Substrate Ubiquitination Controls the Unfolding Ability of the Proteasome

Received for publication, February 8, 2016, and in revised form, June 14, 2016. Published, JBC Papers in Press, July 12, 2016, DOI 10.1074/jbc.M116.720151

Eden L. Reichard, Giavanna G. Chirico, William J. Dewey, Nicholas D. Nassif, Katelyn E. Bard, Nickolas E. Millas, and Daniel A. Kraut
From the Department of Chemistry, Villanova University, Villanova, Pennsylvania 19085

In eukaryotic cells, proteins are targeted to the proteasome for degradation by polyubiquitination. These proteins bind to ubiquitin receptors, are engaged and unfolded by proteasomal ATPases, and are processively degraded. The factors determining to what extent the proteasome can successfully unfold and degrade a substrate are still poorly understood. We find that the architecture of polyubiquitin chains attached to a substrate affects the ability of the proteasome to unfold and degrade the substrate, with K48- or mixed-linkage chains leading to greater processivity than K63-linked chains. Ubiquitin-independent targeting of substrates to the proteasome gave substantially lower processivity of degradation than ubiquitin-dependent targeting. Thus, even though ubiquitin chains are removed early in degradation, during substrate engagement, remarkably they dramatically affect the later unfolding of a protein domain. Our work supports a model in which a polyubiquitin chain associated with a substrate switches the proteasome into an activated state that persists throughout the degradation process.

The ubiquitin-proteasome system is responsible for the bulk of intracellular protein degradation in eukaryotes (1, 2). Misfolded and damaged proteins are recognized by chaperones, tagged with a polyubiquitin chain, and unfolded and degraded by the proteasome. Regulatory proteins such as kinases and transcription factors are also often short-lived due to polyubiquitination and degradation. Increasingly the ubiquitin-proteasome system has been implicated in disease due to its involvement in so many cellular processes (3–6). Proteasome inhibition has successfully been used to treat several types of cancer, and it has been suggested that proteasome activation might be beneficial for diseases involving protein misfolding and aggregation, such as Huntington and Alzheimer diseases (7).

Proteins destined for degradation are typically polyubiquitinated by the action of a series of enzymes (E1, E2, and E3) that first covalently attach the C terminus of ubiquitin to a lysine within the substrate, forming an isopeptide bond, then attach an additional ubiquitin moiety to a lysine within the first ubiquitin, and then continue to extend the chain (8). Ubiquitin contains 7 lysines, resulting in 8 different chain architectures that can result when linkage through the N terminus is factored in (M1, K6, K11, K27, K29, K33, K48, K63), and mixed or branched linkages are also possible. K48-linked chains were the first discovered, and are the canonical degradation signal that targets substrates to the proteasome. K11-linked chains have also been linked to degradation in the cell, and are particularly prevalent in the endoplasmic reticulum-associated degradation pathway and in cell cycle signaling (9). K63-linked chains, on the other hand, are involved in the response to DNA damage, membrane trafficking, and signaling, and are generally not implicated in proteasomal degradation in vivo (10, 11). Nonetheless, K63-linked chains are capable of targeting proteins to the proteasome in vitro (12). The other linkages are less common than these three, and are less well understood (9).

Once polyubiquitinated, substrates are recognized by ubiquitin receptors on the 19S regulatory particle of the proteasome (Rpn10, Rpn13, Sem1, Rpn1, possibly Rpt5) or by adaptor proteins such as Rad23 that bind to both the proteasome and the substrate (13–15) (Fig. 1). After engaging the substrate, typically at an unstructured region near in space to the ubiquitin tag, the proteasome moves processively along its substrate, unfolding domains as they are encountered, and then feeds the unfolded polypeptide chain into the buried protease active sites in the 20S core for degradation to small peptides (16, 17). Proteasomal substrates have their polyubiquitin tag removed and recycled during the degradation process by the deubiquitinase Rpn11, which is poised above the mouth of the Rpt channel so it functions only on an already engaged substrate (2, 18, 19). An additional deubiquitinase, Ubp6, is positioned across from Rpn11, and seems to play a role both in activation and in preventing additional substrates from engaging the proteasome before degradation is complete (20, 21).

Each time the proteasome encounters a folded domain as it moves along the substrate polypeptide chain, there is some chance that rather than successfully unfolding the domain, the proteasome will instead release the substrate. Because the ubiquitin tag is removed for recycling by Rpn11 early on in the degradation process (2), proteasomal failure to unfold a domain within a multidomain protein can lead to the irreversible release of a partially degraded protein, i.e. a failure of processivity. Such failures of processivity are thought to be rare, as they could lead to the formation of potentially toxic byproducts such as an unregulated fragment of a transcription factor. Nonetheless, there are several cases where partial substrate degradation occurs as part of the maturation or repression of a transcription factor or other regulatory protein (22). These bio-

* This work was supported by National Science Foundation Grant 1515229. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains a supplemental Table.

1 To whom correspondence should be addressed: Mendel Hall 214D, 800 Lancaster Ave., Villanova, PA 19085. Tel.: 610-519-3910; E-mail: daniel.kraut@villanova.edu.
Ubiquitination Controls Proteasomal Unfolding Ability

FIGURE 1. Steps in the degradation of polyubiquitinated substrates by the proteasome. Ubiquitin chains bind to ubiquitin receptors on the 19S subunit (pink) and an unstructured region of the substrate (green) is engaged by the Rpt AAA+ ring in the base of the 19S subunit, which begins translocating the substrate toward the 20S core (purple). Ubiquitin chains are removed as Rpn11 (orange) encounters them, folded domains are unfolded as they enter the pore, and the unfolded protein is translocated into the 20S core and degraded into peptides.

logical examples of what has been termed “proteasomal processing” include Spt23, Mga2, and Def1 in yeast, Cubitus interruptus in flies, and the p105 precursor to NfκB and members of the Gli family in mammals (reviewed in Ref. 22). All these proteins are incompletely degraded by the proteasome, resulting in the release of large folded fragments, which go on to have new biological activities distinct from the parent protein. For example, the p105 protein cannot move to the nucleus to turn on transcription of target genes because of internal ankyrin domains that bind to and mask a nuclear localization signal. Upon partial degradation by the proteasome, the ankyrin domains are degraded, exposing the nuclear localization signal and allowing the activation of NfκB responsive genes (23, 24).

Although we are beginning to comprehend some of the mechanisms governing proteasomal processivity and its failure (25–27), our understanding remains limited. We cannot, for example, look at a protein sequence and predict with any confidence if it will be fully or partially degraded by the proteasome. Our ability to determine which proteins will initiate degradation has improved (28–30), and in addition to bringing substrates to the proteasome, ubiquitination may enhance the degradation of substrates by aligning the regulatory and core particles during the initiation of degradation (20, 21). However, as described above ubiquitin is unlikely to remain associated with the proteasome by the time the second or third domain of a multidomain substrate is reached (2, 18, 19, 31). Nonetheless, our results show for the first time that for both the initial domain encountered and subsequent domains the presence and chain architecture of polyubiquitin on a substrate controls the unfolding ability of the proteasome, suggesting that engagement with a polyubiquitinated substrate can shift the proteasome into an activated state that persists throughout the degradation process.

Results

Degradation of GFP Depends on the Method of Ubiquitination—Green fluorescent protein (GFP) is commonly used, both in vivo and in vitro, as a model substrate to follow protein degradation (32–37). However, there are also reports that GFP can be difficult to unfold and degrade, perhaps because of its stable β-barrel structure (12, 38, 39), and in cells degradation of ubiquitin-GFP fusions are dependent on the action of the chaperone p97 (40, 41). In particular, Saecki et al. (12) reported that GFP resists degradation when tagged with K63-linked polyubiquitin chains, whereas other proteins were degraded efficiently. Nonetheless, GFP has been used successfully in recent in vitro proteasomal degradation assays (20, 37, 42).

To isolate the effect of ubiquitin chain linkage on the outcome of GFP degradation, we constructed a model substrate that could be ubiquitinated using either the Rsp5 E3 ligase, which gives exclusively K63-linked chains (12, 43) or a Keap1/Cul3/Rbx1 E3 ligase complex (Keap1 for brevity) previously reported to give primarily K48-linked chains (44). This substrate consisted of an N terminally His-tagged dihydrofolate reductase (DHFR)2 domain followed by an enhanced GFP domain (eGFP) followed by a C-terminal Neh2 domain from the Nrf2 protein, which contains recognition sequences for Keap1 on either side of a 47-amino acid stretch containing 7 lysines (45, 46). A single mutation in the Neh2 domain (G10Y) allowed the introduction of a “PPXY” motif for Rsp5 binding (47) without disrupting the Keap1 binding sites, creating the Neh2Dual degron (Fig. 2a). This substrate was efficiently ubiquitinated using either Rsp5 or Keap1, resulting in both cases in high molecular weight species that only just entered the resolving gel as measured by in-gel GFP fluorescence (Fig. 2b; GFP fusion proteins can be detected by fluorescence directly in SDS-PAGE gels as long as the sample is not boiled, as GFP remains folded under these conditions). Ubiquitination did not change the fluorescence intensity of the substrate.

