The deubiquitinating enzyme Usp14 allosterically inhibits multiple proteasomal activities and ubiquitin-independent proteolysis

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The proteasome-associated deubiquitinating enzyme Usp14/Ubp6 inhibits protein degradation by catalyzing substrate deubiquitination and by poorly understood allosteric actions. However, upon binding a ubiquitin chain, Usp14 enhances proteasomal degradation by stimulating ATP and peptide degradation. These studies were undertaken to clarify these seemingly opposite regulatory roles of Usp14 and their importance. To learn how the presence of Usp14 on 26S proteasomes influences its different activities, we compared enzymatic and regulatory properties of 26S proteasomes purified from wild-type mouse embryonic fibroblast cells and those lacking Usp14. The proteasomes lacking Usp14 had higher basal peptidase activity than WT 26S, and this activity was stimulated to a greater extent by adenosine 5′-O-(thiotriphosphate) (ATP S) than with WT particles. These differences were clear even though Usp14 is present on only a minor fraction (30–40%) of the 26S in WT mouse embryonic fibroblast cells. Addition of purified Usp14 to the WT and Usp14-deficient proteasomes reduced both their basal peptidase activity and the stimulation by ATP S. Usp14 inhibits these processes allosterically because a catalytically inactive Usp14 mutant also inhibited them. Proteasomes lacking Usp14 also exhibited greater deubiquitinating activity by Rpn11 and greater basal ATPase activity than WT particles. ATP hydrolysis by WT proteasomes is activated if they bind a ubiquitinated protein, which is loosely folded. Surprisingly, proteasomes lacking Usp14 could be activated by such proteins even without a ubiquitin chain present. Furthermore, proteasomes lacking Usp14 are much more active in degrading non-ubiquitinated proteins (e.g. Sic1) than WT particles. Thus, without a ubiquitinated substrate present, Usp14 suppresses multiple proteasomal activities, especially basal ATP consumption and degradation of non-ubiquitinated proteins. These allosteric effects thus reduce ATP hydrolysis by inactive proteasomes and nonspecific proteolysis and enhance proteasomal specificity for ubiquitinated proteins.

In eukaryotic cells, 26S proteasomes are the major site for protein degradation. Most proteins digested by proteasomes are first tagged with ubiquitin (Ub)2 chains (1). The 26S proteasome consists of the 20S proteolytic particle and one or two 19S regulatory particles (1, 2). The cylindrical 20S proteasome is a hollow four-ring particle that contains in each of its central β-rings three types of active sites, which are chymotrypsin-like, trypsin-like, and caspase-like in specificity (3). Its outer α-rings contain a gated channel for substrate entry (4, 5). The 19S regulatory particle performs several enzymatic and non-enzymatic functions that are required for the degradation of ubiquitinated proteins, including binding and disassembly of the Ub chain, ATP hydrolysis, and unfolding and translocating the substrate protein (1, 2). The 19S base consists of six homologous AAA ATPases, which form a ring, plus three associated subunits, Rpn1, Rpn2, and Rpn13 (6, 7). The ATPases bind, unfold, and translocate the polypeptide through their central channel and then through the gated entry channel into the inner chamber of the 20S particle where proteolysis occurs (1, 8). The gate of the 20S is opened upon nucleotide binding when the ATPases' C-terminal HbYX (where Hb is a hydrophobic residue) residues bind to intersubunit pockets in the outer α-ring of 20S (9). Most steps in the degradation process are linked to ordered cycles of ATP binding and hydrolysis (10). Initially, the Ub chain binds reversibly to one of the 19S receptor subunits, Rpn10/S5a, Rpn13, or Rpn1 (11–13), but commitment to degradation occurs through an ATP-driven step in which a loosely folded region of the polypeptide becomes tightly bound to the ATPase ring (14).

