The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26S proteasome

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Running title: Differential proteasomal processing of K48 and K63 ubiquitin chains

The role of K63 ubiquitin chains in targeting proteins for proteasomal degradation is still obscure. We systematically compared proteasomal processing of K63 ubiquitin chains with that of the canonical proteolytic signal, K48 ubiquitin chains. Quantitative mass spectroscopic analysis of ubiquitin chains in HeLa cells determines that the levels of K63 ubiquitin chains are insensitive to short-time proteasome inhibition. Also, the K48/K63 ratio in the 26S proteasome-bound fraction is 1.7-fold more than that in the cell lysates, likely because some cellular K63 ubiquitin conjugates are sequestered by K63 chain-specific binding proteins. In vitro, K48 and K63 ubiquitin chains bind the 26S proteasome comparably, while K63 chains are deubiquitinated 6-fold faster than K48 chains. Also, K63 tetraubiquitin-conjugated UbcH10 is rapidly deubiquitinated into the monoubiquitinated form, whereas K48 tetraubiquitin targets UbcH10 for degradation. Furthermore, we found that both the ubiquitin aldehyde- and 1,10-phenanthroline-sensitive deubiquitinating activities of the 26S proteasome contribute to K48- and K63-linkage deubiquitination, albeit the inhibitory extents are different. Together, our findings suggest that, compared with K48 chains, cellular K63 chains have less proteasomal accessibility; and proteasome-bound K63 chains are more rapidly deubiquitinated, which could cause inefficient degradation of K63 conjugates.

Protein ubiquitination is a posttranslational modification catalyzed by a cascade of enzymatic reactions involving a ubiquitin (Ub)1 activating enzyme (E1), a Ub conjugating enzyme (E2) and a Ub ligase (E3) (1). Ub is conjugated onto protein substrates by formation of an isopeptide bond between the carboxyl group of the C-terminal glycine residue of Ub and the ε-amino group of a lysine residue in the substrate. Further, a polyubiquitin (polyUb) chain is formed by conjugating the carboxyl group of the C-terminal glycine residue of Ub and the ε-amino group of a lysine residue in the substrate. In addition, linear polyUbs are linked by amide bonds formed between the C-terminal glycine residue of Ub and the N-terminal methionine residue of a following Ub (2). Thus, at least eight different polyUb linkages exist in cells.

Protein ubiquitination plays diverse roles in regulating cellular activities. Monoubiquitination does not support degradation, but it is involved in regulating membrane trafficking, gene transcription, DNA repair and DNA replication (3). As for polyubiquitination, it seems that different linkages have distinct functions: polyUbs linked through K48 are the primary targeting signals for proteasomal degradation (1), whereas polyUbs linked through K63 recruit other binding partners and execute many functions including kinase activation (4), protein synthesis (5), DNA repair (6;7) and chromosome segregation (8). The functions of other polyUbs have also been suggested such as the involvement of the K6-linkage in regulating DNA repair (9). Interestingly, the Ub linkage on a modified substrate can be
switched in response to different functions. For instance, in TNFα-stimulated activation of the NFκB gene (10), a K63 chain on receptor interacting protein (RIP) functions as a signaling element to recruit the IKK kinase complex, which phosphorylates IκBα (an inhibitor protein of NFκB) and triggers its Ub-dependent degradation. During the stimulation, A20 deubiquitinates K63-linked RIP and then assembles K48 polyUbs on RIP, promoting RIP degradation (10). In addition to K48 polyUbs, a recent proteomic study found that Ub chains linked by K6, K11, K27, K29 or K33 could also serve as proteolytic signals (11). K11 polyUbs were found to mediate degradation of proteins involved in endoplasmic reticulum-associated degradation, cell cycle progression and other functions (11;12), whereas K29 polyUbs may promote Ub fusion degradation (13).

