L8, I44, and V70 (Beal *et al*, 1996; Sloper-Mould *et al*, 2001), and we asked whether the UbL domains are recognized in a similar manner. We mutated the residues corresponding to the ubiquitin hydrophobic patch in the UbL domains of Rad23, Dsk2, Rub1, and Mdy2 to glutamic acid (Fig EV4A), and tested whether the mutations prevented proteasomal degradation. We found that the triple Glu mutations stabilized UbL-His3-tail proteins, as judged by Western blotting against an HA-tag inserted between the His3 domain and the Su9 tail (Fig EV4C). The mutations also allowed the proteins to complement the *his3* mutation under selective growth conditions. Thus, proteasome targeting mediated by UbL domains was disturbed by the mutations and the UbL domains of Rad23, Dsk2, Rub1, and Mdy2 appeared to bind the proteasome through the same interaction surface as ubiquitin in the assays described here.

In summary, the eleven yeast UbL domains we tested were able to target proteins to the proteasome for degradation in at least one of the cell-based assays and degradation depended on the presence of a disordered tail, presumably to allow the proteasome to initiate the degradation.

#### Degradation of natural UbL proteins Mdy2 and Ubp6

The observation that all UbL domains we tested could target artificial proteins for degradation was unexpected because only a subset of UbL domains is thought to function in the UPS physiologically. Degradation of the model proteins in yeast required the presence of an unstructured region in the protein in addition to the UbL domain to allow the proteasome to initiate degradation (Fishbain *et al*, 2011). Perhaps stable natural UbL proteins also escape degradation because they lack sites at which the proteasome can engage them (Prakash *et al*, 2004, 2009; Takeuchi *et al*, 2007; Zhao *et al*, 2010; Fishbain *et al*, 2011, 2015; van der Lee *et al*, 2014). We tested this hypothesis on two UDPs, Mdy2 and Ubp6.

Mdy2 (also known as Get5) catalyzes a step in the guided entry of tail-anchored protein insertion (GET) pathway through which tail-anchored proteins are inserted into the ER membrane (Jonikas *et al*, 2009). It is a 212-amino acid protein with a central UbL domain flanked by an N-terminal domain (NTD) and a C-terminal dimerization domain (Chang *et al*, 2010; Chartron *et al*, 2010, 2012a,b; Simon *et al*, 2013) (Fig 6A). Mdy2 expressed by coupled *in vitro*

 