Degradation by purified proteasome gave dramatically different results depending on how the substrate was ubiquitinated. A substantial portion of the Rsp5-ubiquitinated substrate was only partially degraded. Instead of processively unfolding and degrading GFP, a slightly smaller fluorescent fragment or set of fragments formed during degradation (~30% of full-length protein degraded resulted in fragment formation, and both degradation of the full-length protein and fragment formation were reduced by the addition of the proteasome inhibitor bortezomib). The fragment bands ran slightly faster than ubiquitinated full-length protein, and presumably consisted of the DHFR-eGFP fusion lacking some or all of the Neh2 domain (Fig. 2, c and e). Such a degradation defect would be missed using a standard platereader or fluorimeter assay. In contrast, negligible amounts of fragment (<1%) were formed during the degradation of the Keap1-ubiquitinated substrate (Fig. 2, d and e). Similar results were obtained using the more stable superfolder GFP (sGFP (48)) instead of eGFP (Fig. 2e).

Poor binding of Rsp5-ubiquitinated substrates accompanied by low levels of nonspecific “clipping” by the 20S proteasome could, in principle, explain the observed incomplete

2 The abbreviations used are: DHFR, dihydrofolate reductase; DUB, deubiquitinase; eGFP, enhanced green fluorescent protein; sGFP, superfolder green fluorescent protein; UbA, ubiquitin aldehyde; yODC, yeast ornithine decarboxylase; Ni-NTA, nickel-nitrilotriacetic acid.
Ubiquitination Controls Proteasomal Unfolding Ability

Degradation of GFP. However, Michaelis-Menten plots of Rsp5 and Keap1-ubiquitinated substrate degradation (Fig. 2f) indicate that the apparent affinities of the substrate for the proteasome are similar ($K_m = 600 \pm 200 \text{ versus } 400 \pm 300 \text{ nm}$), and that the Rsp5-ubiquitinated substrate is actually turned over more rapidly ($k_{cat} = 0.7 \pm 0.1 \text{ min}^{-1} \text{ versus } 0.4 \pm 0.1 \text{ min}^{-1}$); substrate concentration did not substantially affect observed fragment levels (Fig. 2g). This modestly faster disappearance of the full-length protein makes sense, because $\approx 30\%$ of the proteasome-engaged substrate is released before unfolding of the GFP domain, speeding up the observed reaction because unfolding is presumably at least partially rate-limiting (49).

One explanation for this difference could be that ubiquitination with Keap1 directly destabilized GFP, whereas ubiquitination with Rsp5 had little or no destabilizing effect. However, the kinetics of guanidine-induced unfolding of GFP were essentially unchanged by ubiquitination with either Keap1 or Rsp5 (Fig. 2h). Additionally, replacement of GFP with mNeonGreen, which is more easily unfolded by the proteasome (39), substantially reduced the amount of fragment detected after Rsp5-ubiquitination and degradation (Fig. 2e), suggesting the stability of the substrate and mode of ubiquitination combine to determine whether a protein is fully unfolded.

To more precisely characterize this partially degraded protein, we used the N-terminal His tag on the eGFP-containing substrate to purify the residual fragment postdegradation. As expected for degradation from the C terminus, the fragment was retained on Ni-NTA beads, indicating that the N terminus was intact. Trypsin digestion followed by LC-MS/MS analysis and comparison to the full-length protein indicated that the fragment terminated in a region stretching from the terminal $\beta$-sheet of GFP (β11) to the first 33 amino acids of the Neh2Dual degron based on a reduced abundance of peptides from those regions followed by a complete lack of peptides detected C-terminal to those regions (Fig. 2i, supplemental Table). It has previously been shown that the bacterial ATP-dependent protease ClpXP can stall while attempting to degrade fragments. f, Michaelis-Menten plots for disappearance of full-length Rsp5-(closed circles) and Keap1- (open squares) ubiquitinated His-DHFR-eGFP-Neh2Dual. Error bars are the S.E. from 3 independent experiments. g, fraction of fluorescent protein containing substrate (red, Rsp5 ubiquitination; blue, Keap1 ubiquitination) that, upon degradation, gives a fragment in the assays used to generate panel g. No bar is shown for Keap1 ubiquitination at 50 nM concentration because fragment band intensity decreased slightly over the time course. h, ubiquitination does not affect guanidine-induced unfolding of sGFP. His-DHFR-sGFP-Neh2Dual was either ubiquitinated or mock-ubiquitinated in the absence of ubiquitin. 40 nM sGFP substrate (red, Keap1; orange, Keap1 mock; blue, Rsp5; purple, Rsp5 mock) was then unfolded in the presence of 5.9 M guanidine (solid lines), and its fluorescence over time (excitation 485 nm, emission 510 nm) was normalized to a guanidine-free control to correct for photobleaching. Average of two experiments is shown. i, mass spectrometry analysis of purified post-degradation fragment from Rsp5-ubiquitinated eGFP containing substrate (red) and full-length substrate (blue). The sum of the peptide intensities for each amino acid position after trypsin digestion followed by LC-MS/MS analysis are shown for the C terminus of the protein. Intensities were greatly reduced for the fragment (relative to the full-length protein) beginning at residue 392 (immediately preceding the β11 strand of GFP), and its fluorescence over time (excitation 485 nm, emission 510 nm) was normalized to a guanidine-free control to correct for photobleaching. Average of two experiments is shown. j, mass spectrometry analysis of purified post-degradation fragment from Rsp5-ubiquitinated eGFP containing substrate (red) and full-length substrate (blue). The sum of the peptide intensities for each amino acid position after trypsin digestion followed by LC-MS/MS analysis are shown for the C terminus of the protein. Intensities were greatly reduced for the fragment (relative to the full-length protein) beginning at residue 392 (immediately preceding the β11 strand of GFP), and its fluorescence over time (excitation 485 nm, emission 510 nm) was normalized to a guanidine-free control to correct for photobleaching. Average of two experiments is shown.
GFP because removal of the β11 strand does not cause complete unfolding of GFP or affect its fluorescence (38), and refolding can occur before degradation is complete. For ClpXP, stalling occurs when pulling is slower than refolding of the strand. Thus, our results suggest that Keap1 ubiquitination leads the proteasome to unfold GFP more quickly than Rsp5 ubiquitination.

Ubiquitin Chain Linkage Affects the Unfolding Ability of the Proteasome—To probe the effect of ubiquitination on the unfolding ability in more detail, including on unfolding and degradation of domains after the ubiquitin chains are removed, we turned to a previously described processivity assay (27). This assay allows us to cleanly distinguish effects on the initiation of degradation (which can be rate-limiting for many substrates (28, 31, 50)) and on unfolding, and examine unfolding of a domain well away from the degron. Briefly, a radiolabeled substrate consisting of the Neh2Dual degron, an easy-to-unfold lysineless barnase domain, and a harder-to-unfold DHFR domain (Fig. 3a) is ubiquitinated and presented to the proteasome under single turnover conditions (proteasome in vast excess of substrate such that each proteasome encounters only a single substrate). Full-length substrate disappears with an apparent rate constant of $k_{\text{deg FL}}$, as the Neh2Dual degron and barnase domains are engaged, unfolded, and hydrolyzed. During this process, Rpn11 removes ubiquitin chains from the Neh2Dual domain (18, 19, 51). When the proteasome reaches the DHFR domain (which is typically stabilized by binding to NADPH), the proteasome will partition between either unfolding and degrading DHFR ($k_{\text{deg frag}}$) or releasing DHFR ($k_{\text{rel frag}}$). Any release will be irreversible, as the ubiquitin modification has already been removed. The ratio of degradation to release ($k_{\text{deg frag}} / k_{\text{rel frag}}$) is defined as the unfolding ability (U), a measure of the processivity of the proteasome, which can also be determined from the fraction of folded DHFR fragment released during degradation (27, 52).

Trace radiolabeled Neh2Dual-Barnase-DHFR substrate was ubiquitinated with either Keap1 or Rsp5, purified (Fig. 3b), and degraded by purified 26S proteasome (Fig. 3, c and d). Significantly less folded DHFR fragment was produced during degradation of the Keap1-ubiquitinated substrate compared with the Rsp5-ubiquitinated substrate, confirming that Keap1 ubiquitination helps the proteasome to unfold DHFR, with an unfolding ability of 12 ± 2 for Keap1 versus 4.7 ± 0.5 for Rsp5 (Fig. 3e). When varying substrate concentrations in this assay, we found that the Rsp5-ubiquitinated substrate contained an inhibitor that the Rsp5-ubiquitinated substrate, confirming that Keap1 ubiquitination of the Keap1-ubiquitinated substrate compared with the d degradation of domains after the ubiquitin chains are removed, (27, 52).