The role of K63 polyUbs in targeting proteins for proteasomal degradation is still unclear. Some studies suggested that K63 polyUbs are competent proteolytic signals. For example, in vitro studies have shown that K63 polyUbs are able to target degradation of several proteins including Sic1, cyclin B1, dihydrofolate reductase and troponin I (14-17). In S. Cerevisiae, partial degradation of the transcription factor Mga2, which releases the N-terminal p90 activator domain, can be processed by overexpression of the UbK48R mutant that promotes the formation of K63 ubiquitinated Mga2 (14). Also, inhibition of the proteasome by MG132 in S. Cerevisiae or mammalian cells causes an increase of both the K48 and K63 Ub conjugates as detected by mass spectroscopic analysis (14). However, to our knowledge, physiological substrates that exclusively depend on the K63-linkage for degradation have not been identified. In contrast to the findings that suggest a role of K63 polyUbs in targeting proteolysis, Xu and colleagues proposed that the K63 polyUbs are not proteolytic signals in S. Cerevisiae based on quantitative proteomic studies. They also suggest that all other Ub linkages can support degradation and have partially redundant functions in proteolysis (11).

In this study we systematically compared proteasomal processing of K63 polyUbs with that of the primary proteolytic signal of K48 polyUbs in the aspects of binding/recognition, deubiquitination and targeting for degradation. Our results suggest that cellular K63 Ub chains have less proteasomal accessibility than K48 chains, likely because some cellular K63 ubiquitin conjugates are sequestered by K63 chain-specific binding proteins, such as NEMO. In vitro K63 and K48 Ub chains bind the 26S proteasome comparably, but K63 chains are deubiquitinated six-time more rapidly than K48 chains. Both the ubiquitin aldehyde (Ubal)- and 1,10-phenanthroline-sensitive deubiquitinating activities of the 26S proteasome contribute to K48- and K63-linkage deubiquitination, albeit their inhibitory extents are different. Moreover, we found that rapid deubiquitination of K63 chains could cause inefficient degradation of their conjugates.

**Experimental Procedures**

**Reagents, plasmids, recombinant protein purification and ubiquitination of UbcH10**

See Supplemental Procedures.

**Proteasomal degradation and deubiquitination assays**

The bovine 26S proteasome and PA700 were purified according to methods established in Dr. DeMartino’s group (18;19). Proteasomal degradation and deubiquitination were performed at 30 °C in degradation buffer (20 mM Tris, pH 7.2, 20 mM NaCl, 5 mM MgCl₂, 2 mM ATP, 1 mM βME and 5% glycerol). Reaction mixtures usually contained 13.5 nM 26S proteasome and 100 nM polyubiquitinated UbcH10 or other substrates as specified in figure legends. Samples were withdrawn at each designated time point and added into 5X SDS sample buffer to stop the reaction immediately. Usually, samples of time 0 represented a reaction of about 15 seconds except in figure 5C, D and figure 6C where samples at time 0 were prepared by adding the substrates directly into 1X SDS sample buffer with the 26S proteasome. For reactions

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containing epoxomicin (100 µM), Ubal (2.5 µM) or 1, 10-phenanthroline (5 mM), the 26S proteasome was preincubated with the corresponding inhibitors for 10 min prior to the supplementation of substrates.

**Size-exclusion spin column assay**

12.5 nM 26S proteasome was preincubated with 2.5 µM Ubal and 5 mM 1, 10-phenanthroline for 10 min at 30 °C in degradation buffer. 80 nM K48 Ub₄ or K63 Ub₄ was then mixed with the preinhibited proteasome and incubated 2 min at room temperature. 60 µl mixtures or Ub₄ alone were loaded into Micro Bio-Spin P-30 chromatography columns (Biorad) and centrifuged according to the manufacturer’s instruction. The flowthrough was eluted directly into a 1.5 ml micro tube with 20 µl 5X SDS sample buffer. 30 µl samples were resolved by SDS-PAGE for immunoblotting assays. To determine the binding between the 26S proteasome (13.5 nM) and Ub₄ (K48 or K63)-Ubch10 (100 nM), we used homemade Sephadex G-100 spin columns (exclusion limit is 80 kDa). After centrifugation, all resulting mixtures (75 µl) were concentrated to about 30 µl by heatin.