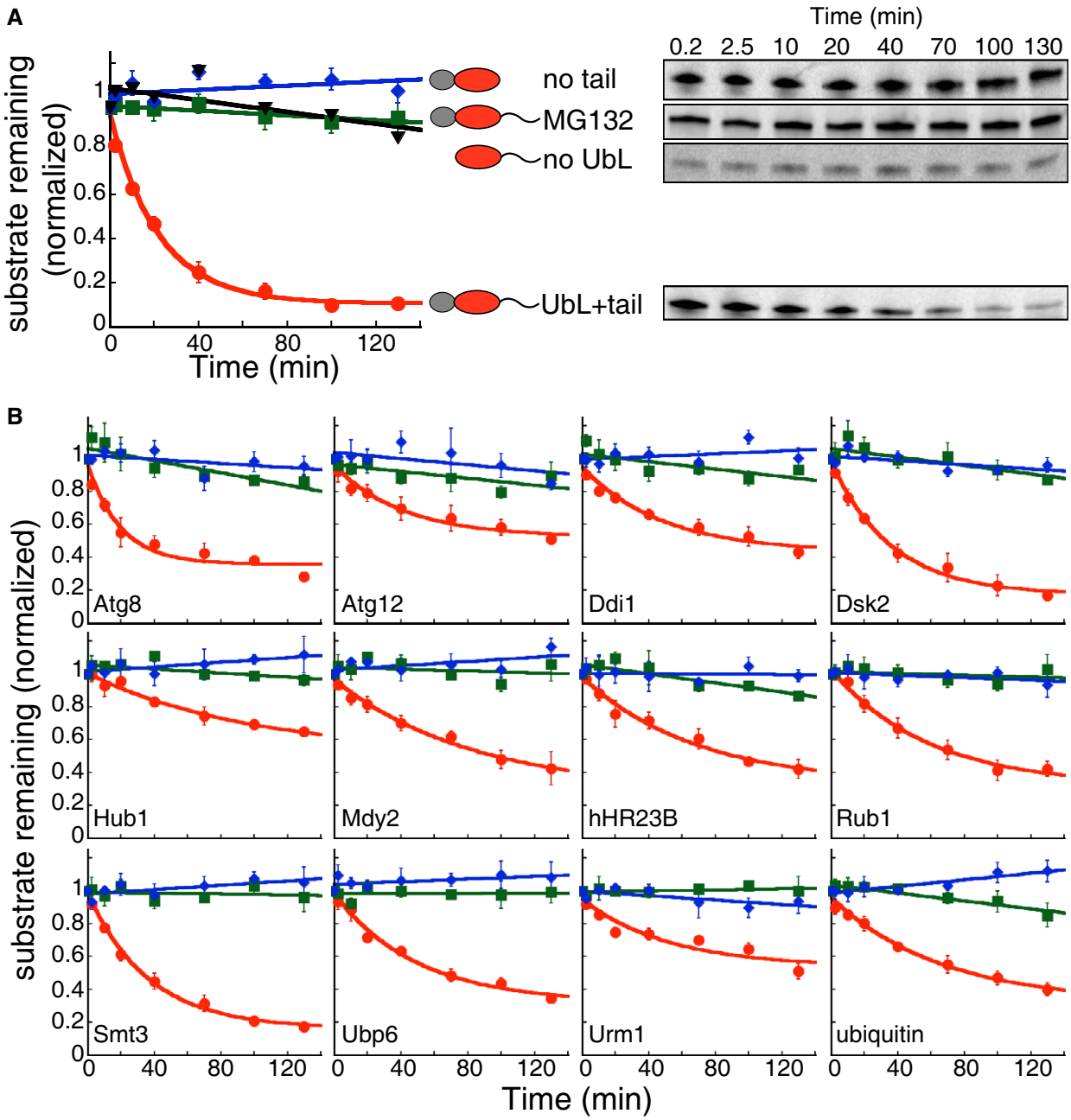


**Figure 3. UbL domains target substrate to proteasome degradation *in vitro*.**

A, B *In vitro* degradation of model proteins by purified *S. cerevisiae* proteasome. The model proteins (UbL-DHFR-95) consisted of an N-terminal UbL domain, followed by an *E. coli* DHFR domain and a 95-amino acid tail derived from *S. cerevisiae* cytochrome $b_2$. UbL-DHFR-95 proteins were degraded by proteasome (red solid circle) and stabilized by removing the UbL domain (no UbL, black triangles), by removing the 95-amino acid tail (no tail, blue diamonds), or by proteasome inhibitor (MG132, green squares). (A) Degradation of model proteins containing the UbL domain of *S. cerevisiae* Rad23. (B) Degradation of model proteins containing other UbL domains or ubiquitin. The graphs plot the amount of substrate estimated by electronic autoradiography in SDS–PAGE gel bands over time as normalized to the initial substrate amount as described in Materials and Methods. Data points represent mean values determined from at least three repeat experiments; error bars indicate s.e.m.

Source data are available online for this figure.

transcription and translation was degraded by purified yeast proteasome (Fig 6B left panel). Degradation was inhibited by the addition of an excess of purified $UbL^{Mdy2}$ domain, indicating that the UbL domain of Mdy2 was required for its degradation (Fig 6B right panel). Degradation also required the N-terminal domain, since deleting the first 73 amino acids of Mdy2 stabilized it against degradation (Fig 6B left panel). The N-terminal domain is largely disordered (Chang *et al*, 2010; Chartron *et al*, 2010) and could be required to allow the

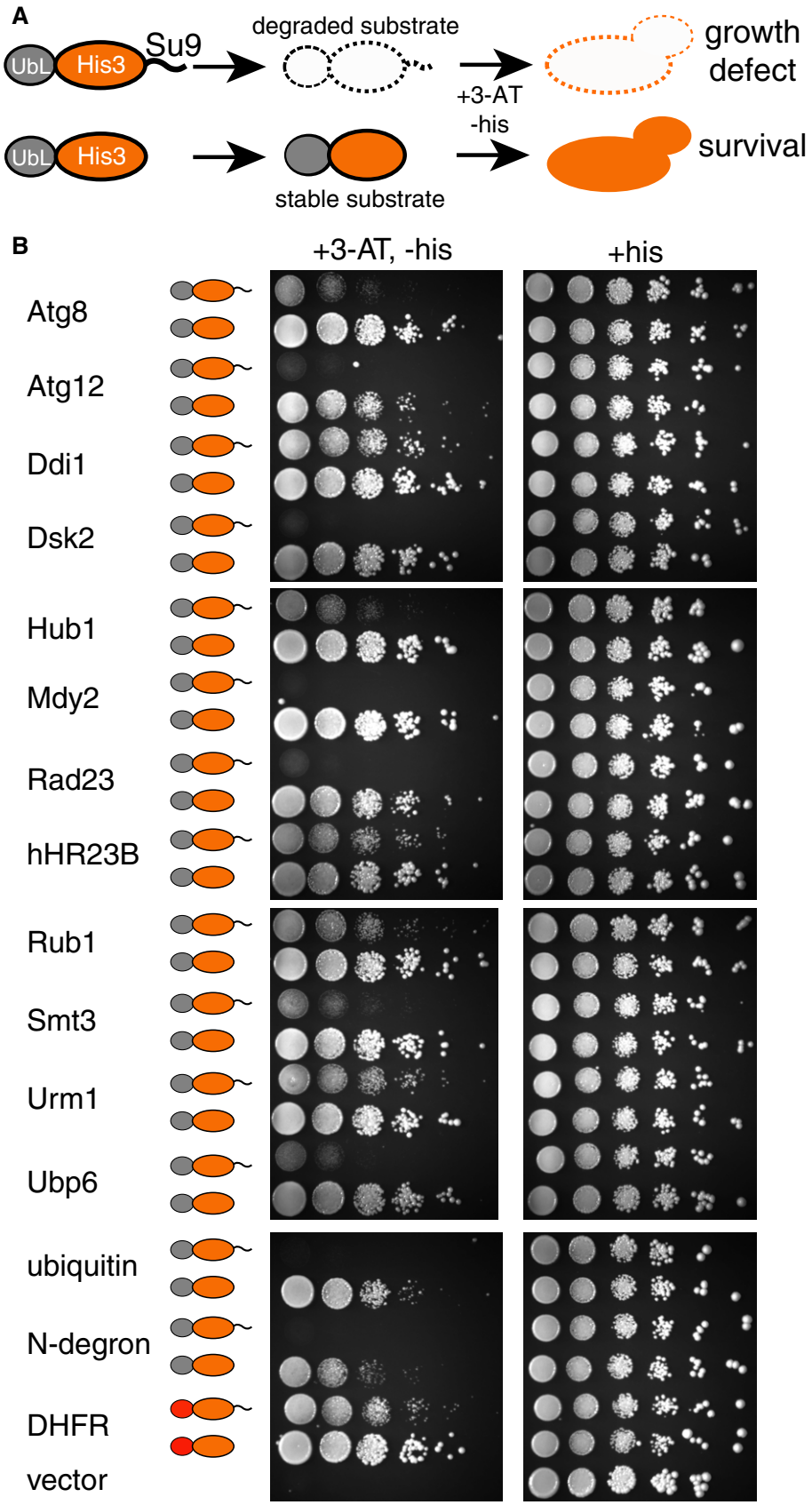


**Figure 4.**

**Figure 4. UbL domains target His3 substrates to proteasome degradation in yeast.**

A Schematic representation of yeast growth assay. The model proteins consisted of an UbL domain and *S. cerevisiae* imidazoleglycerol-phosphate dehydratase (His3), followed by stop codon (no tail) or a 51-amino acid tail derived from subunit 9 of the $F_o$ component of the *Neurospora crassa* ATPase (Su9) at the C-terminus. In *his3* mutant cells, the absence of His3 protein caused a growth defect when grown on selective media. Stable His3 proteins escaped proteasome degradation and rescued the *his3* mutant cells from the growth defect.