To probe the effect of ubiquitination on the unfolding ability in more detail, including on unfolding and degradation of domains after the ubiquitin chains are removed, we turned to a previously described processivity assay (27). This assay allows us to cleanly distinguish effects on the initiation of degradation (which can be rate-limiting for many substrates (28, 31, 50)) and on unfolding, and examine unfolding of a domain well away from the degron. Briefly, a radiolabeled substrate consisting of the Neh2Dual degron, an easy-to-unfold lysineless barnase domain, and a harder-to-unfold DHFR domain (Fig. 3a) is ubiquitinated and presented to the proteasome under single turnover conditions (proteasome in vast excess of substrate such that each proteasome encounters only a single substrate). Full-length substrate disappears with an apparent rate constant of $k_{\text{deg FL}}$, as the Neh2Dual degron and barnase domains are engaged, unfolded, and hydrolyzed. During this process, Rpn11 removes ubiquitin chains from the Neh2Dual domain (18, 19, 51). When the proteasome reaches the DHFR domain (which is typically stabilized by binding to NADPH), the proteasome will partition between either unfolding and degrading DHFR ($k_{\text{deg frag}}$) or releasing DHFR ($k_{\text{rel frag}}$). Any release will be irreversible, as the ubiquitin modification has already been removed. The ratio of degradation to release ($k_{\text{deg frag}} / k_{\text{rel frag}}$) is defined as the unfolding ability (U), a measure of the processivity of the proteasome, which can also be determined from the fraction of folded DHFR fragment released during degradation (27, 52).

Trace radiolabeled Neh2Dual-Barnase-DHFR substrate was ubiquitinated with either Keap1 or Rsp5, purified (Fig. 3b), and degraded by purified 26S proteasome (Fig. 3, c and d). Significantly less folded DHFR fragment was produced during degradation of the Keap1-ubiquitinated substrate compared with the Rsp5-ubiquitinated substrate, confirming that Keap1 ubiquitination helps the proteasome to unfold DHFR, with an unfolding ability of 12 ± 2 for Keap1 versus 4.7 ± 0.5 for Rsp5 (Fig. 3e). When varying substrate concentrations in this assay, we found that the Rsp5-ubiquitinated substrate contained an inhibitor that the Rsp5-ubiquitinated substrate, confirming that Keap1 ubiquitination helps the proteasome to unfold DHFR, with an unfolding ability of 12 ± 2 for Keap1 versus 4.7 ± 0.5 for Rsp5 (Fig. 3e). When varying substrate concentrations in this assay, we found that the Rsp5-ubiquitinated substrate contained an inhibitor derived from the ubiquitination reaction (presumably polyubiquitinated Rsp5 or other polyubiquitinated species) that could be diluted away, greatly accelerating the rate of degradation and permitting the observation of a transient folded DHFR fragment bound to the proteasome before its ultimate degradation or release (Fig. 4a). No or minimal inhibition was seen with Keap1 ubiquitination (Fig. 4b), and dilution of either substrate had no effect on the observed unfolding ability (Fig. 4e). Fits to the model of Fig. 3a enabled us to determine $k_{\text{deg frag}}$ and $k_{\text{rel frag}}$ for Rsp5 and Keap1-ubiquitinated substrates (Fig. 4, a, c, and d, and Table 1), and we found that the increased unfolding ability seen with the Keap1-ubiquitinated substrate was due entirely to an increased rate of degradation. No DHFR fragment was observed in the absence of NADPH to stabilize the DHFR domain (data not shown), suggesting the rate of degradation is limited by the rate of unfolding the domain, at least for stable substrates, and that the increase in $k_{\text{deg frag}}$ corresponds to an increase in the unfolding rate. This increase in the ability to unfold DHFR did not correlate with an increase in the initial degradation rate or the fraction of full-length protein that was successfully engaged (Figs. 3 and 4). In total, these results show that the DHFR domain of a Keap1-ubiquitinated substrate is
Ubiquitination Controls Proteasomal Unfolding Ability

FIGURE 4. The higher unfolding ability due to Keap1 ubiquitination comes from an increased rate of unfolding and degradation. a, an inhibitor is present in Rsp5-ubiquitinated substrate. Radiolabeled ubiquitinated purified Neh2Dual-Barnase-DHFR in PBS + 0.1 mg/ml of bovine serum albumin was used at 1/5th the volume of a degradation reaction (undiluted, blue) or at 1/20th the volume of a degradation reaction (diluted, red) with the extra volume made up by additional PBS + bovine serum albumin. Alternatively, an Rsp5 ubiquitination reaction lacking substrate was purified and added to the diluted substrate (mock ubiquitination, black). The amounts of full-length protein (circles) and DHFR fragment (squares) are shown as a percentage of the ubiquitinated full-length substrate presented to the proteasome at the beginning of the reaction. Curves are fit to single exponentials, except for the diluted substrate, which is fit to the model of Fig. 3a (see “Experimental Procedures”). Error bars are the S.E. from 4 to 6 experiments. b, as in a, but with Keap1 ubiquitination. Error bars are the S.E. from 2 experiments. c, degradation of trace radiolabeled Keap1-ubiquitinated Neh2Dual-Barnase-DHFR by 100 μM yeast proteasome as described in the legend to Fig. 3d but fit to the model of Fig. 3a. The amounts of full-length protein (red circles) and DHFR fragment (blue squares) are shown as a percentage of the ubiquitinated full-length substrate presented to the proteasome at the beginning of the reaction. Solid line is the best fit from the model, dotted line is from forcing the increased unfolding ability observed with Keap1 to derive solely from a faster release rate (k_{rel}^{K48}), dashed line is from forcing the increased unfolding ability observed with Keap1 to derive solely from a faster unfolding and degradation rate (k_{rel}^{rel}), d, expanded view of modeling of fragment from c, indicating that a slower release rate cannot account for the increased unfolding ability. e, comparison of the unfolding abilities from single exponential fits, modeling, and diluted and undiluted substrates. Error bars are the S.E. propagated from curve fitting.

TABLE 1
Rate constants derived from kinetic modeling for Rsp5 and Keap1 ubiquitinated substrates

Degradation assays in Fig. 4, a, c, and d, were fit to the model described in the legend to Fig. 3a for simplicity, and represents any substrate that the proteasome fails to productively engage, either due to deubiquitination of the substrate or substrate to substrate variation that render a small fraction of substrates non-degradable. Ratio is the ratio of the rate constant with Keap1 to the rate constant with Rsp5. Errors are the S.E. from or propagated from curve fitting.

| Parameter | Keap1 | Rsp5 | Ratio |
|-----------|-------|------|-------|
| k_{deg}^{K48} (min⁻¹) | 0.18 ± 0.02 | 0.39 ± 0.03 | 0.46 |
| k_{rel}^{K48} (min⁻¹) | 0.08 ± 0.01 | 0.06 ± 0.01 | 1.3 |
| k_{rel}^{deg} (min⁻¹) | 7.6 ± 0.1 | 1.3 ± 0.1 | 5.9 |
| k_{rel}^{frag} (min⁻¹) | 0.7 ± 0.2 | 0.27 ± 0.04 | 2.6 |
| U^2 | 11 ± 4 | 4.8 ± 0.8 | 2.3 |

*a* U, unfolded abilities.

unfolded more easily by the proteasome than that of an Rsp5-ubiquitinated substrate.