During degradation of a Ub conjugate, the Ub chain is disassembled so that the Ub molecules can be reutilized. Three different deubiquitinating enzymes (DUBs) are associated with the 19S regulatory particle in higher eukaryotes. Two are cysteine proteases, Usp14/Ubp6 (15) and Uch37/UchL5 (16, 17), and the third, Rpn11, is a metalloprotease (18, 19). Rpn11 is an integral 19S subunit, but Usp14 and Uch37 associate reversibly with the proteasome and are usually present at substoichiomet-
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Results

Usp14 deficiency increases 26S content of Uch37 and Rpn13 and overall proteolysis

To understand the regulatory role of Usp14, we compared subunit composition and various enzymatic activities of 26S proteasomes purified from WT mouse embryonic fibroblast (MEF) cells and MEFs lacking Usp14 (Usp14KO). In yeast, Ubp6 is present on a small fraction of the proteasomes (30). In the WT MEF cells, we determined that Usp14 was present on ~40% of the 26S proteasomes based on Western blotting using increasing amounts of purified Usp14 as a standard and a proteasome with molecular mass of 2.5 MDa (Fig. 1A). The proteasomes purified from the WT and Usp14KO MEFs appeared very similar in subunit composition (Fig. 1, B and C) with two clear exceptions. The other cysteine protease DUB, Uch37, and the Ub receptor, Rpn13, to which Uch37 binds were both increased. Interestingly, the total quantity of Uch37 in the two types of cell lysates did not differ, although Rpn13 expression did increase in the Usp14KO cells (Fig. 1D). This increased association of Uch37 and Rpn13 with the proteasome presumably occurred to compensate for the loss of Usp14. In the Δubp6 yeast, degradation of a number of model proteins was increased (25) above control levels. We therefore tested whether overall rates of protein degradation differed in WT and KO MEF cells by pulse-chase methods using [3H]phenylalanine. To follow the breakdown of long-lived proteins, which comprise the bulk of cell proteins (33, 34), cell proteins were radiolabeled for 24 h and regrown in chase medium (DMEM containing non-radioactive phenylalanine and cycloheximide to repress protein synthesis) for 3 h to allow breakdown of short-lived cell components. Then the cells were regrown in the fresh chase medium, and degradation rates were assayed. The total protein degradation rate was about 45% greater in Usp14KO cells (p < 0.05) than in controls (Fig. 1F).

We then determined the contribution of the ubiquitin-proteasome pathway using bortezomib to selectively inhibit the proteasome as described elsewhere (33). Total substrate flux through the proteasome was increased (Fig. 1F) by about 27% in the Usp14KO MEFs (p < 0.01). However, in addition to the enhanced proteasomal proteolysis, there was also greater pro-
tein degradation by lysosomes (calculated by subtracting the proteasomal degradation from the total proteolysis) in the Usp14KO MEFs, which presumably reflects the greater rate of autophagy recently reported in other Usp14-deficient cells (35). However, as shown previously (33), the great majority of the intracellular proteolysis in the MEF cells was by proteasomes.