B UbL domains mediated the degradation of His3 fusion proteins with a Su9 tail and affected yeast growth under selective condition (+3-AT, −his). Replacing UbL domains with DHFR domains rescued the growth defect. Cells expressing His3 fusion proteins in late log phase were serially diluted and stamped on selective plates. The plates were incubated at 30°C for 3 days for imaging.

proteasome to initiate the degradation. Indeed, attaching an unrelated 95-amino acid tail to the C-terminus of Mdy2 restored proteasomal degradation of Mdy2 lacking its N-terminal domain (Fig 6B left panel). In yeast, Get4 binds to Mdy2's N-terminal domain and buries it (Chang *et al*, 2010; Chartron *et al*, 2010). Adding purified Get4 to Mdy2 in the degradation assays protected Mdy2 from proteolysis (Fig 6B right panel). Get4 did not, however, prevent proteasome binding; fusing a 95-amino acid tail to the C-terminus of Mdy2 allowed it to be degraded even in the presence of Get4 (Fig 6B right panel). Thus, Get4 protected Mdy2 from proteasome degradation *in vitro* by preventing the proteasome from initiating degradation.

Next, we studied Mdy2 degradation in yeast cells by expressing Mdy2 under the control of a constitutive promoter (p*TPI1*) (Partow *et al*, 2010), and Get4 under the control of an inducible promoter (p*GAL1*) on the same CEN plasmid. Mdy2 was tagged with a C-terminal HA epitope and Get4 with an N-terminal Flag epitope, so that their abundance in yeast cells could be estimated by Western blotting. Plasmid-expressed Mdy2 was degraded by the proteasome, as demonstrated by the increase in steady-state levels when the proteasome was inhibited with bortezomib (Fig 6C). Overexpressing Get4 at the same time protected Mdy2 from degradation and proteasome inhibition no longer increased Mdy2 steady-state levels (Fig 6C). Mdy2 that lacked the NTD was not degraded by the proteasome as neither proteasome inhibition nor Get4 overexpression affected its steady-state levels (Fig 6C).

Up to this point, we analyzed overexpressed Mdy2 and it was possible that endogenous Mdy2 was protected by other mechanisms such as proteins binding to its UbL domain. However, we found that Mdy2 at physiological levels was also susceptible to proteasomal degradation (Fig 6D). Deletion of *GET4* (yeast strain *get4*Δ, Appendix Table S1) led to the depletion of Mdy2 from cells in a proteasome-dependent manner. Thus, endogenous Mdy2 can be degraded by the proteasome but appears to be protected by the binding of Get4 to a disordered region where the proteasome can initiate the degradation.

Ubp6 binds to the proteasome subunit Rpn1 through an N-terminal UbL domain and through interactions of its catalytic (ubiquitin-specific protease, USP) domain with Rpn1 (Leggett *et al*, 2002; Rosenzweig *et al*, 2012; Aufderheide *et al*, 2015; Bashore *et al*, 2015). By analogy to Mdy2, it seemed possible that Ubp6 escaped degradation because it lacked a region at which the proteasome can initiate the degradation. Indeed, Ubp6 expressed by coupled *in vitro* transcription and translation was not degraded by purified yeast proteasome unless a 95-amino acid disordered tail was fused to its C-terminus (Fig 7A). Deleting the UbL domain inhibited, but did not completely abolish the degradation of Ubp6 with the tail (Fig 7A). Similarly, fusing the 51-amino acid Su9 tail to the C-terminus of Ubp6 allowed the proteasome to degrade the resulting fusion proteins when overexpressed in yeast (Fig 7B). The proteasome inhibitor bortezomib increased the steady-state levels of overexpressed Ubp6 fusions with a tail but not without a tail. Degradation of overexpressed Ubp6 with a tail was not eliminated by the deletion of the UbL domain, presumably because the truncated protein was still able to bind to the proteasome. Degradation of Ubp6 was not simply caused by overexpression. Attaching a short disordered tail (an 11-aa linker followed by the 8-aa Flag epitope) to endogenous Ubp6 also destabilized the protein, and a longer disordered tail (54 aa) caused complete depletion of Ubp6 (Fig 7C). These results suggested that Ubp6 binds to the proteasome through a two-site interaction, yet avoids degradation by the proteasome because it lacks an initiation region.

## Discussion

Ubiquitin and UbL domains mediate protein–protein interactions in the UPS and other cellular processes, but it is not clear how the cell distinguishes between the different signals and assigns them to the appropriate processes. The simplest explanation would be that the proteasome does not recognize ubiquitin signals that direct proteins to processes other than degradation. However, this mechanism seems to be only part of the explanation. For example, polyubiquitin chains formed through both Lys48 and Lys63 are recognized by the proteasome (Saeki *et al*, 2009; Komander & Rape, 2012), yet Lys63-linked chains are thought to function mostly in membrane trafficking and DNA repair (Erpapazoglou *et al*, 2014). One likely basis for targeting specificity is that ubiquitin receptors compete with each other for substrates so that even though the proteasome may be able to recognize a protein, the receptors involved in a competing pathway bind them more effectively because of higher affinity or local concentration (Hicke *et al*, 2005; Grabbe & Dikic, 2009; Dores *et al*, 2010; Nathan *et al*, 2013). Degradation by the proteasome requires a second signal in the target protein in the form of a disordered region at the which the proteasome can initiate degradation (Inobe & Matouschek, 2014). In yeast, the proteins Cdc34, which autoubiquitinates, and Rad23, which contains a UbL domain, escape degradation when overexpressed, but are rapidly degraded by proteasome when disordered tails are fused to their C-termini (Fishbain *et al*, 2011, 2015). More broadly, cellular proteins that contain disordered regions at which the proteasome can initiate degradation have on average shorter half-lives than proteins that lack appropriate initiation regions (van der Lee *et al*, 2014; Fishbain *et al*, 2015).