As the only difference between the Keap1- and Rsp5-ubiquitinated substrates is the means by which they are ubiquitinated, it stands to reason that differential ubiquitination leads to differential outcomes of degradation. Previous literature reports indicated that Keap1 leads to K48-linked chains (with other chains also possible) and that Rsp5 leads to exclusively K63-linked chains (12, 43, 44). Indeed, when Neh2Dual-Barnase-DHFR was ubiquitinated with Rsp5 using K63R ubiquitin incapable of forming K63-linked chains, the majority of the substrate had only 5–7 ubiquitins attached, consistent with monoubiquitination on the lysines in the Neh2Dual region (Fig. 5a). Ubiquitination with a lysine-free ubiquitin (K0) gave a similar pattern on a gel, further supporting the ability of Rsp5 to make only K63-linked chains. However, results with Keap1 were not as clear cut. Neither K48R nor K63R ubiquitin prevented polyubiquitin chains from being formed, indicating that at a minimum, the Keap1 ubiquitination system can synthesize other chain types when it cannot synthesize K48-linked chains (Fig. 5b). We therefore turned to UbiCRest analysis, in which a substrate is presented to linkage-specific deubiquitinases (DUBs) to probe its chain composition (53). An Rsp5- or Keap1-ubiquitinated Neh2Dual-Barnase-DHFR substrate was digested by a nonspecific (vOTU), K11-specific (Cezanne), K48-specific (oTUB1* (54)), or K63-specific (AMSH* (54)) DUB. As expected, the K63-specific DUB gave almost complete deubiquitination (down to multiple monoubiquitins) of the...
Ubiquitination Controls Proteasomal Unfolding Ability

**FIGURE 5.** Rsp5 and Keap1 give rise to different chain linkages. a and b, ubiquitination of Neh2Dual-Barnase-DHFR with Rsp5 (a) or Keap1 (b) in the presence of wild-type, K48R, K63R, or K0 ubiquitin. c and d, deubiquitination of Rsp5- (c) or Keap1- (d) ubiquitinated Neh2Dual-Barnase-DHFR (containing lysines only in the Neh2Dual domain) by vOTU (nonspecific), Cezanne (K11-specific), oTUB1* (K48-specific), AMSH* (K63-specific), all three linkage-specific DUBs at once, or no DUB (control). e, ubiquitination of Neh2DualK53-Barnase-DHFR with either Keap1 or Rsp5. f, unfolding abilities (U) for degradation of Neh2DualK53-Barnase-DHFR substrates. Error bars are the S.E. propagated from curve fitting as described in the legend to Fig. 3e (n = 4). g and h, Neh2Dual-Barnase-DHFR substrates were ubiquitinated with Rsp5 in the presence of pre-assembled K63-linked (g) or K48-linked (h) chains and then deubiquitinated by vOTU (nonspecific), oTUB1* (K48-specific), AMSH* (K63-specific), both linkage-specific DUBs at once, or no DUB (control). Contrast is enhanced in panel h to allow ubiquitination signals to be seen. i, unfolding abilities (U) for degradation of Neh2Dual-Barnase-DHFR substrate ubiquitinated with Rsp5 in the presence of pre-assembled K63-linked or K48-linked chains. Error bars are the S.E. propagated from curve fitting as described in the legend Fig. 3e (n = 4).

Rsp5-ubiquitinated substrate, whereas the K11- and K48-specific DUBs had no effect (Fig. 5c). With the Keap1-ubiquitinated substrate, both the K48- and the K63-specific DUBs, but not the K11-specific DUB, gave some deubiquitination, although some higher molecular weight bands remained even in the presence of all 3 DUBs (Fig. 5d). Thus, Keap1 leads to a mixture of primarily K48 and K63 linkages on the substrate, with additional linkages also possible.

Although polyubiquitin chain linkage is the major difference between substrates ubiquitinated with Rsp5 and Keap1, it also seemed possible that the different E2 and E3 enzymes could lead to other differences in ubiquitination such as the exact placement of ubiquitin modifications or the number of polyubiquitin chains on the substrate, and these, rather than ubiquitin chain linkage, could be responsible for the observed differences in unfolding ability. We tested these possibilities in two ways. First, we removed six of the seven lysines in the Neh2Dual degron, leaving only K53 (Neh2DualK53). This substrate was still efficiently polyubiquitinated by Keap1 and Rsp5 (Fig. 5e), and upon presentation to the proteasome, the DHFR domain of the Keap1-ubiquitinated substrate was unfolded and degraded much more efficiently than that of the Rsp5-ubiquitinated substrate (Fig. 5f; U = 16 ± 3 versus 2.7 ± 0.3). Differences in chain number or positioning on the substrate therefore cannot account for the observed difference in unfolding ability. The slightly lower unfolding ability seen with the Neh2DualK53 substrate with Rsp5 compared with the Neh2Dual substrate containing seven lysines in the degron may indicate that increased numbers of chains or number of ubiquitins attached also modestly increases the unfolding ability for K63-linked chains. Second, we ubiquitinated the Neh2Dual-Barnase-DHFR substrate with pre-assembled unanchored K48-linked or K63-linked polyubiquitin chains. Keap1 was unable to utilize unanchored chains, but Rsp5 was able to ubiquitinate the substrate with K63 or, to a lesser extent, K48-linked chains (Fig. 5, g and h, no DUB lane). Presumably individual K48-linked chains were attached to substrate lysines (albeit with reduced efficiency), but were then largely unable to be extended due to the preference of Rsp5 for forming K63-linked chains. UbiCREST analysis was consistent with incorporation of predominantly K48- or K63-linked chains on the substrate (Fig. 5, g and h).

Upon presentation to the proteasome, the DHFR domain of the K48-ubiquitinated substrate was unfolded and degraded more efficiently than that of the K63-ubiquitinated substrate (Fig. 5i, U = 7 ± 1 versus 3.8 ± 0.3), as was expected if chain linkage is the primary determinant of unfolding ability. The even greater unfolding ability seen with Keap1 ubiquitination may indicate the importance of mixed linkages for maximal unfolding ability, but we cannot rule out effects from chain length or the total quantity of ubiquitin on the substrate (although ubiquitination reactions for shorter periods of time or with reduced quantities of E2 and E3 enzymes, which gave modestly lower levels of ubiquitination, did not substantially change measured unfolding abilities).

Rpn11 removes polyubiquitin from substrates early in the degradation process, probably at the same time the substrate is initially engaged by the proteasomal ATPases (2, 18, 19). By the
Ubiquitination Controls Proteasomal Unfolding Ability

FIGURE 6. Ubp6 inhibition does not cause lower unfolding ability of Rsp5-ubiquitinated substrates. a, deubiquitinase activity (hydrolysis of 0.5 μM Ub) of ubiquitin-AMC of 40 nm proteasome (black squares) in the absence or presence of 100 μM IU1 (red circles) or 5 μM UbA (blue triangles). Data points are the average of two experiments. b, unfolding abilities (U) for degradation of Neh2Dual-Barnase-DHFR substrate ubiquitinated with Keap1 or Rsp5 in the absence (from Fig. 3e) or presence of 5 μM UbA. Error bars are the S.E. propagated from curve fitting as in Fig. 3e (n = 4 in the presence of UbA).

time the proteasome encounters the DHFR domain, all ubiquitin should be removed from the substrate (Fig. 3a), and under appropriate conditions we can kinetically follow the formation and then partial degradation of proteasome-bound DHFR fragment (Fig. 4a). How, then, can the mode of ubiquitination affect the ability of the proteasome to unfold the DHFR? It has been suggested that polyubiquitinated substrates or ubiquitin aldehyde (UbA), but not polyubiquitin alone, can activate proteasomal ATPases and peptidase active sites by triggering a global conformational change in the proteasome (20, 21, 55, 56). Two models seem most likely. Either polyubiquitin stays associated with the proteasome throughout the degradation process, even after removal from the substrate, maintaining an activated state, or the initial binding and recognition of polyubiquitin, either by ubiquitin receptors or deubiquitinases associated with the proteasome, triggers a long-lived activated conformational state, probably during the engagement process. Candidate receptors could include Rpn10, Rpn13, Sem1, Rpn1, Rpt5, or Ubp6, all of which are present in our proteasome preparations as determined by mass spectrometry (27).

The proteasomal deubiquitinase Ubp6 breaks down K63 chains more rapidly than K48-linked chains (11). Although this differential deubiquitination was not obvious in our assays, where we saw essentially no deubiquitination of the substrate that was uncoupled to degradation or partial degradation (Fig. 3), it is possible that the more rapid breakdown of K63-linked chains after their en bloc removal from the substrate by Rpn11 affects the ability of the proteasome to unfold the downstream DHFR domain. If continued polyubiquitin association with the proteasome is required for activation, rapid disassembly might allow the proteasome to switch back to a non-activated state before the DHFR domain is reached. If polyubiquitin binding during the engagement process is important to turn on a long-lived activated state, excessive trimming before the substrate is engaged might prevent this activation from occurring. If trimming leads to a lower unfolding ability for K63-ubiquitinated substrates, inhibiting Ubp6 should increase the unfolding ability observed after Rps5-ubiquitination. We found that although IU1 has been reported to specifically inhibit Usp14, the mammalian homolog of Ubp6 (57), no inhibition of proteasomal deubiquitinase activity was observed at 100 μM IU1 (Fig. 6a). In contrast, a near-complete inhibition of deubiquitinase activity was observed in the presence of 5 μM UbA (Fig. 6a). Rather than increasing the observed unfolding ability, UbA led to a small decrease in unfolding ability (Fig. 6b), suggesting that our observed processivity differences of Ubp6 do not depend on the preferential shortening of K63 versus K48-linked chains.