Figure 1. MEF cells lacking Usp14 have increased content of Rpn13 and Uch37 in the 26S proteasomes. Proteasomes were purified from WT and Usp14KO MEFs using the UBL method (see "Experimental procedures"). Concentrations of each proteasome preparation were determined using the BCA method, and molarities were calculated using 2.5 MDa as the molecular mass of 26S proteasomes. A, Usp14 is a substoichiometric component of WT MEF 26S. The content of Usp14 in 1 pmol of WT MEF 26S proteasomes was determined by Western blotting using recombinant Usp14 as the standard. The content of Usp14 in 1 pmol of WT MEF 26S was calculated as 380 fmol. Upper panel, Western blot. Lower panel, quantitation from upper panel using ImageJ software. B, SDS-PAGE of purified 26S proteasomes from WT and Usp14KO MEFs. Proteins were silver-stained after electrophoresis. C, the contents of Uch37 and Rpn13 were increased in the purified 26S proteasomes from Usp14KO MEFs even though Usp14KO 26S contains the same levels of other subunits as WT 26S. Levels of proteins in 26S proteasomes purified from WT and Usp14KO MEF were determined by Western blotting after SDS-PAGE. D, the expression of Rpn13 was increased in Usp14KO MEFs. Levels of proteasome subunits were determined by Western blotting of cell lysates of WT and Usp14KO MEFs after SDS-PAGE. Unlike the purified proteasomes, only the content of Rpn13, but not that of Uch37, was increased in Usp14KO MEF cells. Therefore, this increase in Uch37 content must be due to increased binding of Rpn13. The content of tubulin was monitored as an input control. E, the cellular content of Ub conjugates did not change significantly in Usp14KO MEFs. The levels of Ub conjugates were determined in lysates of WT and Usp14KO MEFs by Western blotting after SDS-PAGE. F, MEF cells lacking Usp14 degrade long-lived cell proteins faster than WT MEFs. WT and Usp14KO MEF cells were labeled with [3H]phenylalanine (5 μCi/ml) for 24 h. After washing the cells with PBS and then chase medium (DMEM containing 2 mg/ml nonradioactive phenylalanine and 100 μM cycloheximide), cells were grown for a further 2 h to let short-lived labeled proteins be degraded. Then cells were grown in the chase medium with either DMSO (control) or 10 μM bortezomib/Velcade dissolved in DMSO to block proteasome activity. After 1 h of inhibitor treatment, degradation of cellular proteins was measured in the chase medium for up to 4 h. Proteasomal degradation rates were calculated by subtracting the degradation rate with bortezomib treatment from the total degradation rate. Error bars represent S.D. The remaining proteasome-independent proteolysis represents lysosomal proteolysis. *, p < 0.05; **, p < 0.01 compared with WT MEF by Student’s t test. G, MEF cells lacking Usp14 degrade short-lived cell proteins faster than WT MEFs. WT and Usp14KO MEF cells were labeled with [3H]phenylalanine (10 μCi/ml) for 20 min. After washing the cells as in F, cells were incubated for 10 min in the chase medium. Degradation of cellular proteins was then measured in the chase medium for up to 40 min. Error bars represent S.D.* , p < 0.05; **, p < 0.01 compared with WT MEF by Student’s t test. n = 6. IB, immunoblotting.
To also evaluate the degradation rate of short-lived proteins, the MEF cells were initially labeled for 20 min with [3H]phenylalanine. During the subsequent chase period, the most short-lived cell proteins were hydrolyzed about 53% faster in Usp14KO cells (p < 0.05) than in controls (Fig. 1G). Despite the greater rates of proteolysis by the ubiquitin-proteasome and autophagic pathways and despite the loss of a major proteasomal DUB in the Usp14KO MEF cells, the content of ubiquitinated proteins did not differ significantly in the Usp14KO and WT cells (Fig. 1E).

**Binding of Usp14 to the 26S proteasomes inhibits basal peptide hydrolysis**

The chymotrypsin-like activity of the proteasomes from Usp14KO cells was slightly (35%) higher than that of the particles from WT MEFs (Fig. 2A). Because this difference in peptidase activities was seen even though Usp14 is present on less than half the WT particles (Fig. 1A), this difference must underestimate the actual increase in peptide hydrolysis in the Usp14KO cells. We therefore studied the effects of increasing Usp14 content by adding recombinant Usp14 to the purified 26S proteasomes. With WT Usp14 added, the absolute chymotrypsin-like activity of the 26S decreased to a similar extent in WT and KO particles (17–33%) (Fig. 2B). In general, the present inhibition appeared larger in Usp14KO 26S where the basal activity was greater. Addition of the enzymatically inactive Cys→Ala Usp14 mutant also decreased the chymotrypsin-like activity in both WT and Usp14KO proteasomes. Thus, this inhibition is due to allosteric action of Usp14 (Fig. 2B).

Unlike ATP, the non-hydrolyzable ATP analog ATPγS causes a marked stimulation of proteasomal peptide hydrolysis because it induces major conformational changes in the 19S termini (10). Adding increasing amounts of ATPγS stimulates the chymotrypsin-like activities of both the WT and Usp14KO particles (Fig. 2C). However, the maximum stimulation of Usp14KO 26S proteasomes was consistently larger than that of WT particles (Fig. 2C). With ATPγS (100 μM), the chymotrypsin-like activity was stimulated maximally 5–6-fold, the trypsin-like activity was stimulated 11–15-fold, and the caspase-like activity was stimulated 15–20-fold over those activities with ATP (Fig. 2D), and in each case, the maximal activity and the degree of stimulation were greater in the Usp14KO proteasomes than in WT particles (Fig. 1D).