We find here that UbL domains in proteins associated with proteasome-independent processes are recognized by the proteasome with apparent affinities similar to those of *bona fide* proteasome-interacting domains. All the UbL domains we tested can bind to the proteasome and target model proteins for degradation *in vitro*

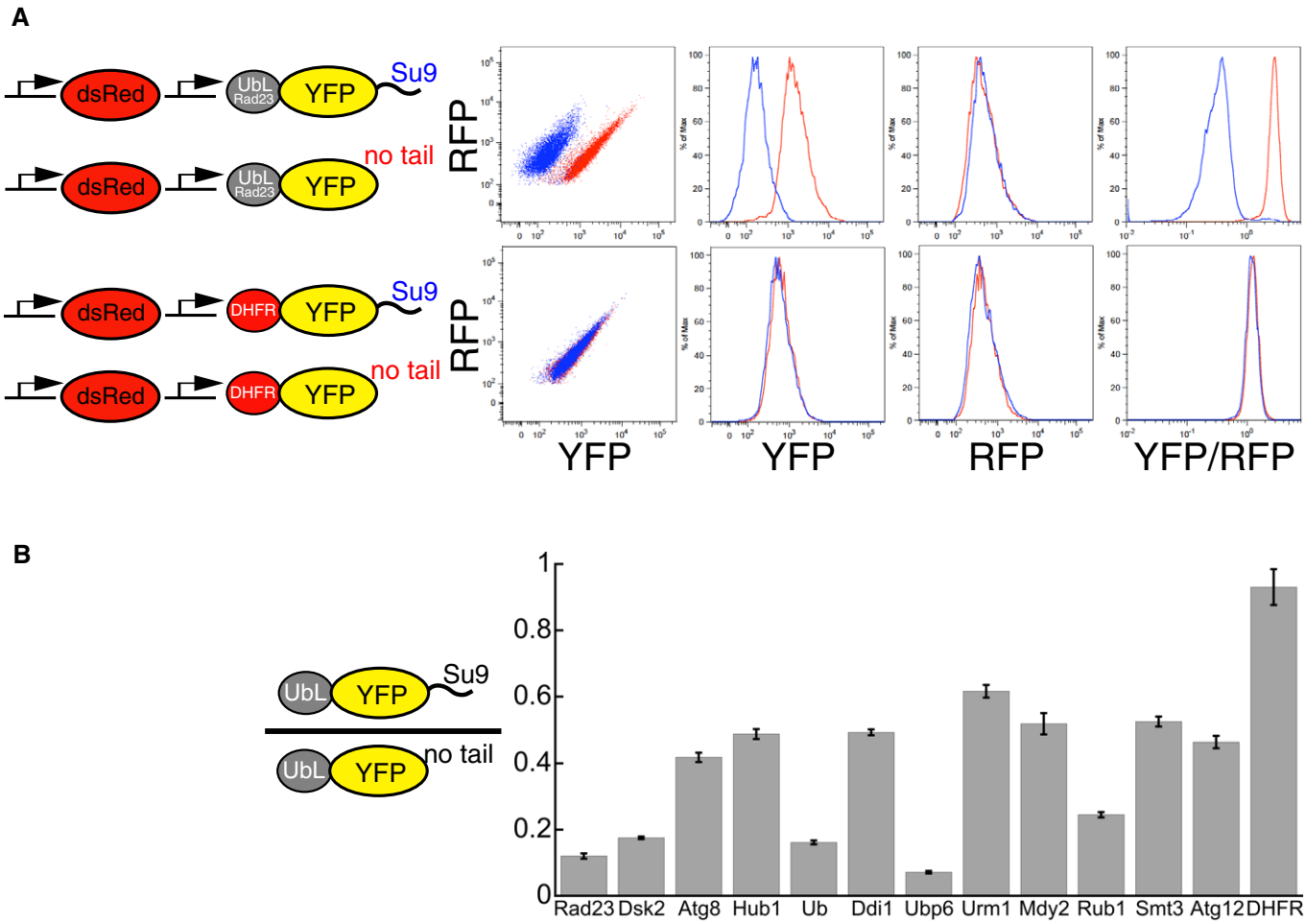


**Figure 5. UbL domains target fluorescence substrates to proteasome degradation in yeast.**

A Fluorescence-based degradation assay in *S. cerevisiae*. The substrate proteins consisted of a UbL domain, followed by a yellow fluorescent protein (YFP) domain and a disordered tail (51 amino acids derived from subunit 9 of the $F_o$ component of the *Neurospora crassa* ATPase, Su9). Control substrates lacked the disordered tail. Substrates and a red fluorescent protein were expressed from consecutive constitutive promoters (pACT1) on the same CEN plasmid (YCplac33). Fluorescence profiles of cells expressing different substrates monitored by flow cytometry. $UbL^{Rad23}$ targeted YFP-Su9 protein for degradation. Removing the Su9 tail or replacing $UbL^{Rad23}$ with a DHFR domain stabilized YFP protein in cells.

B UbL domains targeted YFP substrates to degradation in the presence of an initiation region. The *Y*-axis plots the abundance of the UbL-YFP-tail proteins normalized by a translation control, which is the same UbL-YFP protein without the tail. The ratio is equal to one for domains that are not recognized by the proteasome, and smaller than one if the UbL is recognized by the proteasome and the UbL-YFP-tail protein is degraded. YFP fluorescence was also corrected for plasmid copy number using RFP fluorescence. The median of YFP/RFP ratio for each construct was calculated from 10,000 cells collected in one flow cytometry run and reflected the abundance of YFP substrates in yeast cells as described in Materials and Methods. Data points represent the mean values determined from at least three repeat experiments; error bars indicate s.e.m.

and in yeast. However, in the more stringent proteasome-targeting assay, the degradation of the stable YFP protein, the four highest scoring UbL domains were the ones previously known to bind to the proteasome.