Ubiquitin-independent Substrates Fail to Activate the Unfolding Ability of the Proteasome—The results above indicate either that some ubiquitin chain linkages (K48 and/or mixed linkages) activate the unfolding ability of the proteasome, whereas others (K63) do not, or that polyubiquitination in general activates the unfolding ability of the proteasome, but some chain architectures are capable of more activation than others. Single molecule experiments that reported on the ability of the proteasome to engage its substrates suggested that K48 linkages were superior to K63 linkages (31), whereas in vitro and in vivo binding and degradation studies suggested that branched ubiquitin chains bound better to ubiquitin receptors and led to better degradation (54). Although we found that K63 linkages were, if anything, superior in terms of the initial rate of degradation (Fig. 4), K48-linked chains could be superior for substrate unfolding. We reasoned that if both substrates activate the proteasome, but to differing extents, a non-ubiquitinated substrate would show an even lower unfolding ability. We therefore turned to substrates that could be degraded by the proteasome without being ubiquitinated (58). The N terminus of yeast Rpn4 has been reported to be a transferable ubiquitin-independent degron, capable of targeting fusion proteins such as DHFR to the proteasome in cells by binding to the 19S particle (59). When we replaced the Neh2Dual degron with the Rpn4 degron (Fig. 7a), the outcome of proteasomal degradation was qualitatively different. Instead of a fragment of ~20–25 kDa, a larger fragment was produced in large amounts (~65% of degraded full-length protein is released as a fragment), consistent with the proteasome stalling and falling off the substrate before unfolding the barnase domain (Fig. 7b). Destabilizing the barnase domain by mutagenesis “restored” formation of a DHFR fragment (Fig. 7c), and a different ubiquitin-independent degron derived from the N terminus of yeast ornithine decarboxylase (Fig. 7a, yODC) (60) gave similar results when combined with the destabilized barnase domain (Fig. 7d). The unfolding ability calculated for either ubiquitin independent degron was ~1, much lower than that for either Rsp5- or Keap1-ubiquitinated substrates (Fig. 7f). We added purified polyubiquitin chains in combination with our ubiquitin-independent substrates to see if we could rescue the unfolding ability of the proteasome. Neither polyubiquitin chains nor the deubiquitinase inhibitor UbA (Fig. 7f) were able to significantly increase the observed unfolding ability using the yODC degron, or were able to allow the proteasome to fully unfold barnase without destabilization (data not shown), suggesting activation occurs only when ubiquitin is encountered during the engagement process. However, the proteasome was still capable of degrading more weakly folded proteins using a ubiquitin-independent degron, because omission of NADPH, which binds and stabilizes DHFR, allowed for the complete degradation of the degron-Barn-
Ubiquitination Controls Proteasomal Unfolding Ability

Ubiquitination can be more than just a tag that passively targets a protein for degradation. A polyubiquitin chain can serve as a timer (61), can activate the ATPase and peptidase activities of the proteasome (55, 56, 62, 63), and trigger conformational changes in the proteasome that may prevent additional substrates from binding (20, 21). Computational results have even suggested that polyubiquitin chains can directly destabilize attached proteins (64), although our results suggest GFP is not destabilized by ubiquitination (Fig. 2h). However, it has remained unclear if these effects were relevant to the ability of the proteasome to successfully unfold substrates, especially in the context of multidomain proteins where ubiquitin is removed early in the degradation process. Our results with both GFP and DHFR-based substrates indicate that polyubiquitination activates the unfolding ability of the proteasome even after polyubiquitin chain removal, and that the way the protein is ubiquitinated can also affect proteasomal progressivity, due to differences in chain linkage or architecture.

Differences in ubiquitination could thus be a means to regulate partial substrate degradation or proteasomal processing in the cell (22, 65). For example, the NFκB subunit p50 is created by the partial degradation of the p105 precursor protein, and it has recently been established that ubiquitination by the KPC1 E3 ligase leads to partial degradation, whereas ubiquitination by βTrCP leads to complete degradation (66). Although the chain linkage formed by KPC1 remains unknown, βTrCP forms K48-linked chains with its E2, Cdc34 (67), potentially explaining the more complete degradation observed. The NFκB-related transcription factors in yeast, Spt23 and Mga2, are also partially degraded by the proteasome. Rsp5 ubiquitinates Spt23 and Mga2 with K63-linked chains (68), which presumably lowers the unfolding ability of the proteasome and may therefore contribute to the lack of complete degradation. It will be interesting to look at other proteins targeted by multiple E3 ligases or by both ubiquitin-dependent and independent means to determine whether there are differences in the resulting degradation products.

Our inability to increase the unfolding ability of ubiquitin-independent substrates by adding exogenous polyubiquitin chains suggests that chains do not remain stably associated with the proteasome during degradation, or that if they do, they are unable to trigger activation when either unattached or not directly released from a substrate. Indeed, single molecule studies suggest ubiquitin is released rapidly after substrate engagement, and does not stay associated or re-associate with the proteasome (31). Therefore, we propose that ubiquitin chains are able to promote a long-lived activated conformation of the proteasome during engagement of the substrate, which would require coordinated binding of ubiquitin and an unstructured initiation region on the substrate. Supporting this proposal, proteasomes have been observed to occupy at least three conformations based on cryo-EM studies, and it has been suggested that the s1 state is responsible for substrate binding, whereas the s2 and/or s3 states, in which the ATPase ring changes conformation and the 19S and 20S pores more fully align, are responsible for substrate unfolding and translocation (21, 69).

Discussion

FIGURE 7. The unfolding ability of the proteasome is reduced by ubiquitin-independent targeting. a, schematic depiction of the substrate, which consists of an N-terminal ubiquitin-independent degron (first 80 amino acids of yeast Rpn4 or first 44 amino acids of yeast ODC) followed by a barnase domain and finally a C-terminal DHFR domain. b–e, degradation of trace Rpn4-Barnase-DHFR (b and c) and yODC-Barnase-DHFR (d and e) by 100 nM yeast proteasome. The amounts of full-length protein (open squares) and clipped fragment (blue triangles) or DHFR fragment (red circles) are shown as a percentage of the full-length substrate presented to the proteasome at the beginning of the reaction. In c–e, the barnase domain has been destabilized by mutagenesis (I25A/L89G or L89G), and in e, NADPH has been omitted, allowing complete degradation of DHFR. Curves are fits to single exponentials. Sample gels are shown on the right. Error bars are the S.E. from 24, 4, and 9 independent experiments, respectively. f, unfolding abilities (U) from Fig. 3 and calculated from the curve fits in c and d or from experiments with the addition of ~10 μM polyubiquitin chains (n = 5) or 5 μM UbA (n = 3). Error bars are the S.E. propagated from curve fitting the collected data sets. There is no significant difference (p > 0.1) between any of the Rpn4 or yODC unfolding abilities.
Binding of ubiquitin by Ubp6 during the engagement process has been proposed to stabilize or reinforce a translocation-competent conformation (20, 21), and if this conformation or a similar ubiquitin-triggered conformation persists throughout degradation, it might make back-sliding and release of partially degraded substrate less likely. However, we saw no activation using the Ubp6-inhibitor UbA (Fig. 7f), which also promotes this translocation-competent conformation, suggesting that Ubp6 engagement is not on its own sufficient to activate the proteasome. We speculate that coordinating the timing of ubiquitin recognition with the engagement of a substrate may be critical for determining if the proteasome is fully activated.

Taken as a whole, our results are consistent with the existence of three proteasome conformations with differing unfolding abilities (Fig. 8a). After the substrate is bound and engaged (or simultaneously with engagement) ubiquitin binding to one or more ubiquitin receptors leads to activation (top pathway) or partial activation (middle pathway) of the proteasome via conversion from the s1 to the s2 or s3 conformations (21, 69), depicted as alignment of the central channel running through the purple core particle with the channels running through the pink regulatory particles. The conformation of the proteasome would then persist past chain removal due to the presence of an unfolded polypeptide chain in the channel connecting the regulatory and core particles until the substrate is fully unfolded and degraded or has been released. Full activation would lead to more efficient pulling and a larger observed unfolding activity (as principally seen with Keap1-ubiquitinated substrates), and if this conformation or a similar ubiquitin-triggered conformation persists throughout degradation, it might make back-sliding and release of partially degraded substrate less likely.
Ubiquitination Controls Proteasomal Unfolding Ability

whereas partial activation (as seen with Rsp5-ubiquitinated substrates) leads to an intermediate unfolding ability and more chance of releasing undegraded protein fragments. Alternatively, Rsp5-ubiquitinated substrates could lead to full activation in a subset of proteasomes and fail to activate other proteasomes, or partial and full activation could be in a more rapid equilibrium in which full activation is favored by K48-linked chains. Substrates that lack ubiquitin and therefore do not efficiently activate the proteasome would be unfolded with an even lower efficiency, leading to a lower observed unfolding ability and the increased release of protein fragments, although there would be some probability of activation or partial activation even in the absence of ubiquitin binding, due to intrinsic conformational equilibria of the receptor.