We also tested the effects of increasing Usp14 content on this stimulation of proteasomal peptide hydrolysis by ATPγS. Incubation with the recombinant Usp14 in the presence of ATPγS decreased by 20–26% the chymotrypsin-like activity of both types of proteasomes (Fig. 2E). Thus, although Usp14 increases peptide hydrolysis (21) when it binds a Ub chain, the presence of Usp14 by itself inhibits 20S gate opening and the activation by nucleotides.

**Usp14 inhibits the basal activity of proteasomal ATPases**

A general feature of bacterial ATP-dependent proteases and the archaean PAN ATPase, which are all hexameric complexes homologous to the 26S proteasomal ATPases Rpt1–6, is that their ATPase activity is stimulated upon binding a loosely folded polypeptide (37–39). All these ATPases also bind and translocate polypeptides into an associated proteolytic compartment. Unlike these enzymes, the stimulation of the 26S ATPases requires the simultaneous binding of a Ub chain to Usp14 (or to Uch37) in addition to the unstructured region of
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The polypeptide, although the Ub chain and polypeptide need not be covalently linked (22). To evaluate the role of Usp14 in regulating the ATPases, we compared the activation of ATP hydrolysis by WT and Usp14KO particles (Fig. 3). Surprisingly, the Usp14KO 26S had a 60% higher basal ATPase activity than WT 26S (33.4 versus 20.7 nM ATP/nM 26S/min; p < 0.05) (Fig. 3). When either type of the proteasomes was incubated with linear hexa-Ub chains, there was no significant stimulation of ATPase activity by Usp14KO 26S, but not that by WT 26S, was enhanced by addition of casein alone and reached the same level as seen with 26S upon binding both a Ub chain and casein. 6Ub, linear hexa-Ub chain, *, p < 0.05 compared with control by Student’s t test. Error bars represent S.D.

Figure 3. ATPases of Usp14KO proteasomes, unlike those of WT 26S, are stimulated by unfolded proteins in the absence of a Ub chain. ATP hydrolysis by 26S proteasomes (20 nM) was measured using the malachite green assay (54). The basal ATPase activity of Usp14KO 26S was consistently (60%) higher than that of WT 26S. Upon addition of casein (1 μM) and a hexa-Ub chain (1 μM), which together mimic the binding of a Ub conjugate (22), ATP hydrolysis of WT 26S increased 2–3-fold and resembled the ATPase activity of Usp14KO 26S under the same conditions. However, hexa-Ub alone did not increase ATP hydrolysis of either type of proteasomes. ATP hydrolysis by Usp14KO 26S, but not that by WT 26S, was enhanced by addition of casein alone and reached the same level as seen with 26S upon binding both a Ub chain and casein. 6Ub, linear hexa-Ub chain, *, p < 0.05 compared with control by Student’s t test. Error bars represent S.D.