Several factors may complicate the comparison of *in vitro* and *in vivo* observations quantitatively. *In vivo,* UbL domains could bind the proteasome indirectly. For example, the UbL domains of Rad23, Dsk2, and Ddi1 are thought to bind to their own and each others' UBA domains (Saeki *et al*, 2002a; Heessen *et al*, 2005; Kang *et al*, 2006; Heinen *et al*, 2011). Similarly, other binding partners and receptors for individual UbL domains may modulate their interaction with the proteasome, positively or negatively, as Sgt2 is thought to do for Mdy2 (Chartron *et al*, 2012a), Ufo1 for Ddi1 (Ivantsiv *et al*, 2006), UbL-UBA proteins for Rub1 (Singh *et al*, 2012), Snu66 for Hub1 (Mishra *et al*, 2011), and Shp1 for Atg8 (Krick *et al*, 2010), as may subcellular localization. Under some circumstances, the $K_i$s may underestimate the strengths of the UbL–proteasome interactions. We measured $K_i$s in kinetic competition experiments with the UbL domain of Rad23 so that any interactions that are completely independent of the $UbL^{Rad23}$ binding site would not be detected. Indeed, it was recently reported that the UbL domain of Ubp6 can bind to a site on Rpn1 not recognized by $UbL^{Rad23}$ (Shi *et al*, 2016).

Nevertheless and importantly, even weak proteasome interactions can be physiologically relevant. The UbL domain of Ddi1 has one of the lower proteasome affinities in our competition assay, in agreement with earlier studies (Saeki *et al*, 2002a; Gomez *et al*,

 

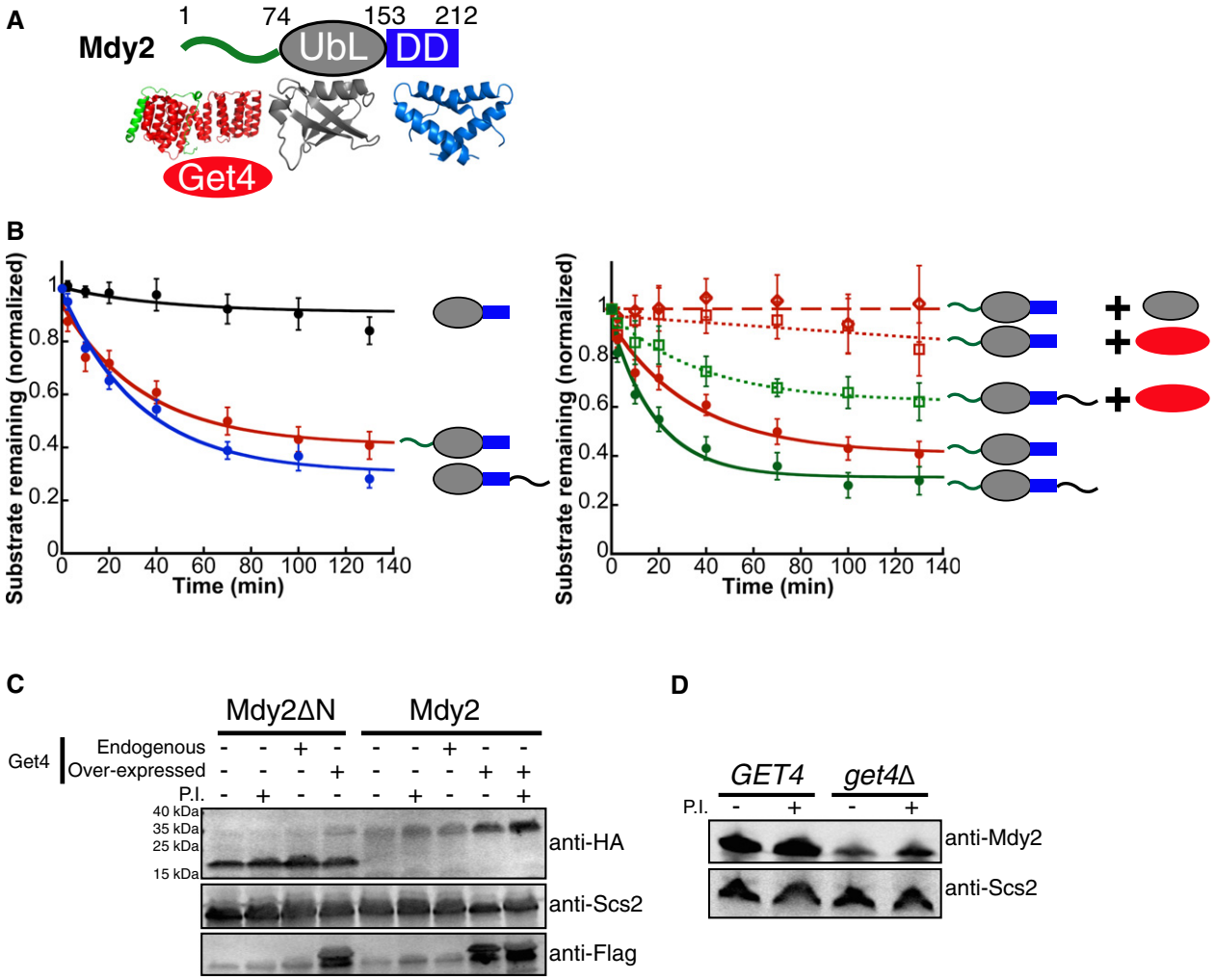


**Figure 6. Degradation of Mdy2 proteins *in vitro* and *in vivo*.**

A Schematic representation of *S. cerevisiae* Mdy2. Structural models of the N-terminal domain (NTD) of Mdy2 (amino acids 1–59, green), with its binding partner Get4 (red, PDB ID: 2WPV), the central UbL domain of Mdy2 (amino acids 74–148, gray, PDB ID: 4GOC), and the C-terminal dimerization domain (DD) of Mdy2 (amino acids 152–212, blue, PDB ID: 2LNZ).