One attractive candidate for a ubiquitin binding partner that triggers activation would be the T1 site of Rpn1, which was just recently determined to be a ubiquitin receptor, and prefers K48-linked chains to K63-linked chains (15). Rpn1 also binds to Ubp6 via its adjacent T2 site (15), providing a potential explanation for the observed effects of Ubp6 on proteasome conformation. Ubp6 itself remains a candidate, although ubiquitin binding to Ubp6 would be necessary but not sufficient for activation to occur.

There is precedent in both simple enzymes and complex molecular machines for long-lived conformational states with varying enzyme activity (70–72). One prediction of our model (Fig. 8a) is that a single proteasome, when degrading a substrate with multiple stable domains, would be likely to either completely degrade each domain (if in an activated conformation) or to release the substrate upon hitting the first stable domain (if in a non-activated conformation), but to only infrequently unfold the first domain and then be stalled by the second domain, as this would require switching from the activated to the non-activated conformation whereas still engaged with the same substrate. On the other hand, in a stochastic model where the proteasome can switch between activated and non-activated forms rapidly, each domain would be treated independently, such that if the first domain were degraded, it would not increase the chance the second domain would be degraded. Using our data for ubiquitin-independent degradation of Rpn4-Barnase-DHFR with either a stable or destabilized barnase domain (Fig. 7, b and c) we can quantitatively test the model. Without destabilizing barnase, ~65% of the full-length protein degraded was released as a barnase-containing fragment (Fig. 7b). Therefore, ~35% of the time, barnase was unfolded and degraded. If barnase was destabilized, ~50% of the full-length protein degraded was released as a smaller DHFR-containing fragment (Fig. 7, c and f). If the ability of an individual proteasome to unfold barnase were unrelated to its ability to then unfold DHFR, we would predict that half of the 35% of the barnase successfully unfolded in Fig. 7b (~17% of the original full-length protein degraded) would stall the proteasome and be released as a DHFR fragment. However, no such fragment was observed (Fig. 8b), providing evidence that a subpopulation of higher unfolding ability proteasomes unfold and degrade both the barnase and DHFR domains. Thus, the proteasome, once activated, appears to stay activated throughout the degradation process. More work is needed to determine how poly-ubiquitinated substrates trigger activation at the molecular level, how the proteasome discriminates between different chain linkages, and how long the activated state of the proteasome persists.

Experimental Procedures

Constructs—GFP constructs (His-DHFR-GFP-Neh2Dual) were made using Gibson or restriction enzyme-mediated cloning in pET44a (Novagen). DHFR was from *Escherichia coli*. mNeonGreen was optimized for *E. coli* expression and synthesized as a gBlock (IDT). The human Neh2 domain (amino acids 1–97) was cloned from pet15b-Nrf2 (gift from Aimee Egger, cloned from cDNA MGC:20033 IMAGE:4548874), and a G10Y mutation was created using oligo-directed mutagenesis to create the Neh2Dualdegron. Constructs for *in vitro* transcription-translation (Degron-Barnase-DHFR-His) were made using Gibson or restriction enzyme-mediated cloning in pIVEX-2.3d (Roche). *Bacillus amyloliquefaciens* barnase contained the inactivating H102A mutation and had all lysines converted to arginine, methionine, or alanine. The Neh2Dual degron was created as described above. A construct containing the Neh2Dual degron with all lysines replaced by arginine except lysine 53 was generated by oligo-directed mutagenesis. A construct containing the Neh2Dual degron with all lysines replaced by arginine except lysine 53 was generated by oligo-directed mutagenesis. The first 80 amino acids of Rpn4 or the first 44 amino acids of ornithine decarboxylase were cloned from yeast genomic DNA to create ubiquitin-independent substrates. The barnase domains of the ubiquitin-independent substrates were destabilized using oligo-directed mutagenesis. The destabilized Rpn4 substrate had both an I25A and L89G mutation, whereas the yODC substrate had just the L89G mutation (73, 74). UbicR est plasmids expressing debiquitinases STAM2-AMSH (AMSH*), UBE2D2-OTUB1 (oTUB1*), Cezanne (Addgene plasmid number 61581), and vOTU (Addgene plasmid number 61589) were gifts from David Komander (53, 54).

pET28-mE1 was a gift from Jorge Eduardo Azevedo (Addgene plasmid number 32534) (75). pET3a-ubiquitin, GST-E2–25K, GST-Ubc13, and GST-Mms2 were gifts from Andreas Matouschek (76). Human UbcH5 and UbcH7 were cloned into pGEX6p-1 (GE Healthcare) from a cDNA library. Ubiquitin-K0 was cloned from pRK5-HA-Ubiquitin-KO (Addgene plasmid number 17603, a gift from Ted Dawson) into pET3a-Ubiquitin to generate pET3a-Ubiquitin-K0.

Proteasome Purification—*Saccharomyces cerevisiae* proteasome was purified from strain YYS40 via a 3× FLAG tag on the Rpn11 subunit essentially as described previously (26) under low salt conditions that maintain the integrity of the proteasome and proteasome-associated proteins such as Ubp6 (27, 77). Cells were grown in YPD for ~24–48 h at 30 °C until an A 600 greater than 1.5. Cells were harvested by centrifugation, resuspended in a buffer containing 25 mM Tris, pH 7.5, 10 mM MgCl₂, 4 mM ATP, 1 mM DTT, 10% (v/v) glycerol and an ATP-regeneration system composed of 0.5 mg ml⁻¹ of creatine phosphokinase and 20 mM phosphocreatine, and then lysed by homogenization. The pH of the lysate was adjusted to 7.5 using 1 M Tris base and clarified by high-speed centrifugation. The
supernatant was supplemented with 5 mM ATP and 1× ATP-regeneration system, filtered, and rotated for 2 h with anti-FLAG M2 affinity gel at 4 °C. The resin was washed with 25 mM Tris, pH 7.5, 5 mM MgCl₂, 2 mM ATP, 1 mM DTT, and 10% (v/v) glycerol, and then was eluted with 100 μg ml⁻¹ of 3× FLAG peptide. Peak elutions were pooled and analyzed using SDS-PAGE. The final concentration of purified proteasome was determined by Bradford assay.

**Protein Expression and Purification—**GFP-containing proteins were expressed in BL21(DE3) cells in either LB or autoinducing media (78). sGFP was expressed at 37 °C, whereas proteins were expressed in BL21(DE3) cells in either LB or autoinducing media as described for GFP-containing substrates. Following elution, PreScission protease was added to remove the His tag and the sample was dialyzed into 50 mM Tris-Cl, 400 mM NaCl, pH 7.4, then passed over a GSH-agarose column to remove PreScission protease.

Mammalian E1 (75) was purified by Ni-NTA chromatography as described for GFP-containing substrates. The enzyme was then buffer exchanged into 10 mM Tris-Cl, 1 mM EDTA, and 1 mM DTT, pH 8.0, and concentrated.

**Substrates—**Radioactive protein substrates were in vitro translated using a Rapid Translation System E. coli High Yield kit (Biotechrabbit), supplemented with [³⁵S]methionine. After centrifugation, substrates were purified via the C-terminal His tag using magnetic Ni-NTA beads (Cube Biotech) and eluted in 50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, 10% (v/v) glycerol, pH 8.0. No further purification was needed for ubiquitin-independent substrates. Ubiquitin-dependent substrates were then in vitro ubiquitinated using either the Cul3/Rbx/Keap1 or Rsp5 system. The Cul3/Rbx/Keap1 ubiquitination reaction contained 130 nM SpUba1 (E1), 310 nM UbcH5 (E2), 500 nM Cul3/Rbx1/Keap1 C151S (E3, where the C151S mutation leads to elevated ubiquitination activity), 2 mg ml⁻¹ of ovalbumin, 5 mM ATP, and 1.45 mg ml⁻¹ of ubiquitin in Keap ubiquitination buffer (45 mM Tris-Cl, 100 mM NaCl, 10 mM MgCl₂, pH 8.0) and was incubated at 25 °C for 1 h. The Rsp5 ubiquitination reactions contained 166 nM SpUba1 (E1), 5.88 μM UbcH7 (E2), 2.82 μM Rsp5 (E3), 4 mM ATP, 1 μM DTT, and 1.33 mg ml⁻¹ of ubiquitin in Rsp5 ubiquitination buffer (25 mM Tris-Cl, 50 mM NaCl, 4 mM MgCl₂, pH 7.5) and was incubated at 25 °C for 1.5 h. For ubiquitination with unanchored polyubiquitin chains, ~50–75 μM of either K48- or K63-linked chains (average size Ub₅) was used in place of ubiquitin, and the reaction was allowed to proceed overnight with K48-linked chains or for 1.5 h with K63-linked chains. Ubiquitinated substrates were purified by size exclusion chromatography in homemade Sephadex G75 (GE Healthcare) spin columns equilibrated in phosphate-buffered saline (PBS) plus 0.1 mM MgCl₂ of bovine serum albumin. Purification and ubiquitination were confirmed by SDS-PAGE analysis. Gels were boiled in 5% trichloroacetic acid, neutralized in 1 M Tris base, dried, exposed to PhosphoImager cassettes, and imaged on a Personal Molecular Imager (Bio-Rad) or a Typhoon FLA 9500 imager with excitation channel 585 nm (H₄) and emission 670 nm (H₄).