Usp14 reduces the degradation of non-ubiquitinated unfolded proteins

These findings predict that proteasomes lacking Usp14 should degrade some non-ubiquitinated proteins faster than the WT 26S. In fact, in the absence of Ub, the intrinsically dis-ordered protein PY-Sic1 (41) is degraded very slowly by WT proteasomes; however, as predicted, Usp14KO 26S degraded Sic1 at least 6-fold faster than WT 26S did (Fig. 4, A and C). By contrast, the addition of linear hexa-Ub chains to mimic substrate ubiquitination stimulated the breakdown of Sic1 by WT 26S about 10-fold and by Usp14KO 26S at most 2-fold (Fig. 4, B and C). Consequently, the maximal rates of degradation achieved by the two types of particles with the Ub chains pres-
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Rapid degradation of Ub conjugates by the proteasome requires the removal of the Ub chain from the substrate (1). Of the three DUBs associated with mammalian 26S proteasomes, only Rpn11 seems essential for the degradation of ubiquitinated proteins (18). Because Usp14 upon binding a Ub chain stimulates proteasome peptide and ATP hydrolysis, we tested whether it may also enhance the proteasome’s other DUB activities by comparing the rates of breakdown of free tetra-Ub chains (chains not conjugated to a protein) by WT and Usp14KO 26S (Fig. 5A). Surprisingly, even though Usp14KO proteasomes lack the important DUB Usp14, they showed a much greater capacity to disassemble Lys-63 Ub chains (Fig. 5B) than WT particles as shown by their more rapid generation of tri-, di-, and mono-Ubs. The loss of Usp14 also mildly increased the rates of disassembly of Lys-48 tetra-Ub (Fig. 5C). Because the Zn²⁺ chelator o-phenanthroline markedly suppressed this accelerated breakdown of tetra-Ub to the level seen with WT 26S, the faster chain hydrolysis in the Usp14KO must be due to activation of the Zn²⁺-containing metalloprotease Rpn11. In fact, Rpn11 is the most active proteasomal DUB against Lys-63 chains (42). Furthermore, although Uch37 and Rpn13 levels were increased in the Usp14KO proteasomes (Fig. 1E), Ub-VS, which inhibits Uch37, did not decrease the rapid breakdown of tetra-Ub chains by Usp14KO 26S (Fig. 5B). Thus, the activity of Rpn11 (but not Uch37) is stimulated by the absence of Usp14 in the 26S. These findings are also consistent with the recent structure of the 26S by cryo-EM, which shows that Ubp6 can normally block substrate access to Rpn11 (31).

Discussion

By helping release Ub from the substrate, especially from proteins bearing multiple Ub chains (23), Usp14/Ubp6 promotes Ub recycling and prevents the degradation of Ub by the proteasome (43). However, in addition to this catalytic role, as emphasized by the present results, Usp14 is a major allosteric regulator of proteasome function that has the unusual capacity to both activate and inhibit multiple steps in Ub conjugate degradation. The first evidence of this regulatory role was the finding in yeast that Ubp6 inhibits the degradation of several proteins (25), leading to the proposal that Usp14/Ubp6 DUB activity serves as a timing device for proteasomal degradation (26). Thus, by removing Ub moieties from the substrate, Usp14 functions in a kinetic competition between substrate release after deubiquitination and substrate destruction (26). Accordingly, low-molecular-weight selective inhibitors of Usp14’s catalytic activity, e.g. IU1, were shown to enhance the degradation of certain proteins (24), presumably by slowing their deubiquitination and providing more time for proteasomal proteolysis. However, unlike a Usp14 substrate or substrate analog (e.g. Ub-al), IU1 does not cause allosteric activation of ATP hydrolysis (22), peptide entry, or degradation of non-ubiquitinated PY-Sic1 (Fig. 4D). Nevertheless, by slowing substrate deubiquitination by Usp14, IU1 may prolong substrate association with Usp14 and thus prolong proteasomal activation.

Because of Usp14’s inhibitory actions, it was surprising to find that binding of a Ub chain to Usp14/Ubp6 allosterically stimulates peptide entry into the 20S (21) as well as 26S ATPase activity provided the substrate contains an unfolded domain (22). The present observations bridge these seemingly contradictory roles and show that Usp14 by itself inhibits at least three key functions in substrate degradation, but upon binding a Ub chain, Usp14 releases this inhibition and activates these processes further.

Interestingly, in Usp14-deficient cells, the proteasomes contain significantly greater amounts of Rpn13 and Uch37 (Fig. 1B) even though the cells contained only slightly increased cellular levels of Rpn13 (Fig. 1C). Although this Ub receptor is often classified as a stoichiometric subunit, several investigators have reported that the Ub receptor Rpn13 is present in proteasomes in substoichiometric levels (44–46). Presumably, the increased Uch37 and Rpn13 levels on the proteasome help compensate for the loss of Usp14’s catalytically activity, but how this loss of Usp14 is recognized and leads to increased Uch37 and Rpn13 binding are interesting questions for future study.