B *In vitro* degradation of Mdy2 proteins by purified *S. cerevisiae* proteasome. Mdy2 (red solid circles) was degraded by the proteasome; removing the NTD (Mdy2ΔN, black solid circles) stabilized the protein but attaching a C-terminal tail to the truncated protein (Mdy2ΔN-95, blue solid circles) restored degradation. Mdy2 (red solid circles) was also stabilized by the addition of its binding partner Get4 (red open squares, dashed line) or $UbL^{Mdy2}$ as a competitor for proteasome binding (red open diamonds, dotted line). When Get4 binding buried the NTD, fusion of a tail to the C-terminus of Mdy2 (green open squares, dotted line) restored degradation. The graph plots the amount of substrate remaining estimated by electronic autoradiography in SDS–PAGE gel bands over time as normalized to the initial amount of substrate as described in Materials and Methods. Data points represent the mean values determined from at least three repeat experiments; error bars indicate s.e.m.

C Degradation of Mdy2 *in vivo*. Steady-state levels of overexpressed C-terminally HA-tagged full-length Mdy2 and a mutant in which the NTD was deleted (Mdy2ΔN) in *S. cerevisiae*, in the absence of Get4 or in the presence of endogenous Get4 or overexpressed N-terminally Flag-tagged Get4. Protein levels were determined by Western blotting for the HA- or Flag-tag, or for the protein Scs2 as a loading control, of SDS-PAGE gels of *S. cerevisiae* protein extracts. The proteasome was inhibited by bortezomib as indicated.

D Steady-state levels of endogenous Mdy2 in Get4 wild-type (*GET4*) or deletion (*get4Δ*) *S. cerevisiae* strains in the presence or absence of the proteasome inhibitor bortezomib. Protein levels were determined by Western blotting for Mdy2, or Scs2 as the loading control, of SDS–PAGE gels of *S. cerevisiae* protein extracts.

Source data are available online for this figure.

2011; Rosenzweig *et al*, 2012), and it targets the model proteins we tested only poorly for proteasomal degradation *in vivo*. However, Ddi1 functions as an extrinsic substrate receptor for the proteasome and is required for the degradation of Ho endonuclease during the regulation of mating type switching in yeast (Kaplun *et al*, 2005). As part of the same process, the UbL domain of Ddi1 also mediates Ddi1's interaction with the ubiquitin ligase Ufo1 through several UIM domains in Ufo1 (Ivantsiv *et al*, 2006). It has been proposed that the UbL domain switches between the two binding partners as it mediates Ho degradation and the lower apparent proteasome affinity may be a reflection of this mechanism (Voloshin *et al*, 2012).

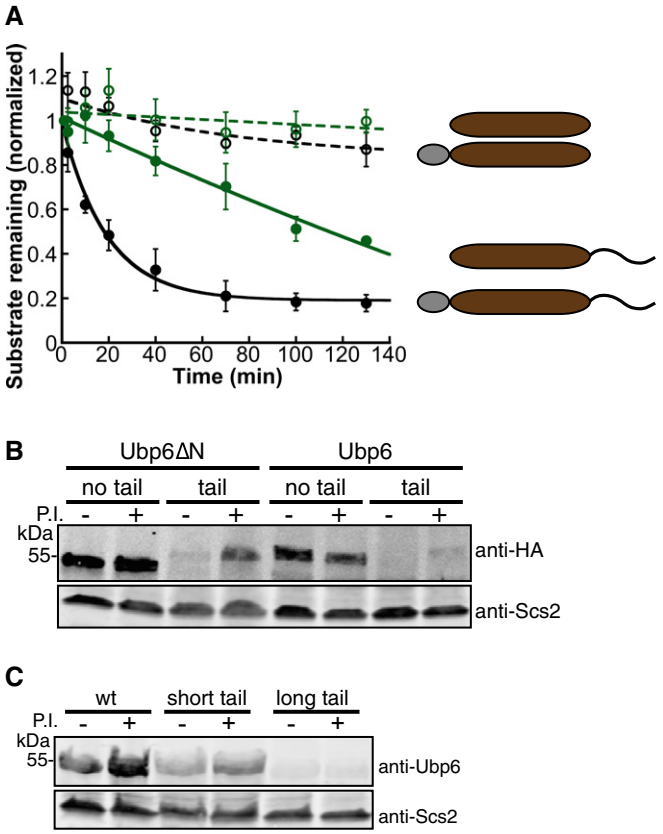


**Figure 7. Degradation of Ubp6 proteins *in vivo* and *in vitro*.**

A *In vitro* degradation of Ubp6 proteins by purified *S. cerevisiae* proteasome. Full-length Ubp6 (Ubp6, black open circles) and a UbL deletion mutant (Ubp6ΔN, green open circles) escaped degradation. Attachment of a C-terminal 95-amino acid tail derived from *S. cerevisiae* cytochrome $b_2$ allowed the proteasome to degrade the Ubp6 proteins (Ubp6-95, black solid circles and Ubp6ΔN-95, green solid circles). The graph plots the amount of substrate estimated by electronic autoradiography in SDS–PAGE gel bands over time as normalized to the initial substrate amount as described in Materials and Methods. Data points represent the mean values determined from at least three repeat experiments; error bars indicate s.e.m.

B Degradation of Ubp6 proteins *in vivo*. Steady-state levels of N-terminally HA-tagged Ubp6 mutants in *S. cerevisiae*. N-terminally HA-tagged Ubp6 and Ubp6ΔN with or without a Su9-tail attached to their C-termini were overexpressed in yeast. Protein levels were determined by Western blotting for the HA-tag, or the protein Scs2 as a loading control, of SDS–PAGE gels of *S. cerevisiae* protein extracts. The proteasome was inhibited by bortezomib where indicated.

C Steady-state levels of endogenous Ubp6 and Ubp6 derivatives with short (19 amino acid) or long (54 amino acid) tails at their C-termini. Protein levels were determined by Western blotting for Ubp6, or Scs2 as a loading control, of SDS–PAGE gels of *S. cerevisiae* protein extracts. The proteasome was inhibited by bortezomib as indicated.

Source data are available online for this figure.