Ubiquitination of GFP proteins was as above, except that for Keap1, 65 nm E1, 50 μM UbcH5, 0.5 μM Cul3/Rbx1, 0.25 μM Keap1 C151S, and 7.25 mg ml⁻¹ of ubiquitin were used and for Rsp5, 17.4 μM UbcH7, 8.46 μM Rsp5, and 2.66 mg ml⁻¹ ubiquitin were used to ubiquitinate 10 μM substrate. Reactions were incubated for 1.5 h at 37 °C prior to spin size exclusion chromatography as described above. Purification and ubiquitination were confirmed by SDS-PAGE followed by direct imaging of the GFP protein on a Typhoon FLA 9500 imager with excitation from the 473 nm laser and emission using the BP510/530DF20 filter. Ubiquitination did not affect the fluorescence of GFP proteins.

3 A. Egger, personal communication.
Degradation Assays—Degradation assays were conducted using 100 nM purified yeast proteasome in excess of radiolabeled substrate or using 50 nM proteasome and varying concentrations of GFP-containing substrate at 30 °C in degradation buffer (50 mM Tris-Cl, 5 mM MgCl₂, 5% glycerol (v/v), 1 mM ATP, 10 mM creatine phosphate, 0.1 mg ml⁻¹ of creatine kinase, and 1% DMSO, pH 7.5). Additionally, 500 μM NADPH was added to radiolabeled substrates to stabilize DHFR unless otherwise indicated. Samples at the designated time points were removed and placed into SDS-PAGE loading buffer to quench the reaction. SDS-PAGE gels were analyzed by fluorescence or phosphorimaging as described above. For mass spectrometry analysis, a large-scale reaction (240 μl) containing 50 nM proteasome and 400 nM Rsp5-ubiquitinated eGFP-containing substrate was allowed to proceed for 2 h at 30 °C. The reaction mixture was then purified using 10 μl of Ni-NTA resin, washed with Ni-NTA lysis buffer, and eluted by the addition of 250 mM imidazole.

LC-MS/MS Analyses and Data Processing—Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed by the Proteomics Facility at the Wistar Institute using a Q Exactive Plus mass spectrometer (Thermo Scientific) coupled with a Nano-ACQUITY UPLC system (Waters). Samples were digested in-gel with trypsin and injected onto a UPLC Symmetry trap column (180 μm inner diameter × 2 cm packed with 5 μm C₁₈ resin; Waters). Tryptic peptides were separated by RP-HPLC on a BEH C₁₈ nanocapillary analytical column (75 μm inner diameter × 25 cm, 1.7-μm particle size; Waters) using a gradient formed by solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Peptides were eluted at 200 nl/min for 5–28% B over 42 min, 28–50% B over 25.5 min, 50–80% B over 5 min, constant 80% B for 7.5 min before returning to 5% B over 1 min. A 30-min blank gradient was run between sample injections to minimize carryover. Eluted peptides were analyzed by the mass spectrometer set to repetitively scan m/z from 400 to 2000. The full MS scan was collected at 70,000 resolution followed by data-dependent MS/MS scans at 17,500 resolution on the 20 most abundant ions exceeding a minimum threshold of 20,000. Peptide match was set as preferred, exclude isotopes option and charge-state screening were enabled to reject singly and unassigned charged ions.

MS data were analyzed with MaxQuant 1.5.2.8 software (PMID:19029910). MS/MS data were searched against the E. coli UniProt protein database plus the recombinant HisDHFR-eGFP-Neh2Dual protein sequence using partial trypsin specificity with up to two missed cleavages, static carbamidomethylation of Cys, and variable oxidation of Met, protein N-terminal acetylation, and diglycine addition to Lys. Consensus identification lists were generated with false discovery rates of 1% at protein and peptide levels. Reverse hits and contaminants were removed from all datasets.

Curve Fitting, Modeling and Data Analysis—Gels were quantified using ImageJ (NIH) or ImageQuant (GE Healthcare). Degradation assays were fit to single exponentials in Igor Pro (Wavemetrics), and amplitudes were used to generate the unfolding ability (U) using Equation 1 (27, 52).

\[
U = \frac{k_{\text{rel}}}{k_{\text{frag}}} = \frac{\text{amplitude full-length degradation}}{\text{amplitude fragment formation}} - 1
\]

(Eq. 1)

To directly determine the degradation and release rate constants, kinetic modeling in COPASI (81) to the scheme shown in Fig. 3a, with an additional term to account for a fraction of full-length protein not successfully engaged by the proteasome, was used as described previously (27).

UbICrest—UbICrest chain linkage assays were done as described previously (53). An Neh2Dual-Barnase-DHFR-His substrate (in some cases containing lysines only in the Neh2Dual region for simplicity) ubiquitinated with either Keap1 or Rsp5 was mixed with 3 μM vOTU, 1 μM oTUB1*, 1 μM AMSH*, or 0.1 μM Cezanne in UbICREST buffer (final composition 62.5 mM Tris, 125 mM NaCl, 10 mM DTT, pH 7.5). After incubation at 37 °C for 30 min, the reaction was quenched in SDS-PAGE loading buffer and analyzed by SDS-PAGE followed by phosphorimaging.

Ubiquitin-AMC Deubiquitination Assay—40 nM Proteasome in 50 mM Tris-Cl, 100 mM NaCl, 5 mM EDTA, 0.1 mM DTT, and 0.1 mg ml⁻¹ of ovalbumin, pH 7.5, was incubated with 0.5 μM ubiquitin-AMC and, in inhibition experiments, 100 μM IU1 (BioVision) or 5 μM UbA in a 384-well Greiner low volume fluorescence plate. Fluorescence over time (excitation 355 nm, emission 460 nm) was monitored in a POLARstar Omega plate reader at 30 °C.

Synthesis and Purification of Unanchored Polyubiquitin Chains—Unanchored K48 polyubiquitin chains were synthesized as described previously (76, 79) by incubating 0.1 μM mammalian E1, 25 μM E2–25K, and 20 mg ml⁻¹ of ubiquitin in K48 buffer (50 mM Tris-Cl, 5 mM MgCl₂, 0.1 mM DTT, 0.6 units ml⁻¹ of pyrophosphatase, 10 mM creatine phosphate, 0.002 mg ml⁻¹ of creatine phosphokinase, and 2.5 mM ATP, pH 8.0) at 37 °C overnight. K63 chains were synthesized as described previously (76, 79) by incubating 0.1 μM mammalian E1, 4 μM Ub13 (E2), 4 μM Mms2, and 20 mg ml⁻¹ of ubiquitin in K63 buffer (50 mM Tris-Cl, 5 mM MgCl₂, 0.1 mM DTT, 0.6 units ml⁻¹ of pyrophosphatase, 10 mM creatine phosphate, 0.002 mg ml⁻¹ of creatine phosphokinase, and 2.5 mM ATP, pH 7.6) at 37 °C overnight. Both K48 and K63 synthesis reactions were treated with 5 mM DTT, 1 mM EDTA, and protease inhibitor for 20 min at room temperature and then purified as previously described (76, 79). Briefly, the ubiquitination reaction was applied to a HiTrap-Q column (GE Healthcare) and the flow-through was collected. The flow-through was acidified to pH 4.0 using 2 M acetic acid and further purified on a Mono S column (GE Healthcare). Fractions were analyzed using SDS-PAGE and those containing Ub₄ or higher were pooled and buffer exchanged into 10 mM Tris-Cl, pH 7.5, using Amicon or VivaSpin concentrators. Chain concentration was approximated using a molar absorptivity of 1370 M⁻¹ cm⁻¹ per ubiquitin monomer and the approximate average number of ubiquitins in the final preparation (seven).

Fluorescence Unfolding—His-DHFR-sGFP-Neh2Dual was either ubiquitinated or mock-ubiquitinated (ubiquitination mix lacked ubiquitin) as described above. 40 nM sGFP substrate
was then unfolded by 50-fold dilution into unfolding buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 6 M guanidine Cl, 10% glycerol, 1 mM DTT) or folding buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM DTT) and its fluorescence over time was monitored (excitation 485 nm, emission 510 nm) in a FluoroMax fluorimeter at room temperature.