**Quantitative mass spectroscopic analysis**

The whole cell lysates and the purified 26S proteasome described above were resolved in SDS-PAGE. The gel regions containing the vast majority of ubiquitinated proteins (>70 kDa), judged by immunoblotting, were excised followed by in-gel trypsin digestion. Because trypsin cleaves ubiquitin to a small GG tag on modified lysine residues, the abundance of polyUb linkages is represented by the level of the GG peptides. Stable isotope labeled GG peptides were chemically synthesized, quantified and added into the samples as internal standards (21;22). The samples were fractionated by reverse-phase liquid chromatography coupled with a hybrid mass spectrometer (LTQ-Orbitrap; Thermo Fisher Scientific). The instrument was operated in the mode of selective reaction monitoring (SRM) to increase sensitivity. The native GG peptides and the labeled internal standards were eluted together and separated by mass spectrometry due to mass difference, enabling relative quantification.

**Purification of the 26S proteasome-bound ubiquitinated proteins**

We obtained the pQCXIP viral expression plasmid that expresses S13/Rpn11-HTBH or the HTBH tag alone from Dr. L. Huang at the University of California at Irvine. The HTBH tag includes a 6X His-tag, TEV protease site, in vivo biotinylation sequence and another 6X His-tag. Stable HeLa cell lines that express S13-HTBH or the HTBH tag were established according to a published method by using HEK293 10A1 packaging cells (20). To purify the 26S proteasome, HeLa cells were grown in DMEM containing 10% FBS and 1% penicillin/streptomycin. At 90% confluency, three 10 cm plates of cells were treated with 30 µM MG132 or 0.3% DMSO for 45 min, then washed twice with PBS buffer before harvest. Cells were lysed with lysis buffer (20 mM Tris, pH 7.2, 50 mM NaCl, 10% glycerol, 2 mM ATP, 5 mM MgCl₂, 2 mM βME, 10 mM iodoacetamide, 2 mM 1,10-phenanthroline and the protease inhibitor cocktail from Roche). The lysates were cleared by centrifugation and the supernatants were incubated with 50 µl of streptavidin agarose for 2 hrs at 4 °C. The resins were then washed three times with the lysis buffer, followed by two additional washes with the lysis buffer without iodoacetamide. Finally, the streptavidin-bound 26S proteasome was released by incubation with 50 ng/µl TEV overnight at 4 °C.

**Supplemental Data**

Supplemental Data include nine figures and figure legends.
Results

Neither the cellular level nor the 26S proteasome-bound level of K63 polyUbs increased in response to short-time proteasome inhibition

If a specific Ub linkage targets proteins for proteasomal degradation, its cellular level and the 26S proteasome-bound fraction should elevate in response to short-time proteasome inhibition. To test this idea, we first generated stable HeLa cell lines that express S13/Rpn11-HTBH or the HTBH tag alone according to a previous report (20) (data not shown). This allowed us to rapidly purify the 26S proteasome using affinity purification. We next examined whether treating these stable HeLa cell lines with 30 µM MG132 for 45 minutes would increase cellular Ub conjugates. We found that the high molecular weight Ub conjugates in the whole cell lysates significantly increased as determined with immunoblotting of Ub (figure 1A). Quantitative mass spectroscopy (21) that uses heavy isotope-labeled Ub linkage peptides as internal standards identified K11-, K48- and K63-linked polyUbs in both the DMSO- (control) and MG132-treated whole cell lysates. Interestingly, the relative amounts of the K11- and the K48-linkages increased 1.8- and 2.3-fold, respectively, upon MG132 treatment (figure 1B). In contrast, the amount of the K63-linkages did not change (figure 1B). The other polyUb linkages were undetectable in the whole cell lysates. Thus, the cellular level of the K63-linkages does not elevate in response to short-time proteasome inhibition, whereas the K11- and K48-linkages promptly increase.