The UbL domain in Mdy2 appears to bind the proteasome even less tightly than the UbL of Ddi1 ($K_i$ = 33 ± 3 μM for $UbL^{Mdy2}$ vs. $K_i$ = 17 ± 2 μM for $UbL^{Ddi1}$), but it can target model proteins as well as Mdy2 itself to the proteasome for degradation. Indeed, even endogenous Mdy2 can be degraded by the proteasome. The first 73 amino acids of Mdy2 are disordered in structures of Mdy2 alone and the length and amino acid composition of this region are in the range observed in sequences that allow the proteasome to initiate degradation efficiently. In yeast, the Get4 protein binds tightly to this region and buries it entirely (Chang *et al*, 2010; Chartron *et al*, 2010). There are no other disordered regions in Mdy2, so that the proteasome is unable to initiate degradation when it binds to Mdy2. The UbL domain of Mdy2 also interacts with Sgt2, another component of the GET pathway (Chartron *et al*, 2012a). Why would the cell use a UbL domain that interacts with both its binding partner in the GET pathway and the proteasome? The arrangement may keep Mdy2 abundance in a specific range. Mdy2 that accumulated above the amount of Get4 would be subject to degradation by the proteasome. An upper concentration threshold might prevent competition with interaction partners, such as Sgt2, that are shared with other pathways.

At the same time, tight proteasome interaction does not necessarily lead to degradation. Ubp6 binds the proteasome with high affinity (Leggett *et al*, 2002; Rosenzweig *et al*, 2012; Aufderheide *et al*, 2015; Bashore *et al*, 2015; Shi *et al*, 2016) but escapes proteolysis because it lacks a site at which the proteasome is able to initiate degradation. We have found previously that the UbL-UBA protein Rad23 also escapes the degradation because it lacks effective proteasomal initiation sites and it is likely that the other two UbL-UBA proteins in yeast, Dsk2 and Ddi1, are protected by a similar mechanism (Fishbain *et al*, 2011, 2015). The last remaining UbL domain protein in yeast, Atg12, contains a C-terminal UbL domain, and its N-terminal half is predicted to be disordered. We were not able to purify Atg12's UbL domain and therefore do not know whether it is able to bind to the proteasome directly.

Of the eleven yeast UbL proteins, five (Atg8, Hub1, Rub1, Smt3, and Urm1) are ubiquitin-like modifiers that become attached to various macromolecules. Two of these, Atg8 and Hub1, bind the proteasome more tightly than the UbL of Ddi1 in our *in vitro* assay. Atg8 ($K_i$ = 3.3 ± 0.3 μM) competed as effectively for proteasome binding as the UbL of the proteasomal substrate receptor Dsk2 ($K_i$ = 3.5 ± 0.2 μM) and Hub1 competed only slightly less effectively ($K_i$ = 9.8 ± 0.8 μM). Atg8 becomes attached to phosphatidylethanolamine in the autophagy pathway (Ichimura *et al*, 2000), and Hub1 is thought to bind to proteins non-covalently (Lüders *et al*, 2003; Yashiroda & Tanaka, 2004; Mishra *et al*, 2011). The interaction of Hub1 with its binding partner Snu66 leaves the likely proteasome interaction surface free (Mishra *et al*, 2011) so that Hub1 might still be able to bind to the proteasome when in complex with Snu66. Snu66 is predicted to contain long disordered regions yet may escape the degradation because of competition with other binding partners or its localization. It is also possible that ubiquitin-like modifiers destabilize target proteins under some circumstances. In human cells, the ubiquitin-like modifier FAT10 is incorporated into proteasome degradation signals (Hipp *et al*, 2005). Future work will reveal how common this mechanism is and whether ubiquitin-like modifiers also target proteins to degradation in yeast.

In summary, we find that 10 of the 11 ubiquitin-like domains encoded in the yeast genome are able to bind to the proteasome and most of them are able to target proteins for degradation. Thus, protein stability is not solely controlled by proteasome affinity as determined by the UbL domains but also by the presence or absence of sites at which the proteasome can engage its substrates to initiate the degradation.

 

## Materials and Methods

### Substrate proteins

Protein substrates were derived from *E. coli* DHFR, *N. crassa* ATPase $F_o$ component subunit 9, *S. cerevisiae* cytochrome $b_2$, UbL proteins, His3, and *Homo sapiens* hHR23B. Fluorescent proteins were gifts from B. S. Glick (University of Chicago). The coding sequences were cloned into the plasmid pGEM-3Zf (+) (Promega) for expression *in vitro* and in *E. coli* for protein purification as described previously (Fishbain *et al*, 2011, 2015), or into the yeast CEN plasmid YCplac33 or 2-micron plasmid pYES2 for *in vivo* experiments. In model proteins, N-terminal UbL domains were connected to *E. coli* DHFR, *S. cerevisiae* His3, GFP, or YFP by the linker sequence (VDGGSGGGS) as described previously (Kraut & Matouschek, 2011). C-terminal tails derived from cytochrome $b_2$ or subunit 9 were attached to DHFR and the other proteins through a two-amino acid linker (PR), and lysine residues in the cytochrome $b_2$ tails were replaced by arginine. Model proteins without a tail or with cytochrome $b_2$ tails had a hexahistidine tag at the very C-terminus. Isolated individual UbL domains all ended with the linker VDGGSGGGS followed by a C-terminal hexahistidine tag for purification.

### Protein expression and purification

Yeast proteasome was purified from *S. cerevisiae* strain YYS40 (Appendix Table S1) by immunoaffinity chromatography using anti-FLAG antibodies (M2 agarose affinity beads, Sigma) as described previously with modifications (Saeki *et al*, 2005). Proteasome preparations were analyzed by SDS–PAGE and compared to published compositions (Lander *et al*, 2012). Each proteasome preparation was checked for activity by testing the degradation of the proteasome substrate $UbL^{Rad23}$-DHFR-95 and for contamination by proteases by testing the stability of proteins that lacked a proteasome-binding tag (DHFR-95) as described previously (Fishbain *et al*, 2011). For *in vitro* degradation experiments, radioactive substrates were expressed from a T7 promoter by a coupled *in vitro* transcription–translation reaction using the RTS 100 *E. coli* HY Kit (Roche) or TNT® Coupled Reticulocyte Lysate Systems (Promega) containing [$^{35}$S]-methionine following the manufacturer's protocol. After synthesis, the substrates were either partially purified by high-speed centrifugation followed by precipitation in two volumes of saturated $(NH_4)_2SO_4$ or affinity-purified using Talon magnetic beads (Clontech) as described previously (Kraut & Matouschek, 2011).