In the absence of a substrate and thus independently of its role as a DUB, Usp14 was found to inhibit multiple proteasomal processes. For example, the 26S lacking Usp14 has a 20–30% greater peptidase activity than WT 26S (Fig. 2A). Because at most 30–40% of the WT proteasomes contain Usp14 (Fig. 1E), this greater activity must underestimate the actual effect of Usp14 on peptide entry into the 20S particle and ATP hydrolysis. In addition, all three peptidase activities of the Usp14KO 26S were consistently stimulated by ATPγS more than the WT (Fig. 2D). Accordingly, addition of recombinant WT or Cys →
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Ala Usp14 further represses allosterically both the basal and ATPγS-stimulated peptidase activities (Fig. 2, B and E). Nucleotides, like ATPγS, open the gated entry of the 20S particle by stimulating interaction of ATPases’ C-terminal HbYX motifs with intersubunit pockets in the 20S α-ring (47), but recent cryo-EM studies showed that ATPγS also induces structural reorganization of the 19S complex that aligns the central pore in the ATPase ring with the 20S entry channel (36). Because Usp14 suppresses all three peptidase activities in the presence of ATPγS (Fig. 2D), Usp14 must inhibit substrate entry rather than altering the catalytic activity of the three sites. Although ATPγS was previously assumed to cause maximal increases in substrate entry, Usp14KO can be stimulated further than WT 26S in the presence of ATPγS. Thus, Usp14 must influence substrate entry by additional mechanisms distinct from those activated by ATPγS.

One important surprising finding was that after loss of Usp14 the 26S exhibited a greater ability to disassemble tetra-Ub chains due to increased activity of Rpn11 (Fig. 5). Thus, under basal conditions, Usp14 allosterically inhibits Rpn11 and reduces its capacity to digest Ub conjugates. Recently, the catalytic domain of Usp14 (the “USP domain”) upon association with Ub-VS was shown by cryo-EM to block sterically substrate access to Rpn11 (31). Therefore, the loss of Usp14 probably exposes the active site of Rpn11 and enables it to digest Ub chains more readily. However, it seems likely that in addition to this steric effect Usp14 may allosterically inhibit Rpn11’s catalytic activity just as it inhibits the particle’s other proteasomal activities.

In the Usp14KO proteasomes, ATPase activities are also regulated differently than in WT (Fig. 3). Unlike the WT, which requires the binding of both a Ub chain and an unfolded protein to stimulate ATP hydrolysis (22), in the Usp14-deficient 26S, such a protein can stimulate this process even without a Ub chain present (Fig. 3). In this respect, the ATPases in the Usp14KO proteasomes resemble the homologous, ATP-dependent proteases from bacteria (Lon, ClpAP, ClpXP, and HslUV) and the direct 26S ancestor, PAN, which regulates the activity of archaeal 20S proteasomes. These enzymes are all protein substrate-activated AAA ATPases (38, 39). During the evolution of eukaryotes, ubiquitin conjugation became linked to proteolysis, and through Usp14, the 26S proteasome evolved the requirement for Ub chain binding to activate peptide entry and ATP hydrolysis. This enhancement of ATPase activity is a critical event because it drives substrate translocation, and the amount of ATP consumed is directly proportional to the amount of Ub conjugates degraded (48). Although the structural basis for these different allosteric effects are not yet clear, it is noteworthy that Ubp6 binds to Rpn1, but when Ub aldehyde occupies the active site on Usp14, these proteins also interact with the ATPases Rpt1 and -2 (30–32).

The inhibition of multiple proteasomal activities by Usp14 can account for the finding that MEF cells lacking Usp14 degrade cellular proteins, both short-lived and long-lived components (Fig. 1, F and G), faster than WT MEFs. This observation of enhanced degradation of cell proteins generally by proteasomes extends the findings on breakdown of several model proteins in Δubp6 yeast (25). Surprisingly, protein degradation in lysosomes was also enhanced in these Usp14KO cells (Fig. 1F), and the lack of this DUB has been reported recently to stimulate autophagy in other cell types (35). These observations are also intriguing because they are further evidence that under physiological conditions the ubiquitin-proteasome pathway and autophagy are coordinately regulated even though the proteasomes remain the primary site of protein hydrolysis (33, 49).