Next, we examined the amount of 26S proteasome-bound polyUb linkages to evaluate which Ub linkages target substrates to the 26S proteasome. To this end, we purified the 26S proteasome from established stable HeLa cells by utilization of the HTBH tag according to a previous report (20). To protect the cellular polyUbs from deubiquitination, the whole cell lysates were treated with inhibitors of deubiquitinating enzymes (iodoacetamide and 1,10-phenanthroline). Coomassie-stained SDS-PAGE and immunoblotting confirmed that the 26S proteasome was purified from HeLa cells expressing S13-HTBH, but not from the HTBH tag alone (figure 1C and D). Immunoblotting of the purified 26S proteasome with an anti-Ub antibody found that the purified 26S proteasome contained significant amounts of high molecular weight Ub conjugates, with more in the MG132-treated preparation (figure 1E). Next, we used mass spectroscopy to quantitate the proteasome-bound polyUb linkages. After enriching on the proteasome, the K6-, K11-, K27-, K29-, K48- and K63-linkages were detectable and their relative amounts were quantitated (figure 1F). MG132 treatment increased the 26S proteasome-bound Ub conjugates of the K6-, K11-, K27-, K29- and K48-linked at a range of 1.7- to 2.5-fold, whereas the K63-linked only had a slight increase (figure 1F). Thus, the K63-linkage is the only detectable form in the 26S proteasome-bound fraction that does not significantly increase in response to short-time proteasome inhibition. Moreover, the amount of K48 polyUbs bound to the 26S proteasome is 6- and 12.7-fold more than the K63 polyUbs in the DMSO- and MG132-treated preparations, respectively (figure 1G). This could explain why immunostaining of HeLa cells with Ub linkage-specific antibodies found that K48 polyUbs colocalize with the 26S proteasome, whereas the K63 polyUbs do not (23). Furthermore, the K48/K63 ratios in the 26S proteasome-bound fraction are 1.7-fold higher than those in the whole cell lysates in both DMSO- and MG132-treated preparations (figure 1G). Thus, cellular K48 polyUbs appear to have more proteasome accessibility than the K63 polyUbs.

Both the K48 and K63 polyUbs bind the 26S proteasome comparably, but the K63 polyUbs are rapidly deubiquitinated

In the DMSO-treated HeLa cells, 11% and 68% of the 26S proteasome-bound Ub conjugates are the K63- and K48-linked forms, respectively (data not shown). This indicates that both Ub linkages can bind the 26S proteasome. We therefore evaluated the ability of the 26S proteasome to process both Ub link-
ages from the aspects of proteasomal binding, deubiquitination and degradation of conjugates. To obtain large quantities of the 26S proteasome for biochemical characterization, we purified the 26S proteasome from bovine red blood cells according to our previous report (18) (supplementary figure 1). Mass spectroscopy and immunoblotting determined that the purified 26S proteasome contained three deubiquitinating enzymes: Uch37, Usp14 and S13 (Rpn11) (supplementary figure 1C-F and data not shown). Notably, purified PA700 (19) contained both Uch37 and S13, but no Usp14 (supplementary figure 1C). Usp14 is a proteasome-associating protein and its association is salt concentration-sensitive (24). It is likely that Usp14 dissociates from PA700 during the anion exchange purification step.

To examine the ability of the mammalian 26S proteasome to bind K48 and K63 polyUbs, we performed a size-exclusion spin column assay. We found that both K48 and K63-Ub4 had comparable binding efficiency to the 26S proteasome (figure 2A). In the spin column assay, K63- or K48-Ub4 are trapped inside the P-30 spin column (40 kDa exclusion limit) after centrifugation (lane 2 in figure 2A). Coelution of Ub4 with the 26S proteasome in the flow-through indicates a direct binding between them (lane 3 in figure 2A). It is noteworthy that the 26S proteasome was incubated with both Ubal and 1,10-phenanthroline to inhibit its deubiquitinating activities in these binding assays. The more rapid deubiquitination of K63-Ub4 correlated with its higher Vmax value as compared to K48-Ub4, while both chains had similar Km values (figure 2C and supplementary figure 3). Together, these results indicate that the 26S proteasome binds both K48 and K63 polyUbs comparably, but it deubiquitinates K63 polyUbs much more rapidly.

**Both the Ubal- and 1,10-phenanthroline-sensitive deubiquitinating activities contribute to deubiquitination of K48 and K63 polyUbs**

We next aimed to determine which of the deubiquitinating activities of the 26S proteasome is responsible for deubiquitination of K48 or K63 polyUbs. The 26S proteasome contains two cystine-protease deubiquitinating enzymes (Uch37 and Usp14) which are sensitive to Ubal inhibition. It also contains a Zn2+-dependent metalloprotease (S13/Rpn11) which is inhibited by the metal chelator 1,10-phenanthroline. Uch37 was shown to have a Ub chain trimming activity which initiates cleavage from the distal site (25). S13 has a proximal deubiquitinating activity which cleaves an entire polyUb from the substrates (26). Ubal had a concentration-dependent inhibitory effect on 26S proteasome-catalyzed deubiquitination (supplementary figure 4).