### Proteasomal degradation assays

Degradation assays with radiolabeled substrates were performed as described previously (Kraut & Matouschek, 2011). Briefly, the assays were carried out at 30°C by adding radiolabeled substrates to 40 nM of purified yeast proteasome in a reaction buffer containing a creatine phosphate and creatine kinase ATP-regenerating system. Samples were removed at designated times, added to SDS–PAGE sample buffer to stop the reaction, and analyzed by SDS–PAGE. Protein amounts were determined by electronic autoradiography (Instant Imager, Packard); 100 μM MG132 was used to inhibit the proteolytic activity of proteasome where indicated. The degradation assays monitoring fluorescence intensity were performed in 384-well plates in a plate reader (Infinite M1000 PRO, Tecan). Assays were carried out at 30°C by adding fluorescent substrates to 40 nM of purified yeast proteasome in a reaction buffer containing a creatine phosphate and creatine kinase ATP-regenerating system. Fluorescence intensity was read every 30 s (excitation: 485 nm, 5 nm bandwidth; emission: 525 nm, 10 nm bandwidth) for a total reaction time of one hour. Protein amounts were corrected based on fluorescence intensity of the reaction in each well using standard curves describing the relationship between fluorescence intensity and protein concentration. Each assay was repeated at least three times. Initial degradation rates are given by the slope of the decay curves at time zero and were calculated as the product of the amplitude and the rate constant of the decay curve determined by nonlinear fitting to a single exponential decay to a constant offset using the software package Kaleidagraph (version 4.1, Synergy Software).

### Yeast transformation and strain construction

His3 and fluorescent substrates were expressed in *S. cerevisiae* from a *CEN* plasmid (YCplac33) or a 2-micron plasmid (pYES2) with a *URA3* selection marker. The plasmids were transformed into *S. cerevisiae* strains (Appendix Table S1) using the Frozen-EZ Yeast Transformation II Kit (Zymo Research). To attach a tail to endogenous Ubp6, linearized DNA encoding the tail sequence followed by a *LEU2* selection cassette and flanked by sequences homologous to the insertion site was transformed into the host strain *UBP6-GFP* (Appendix Table S1). The constructs were confirmed by Sanger sequencing.

### *In vivo* protein abundance determination

HA-tagged Mdy2 protein derivatives were expressed from the constitutive triosephosphate isomerase (*TPI1*) promoter and Flag-tagged Get4 from the inducible *GAL1* promoter. Both proteins were expressed from the *CEN* plasmid YCplac33 in *S. cerevisiae* strains *pdr5*Δ or *get4*Δ (Appendix Table S1). HA-tagged Ubp6 protein derivatives were expressed under the control of the inducible *GAL1* promoter on the *CEN* plasmid YCplac33 in *S. cerevisiae* strain *pdr5*Δ. HA-tagged UbL-His3 proteins were expressed from the inducible GAL1 promoter on the 2-micron plasmid pYES2 in *S. cerevisiae* strain *pdr5*Δ. Cells with overexpressed or endogenous Mdy2 and Ubp6 proteins were grown to mid–log phase with glucose or galactose as a carbon source and lysed by vortexing with glass beads (BioSpec Products). Protein extracts were prepared and analyzed by Western blotting using standard protocols as described (Fishbain *et al*, 2015). HA-tagged Mdy2 or Ubp6 proteins were detected with a monoclonal anti-HA antibody (1:500, Roche Life Science, #12158167001); Flag-tagged Get4 was detected by a monoclonal anti-Flag antibody (Cell Signaling, #8146). Scs2p was detected by an anti-Scs2 polyclonal antibody (1:1,000, gift from J. Brickner, Northwestern University). Endogenous Mdy2 was detected by an anti-Mdy2 polyclonal antibody (Liou *et al*, 2007) (1:10,000, gift from C. Wang, Institute of Molecular Biology, Academia Sinica). Endogenous Ubp6 was detected by an anti-Ubp6 antibody (Leggett *et al*, 2002) (1:500, gift from D. Finley, Harvard

Medical School). Alexa-680-labeled goat anti-rabbit antibody (1:20,000, Invitrogen, #A21109) and Alexa-800-labeled goat anti-mouse antibody (1:20,000, Rockland Immunochemicals, #610-132-121) were used as secondary antibodies. Protein amounts were estimated by direct infrared fluorescence imaging (Odyssey, LI-COR Biosciences). 100 μM bortezomib was used to inhibit proteasome activity where indicated.

#### Flow cytometry

Yeast cells were grown at 30°C to early log phase before harvesting for analysis. Fluorescence signals in dsRed and YFP channels were collected on an LSR Fortessa (BD Biosciences) flow cytometer and analyzed by FlowJo to calculate the median YFP over RFP fluorescence ratio of 10,000 cells in each population. Assays were repeated at least three times; 100 μM bortezomib was used to inhibit proteasome activity where indicated.

#### Yeast growth assay

His3 proteins were expressed under the control of the inducible *GAL1* promoter on a 2-micron plasmid (pYES2) in *S. cerevisiae* strain *pdr5*Δ (Appendix Table S1). Cells were grown with galactose as a carbon source at 30°C to late log phase, serially diluted ($OD_{600}$ from $10^{-1}$ to $10^{-6}$), and stamped on synthetic media plates. The plates were incubated at 30°C for 3 days before imaging.

**Expanded View** for this article is available online.


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### Author contributions

HY, GK, and CMY performed experiments, analyzed data, and co-wrote the paper. AM directed experiments, analyzed data, and co-wrote the paper.

### Conflict of interest

The authors declare that they have no conflict of interest.

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