It is also noteworthy that, unlike the WT, proteasomes lacking Usp14 can efficiently degrade certain unstructured proteins without ubiquitination (Fig. 4A). This degradation of non-ubiquitinated proteins must result from enhanced translocation of such unstructured proteins into the 20S particle and presumably from the ability of such non-ubiquitinated proteins to activate ATP hydrolysis. The inhibition of Ub-independent proteolysis by Usp14 must also be an allosteric effect because there is no Ub chain on these substrates to hydrolyze and because the inactive Cys → Ala mutant Usp14 also inhibited degradation (Fig. 4E).

Although these several inhibitory actions of Usp14 probably contribute to its ability to suppress overall protein degradation in vivo (25), its inhibition of ATPase activation by non-ubiquitinated proteins appears to be critical in preventing Ub-independent proteolysis in vivo. These findings together also suggest a clear rationale for these bidirectional effects of Usp14 on proteasome function (Fig. 6). In the absence of a substrate, Usp14 helps prevent wasteful ATP consumption and non-selective proteolysis by the proteasome, especially degradation of non-ubiquitinated proteins. The activation of the proteasome upon binding a Ub conjugate thus enhances the selectivity for ubiquitinated substrates, especially those with unfolded domains.

As noted above, the greater activity of the 26S from cells lacking Usp14 is striking because Usp14 is present on only a
fraction of the WT particles. It seems likely, therefore, that additional factors can suppress the basal activities of the particles lacking Usp14. One possible inhibitory factor might be Uch37 because its content on the 26S is increased in the Usp14KO MEFs and because the interaction of Ub chains with Uch37 can activate gate opening and ATP hydrolysis (22). Recent cryo-EM studies suggest that only a small fraction of 26S proteasomes in cells is active in proteolysis, and it is probably the fraction containing Usp14 (50). Because our recent studies show that the binding of Ub conjugates to the proteasome stimulates the association of cytosolic Usp14 with the particles,3 the subsequent interaction between the Ub chain and Usp14 can lead to more efficient and selective breakdown of the ubiquitinated substrates.

**Experimental procedures**

**Purification of proteins**

GST-UBL derived from hHR23B was expressed in *E. coli* and purified with GSH-Sepharose as described previously (51). GST-fused Usp14 was also expressed in *E. coli* and purified using GSH-Sepharose (24). Resin-bound Usp14 was treated with thrombin to cut off GST from Usp14. Residual thrombin was cleared from Usp14 using benzamidine-Sepharose. His-UIM derived from UIM2 of S5a (51) and His-tagged PY-Sic1 (52) were expressed in *E. coli* and purified with a Ni-NTA resin.

**Purification of 26S proteasomes**

WT and Usp14KO MEFs (24) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. Affinity purification of 26S proteasomes was carried out as described previously (53). Briefly, cell lysates prepared with sonication (15 s six times at 18 watts) were spun for 1 h at 100,000 × g. The soluble supernatants were incubated at 4 °C with GST-UBL derived from hHR23B and a corresponding amount of GSH-Sepharose. The slurry containing 26S proteasomes bound to GST-UBL was poured into an empty column, washed, and incubated with His-UIM. The eluate was collected and incubated with Ni-NTA-agarose for 20 min at 4 °C. The Ni-NTA-bound His-UIM was spun out, and the remaining supernatant contained purified 26S proteasomes. Protein concentration was determined using a BCA reagent. The molarity of 26S proteasome particles was calculated based on an average molecular mass of 2.5 MDa.

**Antibodies**

Polyclonal rabbit anti-Ub (A-100) was purchased from Boston Biochem. Anti-Rpn1 (sc-68332), anti-Rpn2 (sc-166038), and anti-Sic1 (sc-50441) were from Santa Cruz Biotechnology. Anti-Rpn6 (14303), anti-Rpn11 (4197), and anti-Rpt5 (13923) were from Cell Signaling Technology. Anti-β5 (A303-847A), anti-Usp14 (A300-920A), and anti-Rpn13 (A302-554A) were purchased from Bethyl Laboratories. Anti-Uch37 (3904-1) was from Epitomics. HRP-conjugated secondary antibodies were purchased from Promega.

3 C.-L. Kuo and A. L. Goldberg. (2017) Ubiquitinated proteins promote the association of proteasomes with the deubiquitinating enzyme Usp14 and the ubiquitin ligase Ube3c. *Proc. Natl. Acad. Sci. U.S.A.* 114, E3404–E3413.