Accordingly, we used 2.5 µM Ubal to block the activity of Uch37/Usp14 and 5 mM 1,10-phenanthroline to inhibit the activity of S13. For K48 Ub4, at a reaction time point when more than 85% of Ub4 was degraded if no Ubal or 1,10-phenanthroline was added, adding Ubal exhibited nearly complete inhibition of both the 26S proteasome- and PA700-mediated deubiquitination, while 1,10-phenanthroline had a more mild inhibitory effect (upper panels in figure 3A and B). For K63 Ub4, neither Ubal nor 1,10-phenanthroline alone completely inhibited deubiquitination, with Ubal having a stronger effect (lower panels in figure 3A and B). In contrast, deubiquitination of K63 Ub4 was abolished by a combination of these two inhibitors (lanes 10 and 11 in figure 3B). Thus, both the Ubal- and 1,10-phenanthroline-sensitive deubiquitinating enzymes contribute
to deubiquitination of K48 and K63 polyUbs, but they have different inhibitory extents.

**The 26S proteosome catalyzes Usp14-dependent deISGylation activity**

To investigate whether both of the thiol proteases (Uch37 and Usp14) of the proteasome contribute to the Ubal-sensitive deubiquitination, we prepared Usp14-depleted 26S proteasome by taking advantage of the fact that its residence on the 26S proteasome is salt concentration-sensitive (24). To remove Usp14, we incubated our purified 26S proteasome with 160 mM NaCl. Dissociated salt-sensitive binding proteins were further separated by gel filtration (data not shown). We found that, compared to the purified intact 26S proteasome, the salt-treated 26S proteasome contained 9% Usp14 and 66% Uch37 (figure 4A). Other intrinsic proteasomal subunits including the S13 subunit remained intact (figure 4A and supplementary figure 5A). Thereafter, the salt-washed 26S proteasome was referred to as the 26S (SW) proteasome. Importantly, salt-treatment didn’t disrupt proteasome integrity as both the regularly purified 26S and the 26S (SW) proteasomes consisted of approximately 50% doubly-capped and 50% singly-capped proteasomes (figure 4B). Also, salt-treatment didn’t affect the peptidase activity of the proteasomes (values listed under the gel in figure 4B and supplementary figure 5B). Next, we examined whether adding back recombinant Usp14 increases the deubiquitinating activity of the 26S (SW) proteasome. The size exclusion spin column assay demonstrated that the recombinant Usp14 bound the 26S (SW) proteasome and PA700 (figure 4C). Moreover, the 26S and PA700-bound Usp14 was activated, at least partially, as probed by Ub vinyl sulfone (figure 4D). However, binding of Usp14 did not promote the 26S (SW) proteasome or PA700 to deubiquitinate Ub-Amc and K48-Ub4 (figure 4E and supplementary figure 5C and D). Consistent with these findings, the loss of the deubiquitination activity of the 26S (SW) proteasome (39%) when using Ub-Amc as the substrate, is proportional to the loss of the Uch37 subunit (34%), but not Usp14 (91%) (supplementary figure 5D). Uch37 associates with the 26S proteasome through interacting with Adrm1. Moreover, Adrm1 activates Uch37 deubiquitinating activity (27-29). In contrast to Usp14, adding back purified Adrm1/Uch37 to the 26S (SW) proteasome significantly stimulated deubiquitination of Ub-Amc (supplementary figure 5D), although no effect was observed for deubiquitination of K48- or K63-Ub4 (data not shown). The deubiquitination discrepancy between Ub-Amc and polyUbs is unclear and under investigation. A recent study found that proteasome-bound Usp14 is reactive to the catalytic site probes of both Ub-vinylmethyl ester and ISG15-vinyl sulfone (30). ISG15 is an Ub-like modifier found only in vertebrates and its expression is induced by type I interferons and viral or bacterial infection. We therefore examined if Usp14 catalyzes deISGylation. PA700 did not catalyze deISGylation of ISG15-Amc. Adding back recombinant Usp14 to PA700 stimulated deISGylation of ISG15-Amc (figure 4G). Similarly, adding back recombinant Usp14 stimulated the 26S (SW) proteasome’s deISGylation activity (figure 4G). Thus, Usp14 catalyzes the deISGylation activity of the 26S proteasome.