**Author Contributions**—D. A. K., E. L. R., G. G. C., and W. J. D. wrote the manuscript. D. A. K., E. L. R., G. G. C., W. J. D., and N. D. N. conceived, performed, and analyzed the experiments. K. E. B. and N. E. M. created constructs used in these experiments and performed preliminary experiments. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank Aimee Eggler, Anita Engh, Justin Hines, Jennifer Palencher, and Barry Selinsky for helpful discussions, Aimee Eggler for providing reagents, and Dr. Hsin-Yao Tang at the Wistar Institute Proteomics Facility for his assistance in the analysis of proteomics data.

**References**

1. Voges, D., Zwickl, P., and Baumeister, W. (1999) The 26s proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* **68**, 1015–1068.

2. Finley, D. (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu. Rev. Biochem.* **78**, 477–513.

3. Hoeller, D., and Dikic, I. (2009) Targeting the ubiquitin system in cancer therapy. *Nature* **458**, 438–444.

4. Li, Y.-F., and Wang, X. (2011) The role of ubiquitinated proteasome-mediated processing in viral disease system. *Biochem. Biophys. Acta* **1809**, 141–149.

5. Hegde, A. N., and Upadhye, S. C. (2011) Role of ubiquitin-proteasome-mediated proteolysis in nervous system disease. *Biochem. Biophys. Acta* **1809**, 128–140.

6. Baraibar, M. A., and Friguet, B. (2012) Changes of the proteasomal system during the aging process. *Prog. Mol. Biol. Transl. Sci.* **109**, 249–275.

7. Huang, L., and Chen, C.-H. (2009) Proteasome regulators: activators and inhibitors. *Curr. Med. Chem.* **16**, 931–938.

8. Finley, D., Ulrich, H. D., Sommer, T., and Kaiser, P. (2012) The ubiquitin-proteasome system of Saccharomyces cerevisiae. *Genetics* **192**, 319–360.

9. Kulathu, Y., and Komander, D. (2012) Atypical ubiquitylation: the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nat. Rev. Mol. Cell Biol.* **13**, 508–523.

10. Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* **137**, 133–145.

11. Jacobson, A. D., Zhang, N.-Y., Xu, P., Han, K.-J., Noone, S., Peng, J., and Liu, C.-W. (2009) The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome. *J. Biol. Chem.* **284**, 35485–35494.

12. Saeki, Y., Kudo, T., Sone, T., Kikuchi, Y., Yokosawa, H., Toh-e, A., and Tanaka, K. (2009) Lysine 63-linked polyubiquitin chain may serve as an “aide memoire” for targeting the 26s proteasome. *EMBO J.* **28**, 359–371.

13. Ciechanover, A., and Stahili, A. (2014) The complexity of recognition of ubiquitinated substrates by the 26S proteasome. *Biochem. Biophys. Acta* **1843**, 86–96.

14. Paraskevopoulos, K., Kriegenburg, F., Ratham, M. H., Rössner, H. I., Medina, B., Larsen, I. B., Brandrup, R., Hardwick, K. G., Hay, R. T., Krage-lund, B. B., Hartmann-Petersen, R., and Gordon, C. (2014) Dsl1 is a 26S proteasome ubiquitin receptor. *Mol. Cell* **56**, 453–461.

15. Shi, Y., Chen, X., Elsasser, S., Stocks, B. B., Tian, G., Lee, B.-H., Shi, Y., Zhang, N., de Poot, S. A. H., Tuebing, F., Sun, S., Vannoy, I., Tarasov, S. G., Engen, J. R., Finley, D., and Walters, K. J. (2016) Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome. *Science* **351**, 716a9421.
Ubiquitination Controls Proteasomal Unfolding Ability

37. Beckwith, R., Estrin, E., Worden, E. J., and Martin, A. (2013) Reconstitution of the 26S proteasome reveals functional asymmetry in its AAA⁺ unfoldase. Nat. Struct. Mol. Biol. 20, 1164–1172.

38. Nager, A. R., Baker, T. A., and Sauer, R. T. (2011) Stepwise unfolding of a β barrel protein by the AAA⁺ ClpXP protease. J. Mol. Biol. 413, 4–16.

39. Khmelinskii, A., Meurer, M., Ho, C. T., Patricelli, M. P., Dodder, P., Rosen, H., and Deshaies, R. J. (2011) Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic protein clearance pathways. Proc. Natl. Acad. Sci. U.S.A. 108, 4834–4839.

40. Wojcik, C., Rowicka, M., Kudlicki, A., Nowis, D., McConnell, E., Kujawa, M., and DeMartino, G. N. (2006) Valosin-containing protein (p97) is a regulator of endoplasmic reticulum stress and of the degradation of N-end rule and ubiquitin-fusion degradation pathway substrates in mammalian cells. Mol. Cell Biol. 17, 4606–4618.

41. Chou, T.-F., Brown, S. J., Minond, D., Nordin, B. E., Li, K., Jones, A. C., and Demartino, G. N. (2009) Complete subunit architecture of the proteasome regulatory particle. Nature 482, 186–191.

42. Kim, H. C., and Huibregtse, J. M. (2009) Polyubiquitination by HECT E3s and the determinants of chain type specificity. Mol. Cell. Biol. 29, 3307–3318.

43. Zhang, D. D., Lo, S.-C., Sun, Z., Habib, G. M., Lieberman, M. W., and Hannink, M. (2005) Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3 targets Keap1 for degradation by a proteasome-independent pathway. J. Biol. Chem. 280, 30091–30099.

44. Zhang, D. D., Lo, S.-C., Cross, J. V., Templeton, D. J., and Hannink, M. (2004) Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol. Cell Biol. 24, 10941–10953.

45. Baird, L., Swift, S., Llères, D., and Dinkova-Kostova, A. T. (2014) Monitoring Keap1-Nrf2 interactions in single live cells. Biotechnol. Adv. 32, 1133–1144.

46. Saeki, Y., Isono, E., and Toh-E, A. (2005) Preparation of ubiquitinated proteins activate the proteasomal ATPases by binding to Usp14/Ubp6, which causes 20S gate opening. Mol. Cell 36, 794–804.

47. Pédelaqu, J.-D., Cabantous, S., Tran, T., Terwilliger, T. C., and Waldo, G. S. (2011) Stepwise unfolding of a cent protein. Mol. Cell. Proteom. 10, 399, 7841–7850.

48. Lander, G. C., Estrin, E., Matouschek, A., Bogas, E., and Martin, A. (2012) ATP-dependent proteases differ substantially in their ability to unfold globular proteins.

49. Henderson, A., Erales, J., Hoyt, M. A., and Coffino, P. (2011) Dependence of proteasome processing rate on substrate unfolding. J. Biol. Chem. 286, 17495–17502.

50. Inobe, T., Fishbain, S., Prakash, S., and Matouschek, A. (2011) The folding of an enzyme: II. substructure of barnase and the conformational change profile of a unique 19S RP subunit. J. Mol. Biol. 407, 40–50.

51. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., 3rd, Koonin, E. V., and Deshaies, R. J. (2002) Role of Rpn11 metalloprotease in geometry of the two-component proteasome degron.

52. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., 3rd, Koonin, E. V., and Deshaies, R. J. (2002) Role of Rpn11 metalloprotease in geometry of the two-component proteasome degron. Mol. Biol. Cell 13, 399–408.

53. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., 3rd, Koonin, E. V., and Deshaies, R. J. (2002) Role of Rpn11 metalloprotease in geometry of the two-component proteasome degron.
Baker, R. T., Walz, T., Ploegh, H., and Finley, D. (2002) Multiple associated proteins regulate proteasome structure and function. *Mol. Cell* 10, 495–507

78. Studier, F. W. (2005) Protein production by auto-induction in high-density shaking cultures. *Protein Expr. Purif.* 41, 207–234

79. Raasi, S., and Pickart, C. M. (2005) Ubiquitin chain synthesis. *Methods Mol. Cell Biol.* 301, 47–55

80. Small, E., Eggler, A., and Mesecar, A. D. (2010) Development of an efficient *E. coli* expression and purification system for a catalytically active, human Cullin3-RINGBox1 protein complex and elucidation of its quaternary structure with Keapl. *Biochem. Biophys. Res. Commun.* 400, 471–475

81. Hoops, S., Sahle, S., Gauges, R., Lee, C., Pahle, J., Simus, N., Singhal, M., Xu, L., Mendes, P., and Kummer, U. (2006) COPASI: a COmplex PAthway Simulator. *Bioinformatics* 22, 3067–3074