**Immunoblot analysis**

Samples for immunoblotting were run on 4–12% Bis-Tris gels (Life Technologies) with MES buffer (NP0002). Proteins were analyzed following SDS-PAGE and transferred onto 0.45-μm PVDF membranes (Whatman). Immunoblots were blocked and incubated with appropriate primary and secondary antibodies. Membranes were developed with enhanced chemiluminescence reagent (Immobilon Western HRP substrate and Luminol reagent WBKLS0500, Millipore) onto X-ray film. The ImageJ program (W. S. Rasband, National Institutes of Health) was utilized to quantify the signal from the film.

**Proteasome activity assays**

As indicated, proteasomes were generally incubated with or without casein, linear hexa-Ub chains, Usp14, or a DUB inhibitor at room temperature for 15 min before the start of reactions. Peptide hydrolysis by MEF 26S proteasomes was measured with 10 μM Boc-Leu-Arg-Arg-amc (Boc-LRR-amc), Suc-Leu-Leu-Val-Tyr-amc (Suc-LLVY-amc), or Ac-Nle-Pro-Nle-Asp-amc (Ac-nLPnLD-amc) (Bachem) (λex 380 nm; λem 460 nm) and the indicated concentration of 26S proteasomes at 37 °C. Proteasomal activities were calculated from 30 to 60 min after the start of the reaction. The reaction mixture was composed of 50 mM Tris (pH 7.6), 0.1% Triton X-100, 0.1 mM ATP or ATPγS, 0.5 mM MgCl2, 1 mM DTT, and 25 ng/μl BSA (Sigma). Degradation of PY-Sic1 (100 nM) by 26S proteasomes (2 nM) was carried out in the presence of 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM ATP, 1 mM DTT, and 0.01 mg/ml BSA (Sigma) for the indicated time (0–4 h) at 37 °C and measured by Western blotting with an anti-Sic1 antibody. ATP hydrolysis by proteasomes was measured using the malachite green assay (54). Deubiquitination by 26S particles (5 nM) was assayed with tetra-Ub chains (368 nM) in HEPES buffer (25 mM) containing 100 mM KCl, 5 mM MgCl2, 1 mM ATP, 1 mM DTT, and 0.01 mg/ml BSA (Sigma) for the indicated time (0–4 h) at 37 °C and measured by Western blotting with anti-Ub antibodies.

**In vivo proteolysis**

The overall rates of degradation of long-lived proteins in WT and Usp14KO MEF cells were determined as described previously (33, 49). MEF cells were grown with [3H]phenylalanine (5 μCi/ml) for 24 h to label cell proteins and then washed twice with PBS and once with chase medium (i.e. fresh DMEM containing 2 mM nonradioactive phenylalanine and 100 μM cycloheximide). Cells were grown in chase medium for 2 h to let short-lived proteins be degraded. Then fresh chase medium containing DMSO or 10 μM bortezomib/Velcade was added. One hour after addition of the inhibitors, samples of the medium were collected for up to 4 h. Then each sample was mixed with 10% trichloroacetic acid (final concentration) to precipitate proteins. The acid-soluble radioactivity reflects the amount of prelabeled, long-lived cell proteins degraded at each time and was expressed relative to the total amount of radioactivity initially incorporated into protein. The absolute rate of proteasomal proteolysis was determined by subtracting the rates of proteolysis in cells treated with bortezomib from that in untreated cells. Lysosomal proteolysis was determined by sub-
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tracting the rates of proteasomal degradation from that of overall proteolysis.

The overall rates of degradation of short-lived proteins were determined by a pulse-chase protocol by incubating MEF cells with [3H]phenylalanine (10 μCi/ml) for 20 min to label cell proteins. The cells were then washed twice with PBS and once with the chase medium containing large amounts of nonradioactive phenylalanine to block incorporation of labeled amino acids. After incubation in chase medium for 20 min more, samples were collected every 10 min for up to 40 min. The degradation rate was calculated using the same method as used for evaluating the degradation rate of long-lived proteins.

Author contributions—H. T. K. and A. L. G. designed the studies and wrote the manuscript. H. T. K. performed the experiments.

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