**Rapid deubiquitination causes inefficient degradation of some K63 polyUb substrates**

Since K48 and K63 polyUbs have obviously different deubiquitination rates catalyzed by the 26S proteasome, we next compared the ability of K48- and K63-Ub4 to target proteins for degradation. To do this, we conjugated K48- or K63-Ub4 to the physiological substrate, UbcH10 (31), using immunoprecipitated Xenopus anaphase-promoting complex/cyclosome (APC/C) as the E3 ligase (18). The size exclusion spin column assay demonstrated that both K48 and K63 Ub4-UbcH10 efficiently bound to the 26S proteasome (figure 5A). Consistent with our earlier report (18), K48 Ub4-UbcH10 was efficiently degraded by the purified 26S proteasome as judged by the fact that UbcH10 only accumulated in the reaction containing the proteasome inhibitor, epoxomicin (comparing lanes 1 and
3 in figure 5B). Concomitantly, more K48 Ub4-UbcH10 remained in the reaction containing epoxomicin (lane 1 in figure 5B), suggesting that deubiquitination is impaired as a secondary consequence of inhibited proteolytic activity (18;32). Surprisingly, K63 Ub4-UbcH10 was rapidly deubiquitinated by the purified 26S proteasome and mainly accumulated as mono-ubiquitinated UbcH10 (Ub-UbcH10) (figure 5C). Since monomeric Ub can’t efficiently bind the 26S proteasome, rapid deubiquitination into Ub-UbcH10 could cause the substrate to dissociate from the 26S proteasome without degradation. The rapid deubiquitination of K63 Ub4-UbcH10 was inhibited by the addition of both Ubal and 1,10-phenanthroline (lane 5 in figure 5C). Consistent with the results from previous deubiquitination inhibition assays (figure 3B), Ubal had a stronger effect than 1, 10-phenanthroline on inhibition of the deubiquitination of K63 Ub4-UbcH10 (lanes 6 and 7 in figure 5C). Notably, about 12% of K63 Ub4-UbcH10 was degraded as determined by densitometric analysis (comparing lanes 4 and 8 in figure 5C). Together, these results show that rapid deubiquitination of polyUbs could cause inefficient degradation of some of its conjugates.

Since the majority of K63 Ub4-UbcH10 was rapidly deubiquitinated into Ub-UbcH10 without degradation, we hypothesized that the 26S (SW) proteasome, which has decreased deubiquitination activity, would retain K63 Ub4-UbcH10 on the proteasome long enough to promote degradation. This idea turns out to be true as we found that the 26S (SW) proteasome efficiently bound K63 Ub4-UbcH10 (supplementary figure 6) and catalyzed efficient degradation of K63 Ub4-UbcH10 (figure 5D). Degradation was abolished by adding either Ubal or 1,10-phenanthroline (lanes 5 and 6 in figure 5D), indicating that both of the deubiquitination activities are required for mediating degradation-coupled deubiquitination. Interestingly, degradation of K63 Ub4-UbcH10 by the 26S (SW) proteasome occurred even more rapidly than the degradation of K48 Ub4-UbcH10 by either the 26S (SW) (supplementary figure 7) or the regular 26S proteasome (comparing figure 5D to figure 5B), indicating that deubiquitination could be the rate limiting step in degradation of some proteins.

**NEMO protects K63-linked polyUbs from proteasomal deubiquitination in vitro**

The above studies indicate that both K48 and K63 polyUbs bind the 26S proteasome equally well in vitro (figure 2A and 5A), while K48 polyUbs are found to be more abundant at the proteasome in vivo (figure 1G). Since K63 polyUbs often form a complex with their binding partners when performing non-proteolytic functions, we speculated that K63 polyUb-interacting proteins sequester K63 polyUbs and limit their accessibility to the 26S proteasome or other deubiquitinating enzymes. In this regard, in TNFα-stimulated activation of the NFκB gene, IKKγ (NEMO), the non-catalytic subunit of the IKK kinase complex, binds the K63 polyUb chain(s) on RIP (33) and this interaction is required for stabilization of RIP (33). This stabilization effect is likely mediated by blocking deubiquitination of K63 polyubiquitinated RIP by A20 (33). Accordingly, we hypothesized that NEMO is capable of protecting K63 polyUbs from deubiquitination by the 26S proteasome and other deubiquitinating enzymes such as A20. To test this hypothesis, we synthesized K63 polyUb mixtures, Ub$_{n>6}$. GST-NEMO pulldown experiments demonstrated that NEMO preferred to bind long K63 chains (figure 6A). Consistent with our hypothesis, preincubation of K63 Ub$_{n>6}$ with NEMO blocked 26S proteasome-mediated deubiquitination and this effect was more pronounced on longer polyUbs (figure 6B). We next conjugated K63 Ub$_{n>6}$ to UbcH10 using immunoprecipitated Xenopus APC/C as the E3 Ub ligase. K63 Ub$_{n>6}$-UbcH10 was rapidly deubiquitinated into Ub-UbcH10 by the 26S proteasome without obvious degradation (comparing lanes 2 and 3 in figure 6C), consistent with the result obtained from K63 Ub$_{4}$-UbcH10 (figure 5C). Preincubation of Ub$_{n>6}$-UbcH10 with NEMO blocked proteasome-mediated deubiquitination (lanes 4-6 in figure 6C). A longer time course experiment showed that inhibition of the deubiq-
utination of K63 Ub<sub>n=6</sub>-UbcH10 by NEMO was extremely effective (supplementary figure 8). Furthermore, the inhibitory effect was due to the specific interaction between NEMO and K63 polyUbs because a single residue substitution (L329P) in NEMO that disrupts the interaction between NEMO and K63 polyUbs (33), abrogated the inhibitory effect on deubiquitination of K63 Ub<sub>n=6</sub>-UbcH10 (lanes 7-9 in figure 6C). Moreover, we found that NEMO protected K63 Ub<sub>n=6</sub>-UbcH10 from A20-mediated deubiquitination (supplementary figure 9). Together, these results demonstrate that K63 polyUb-interacting proteins can protect K63 polyUbs from deubiquitination by the 26S proteasome and other deubiquitinating enzymes.

**NEMO protects its bound linear polyUbs from deubiquitination in vivo**

Next, we sought to examine whether NEMO protects its bound polyUbs from deubiquitination in vivo. Linear Ub chains were recently discovered to regulate activation of the NFκB pathway by modification of NEMO (34). Structural studies revealed that NEMO binds linear di-Ub through its CC2-LZ (also called UBAN) domain (35;36). We therefore examined whether NEMO can stabilize linear Ub<sub>6</sub> in vivo. HEK293 cells were transfected with HA-Ub<sub>6</sub> and Flag-NEMO, Flag-NEMO (L329P) or a combination of both. Immunoblotting the whole cell lysates with an anti-HA antibody found no obvious accumulation of HA-Ub<sub>6</sub> in cells when it was transfected alone (lane 2 in figure 6D). In contrast, HA-Ub<sub>6</sub> and its conjugates accumulated when cotransfected with NEMO but not NEMO (L329P) (lanes 5 and 6 in figure 6D). These results suggest that NEMO protects linear Ub<sub>6</sub> from deubiquitination or degradation in cells.

**Discussion**

**K48 and K63 Ub chains have different proteasomal accessibility, deubiquitination rates and abilities to target proteins for proteolysis**

In this study we systematically compared K63 polyUbs with K48 polyUbs in proteasomal binding, deubiquitination and in targeting proteins for degradation. In vitro, K48-linked and K63-linked tetraubiquitin bound the 26S proteasome equally well (figure 2A). In contrast, quantitative mass spectroscopy determined that the 26S proteasome-bound Ub chains had a higher K48/K63 ratio than that in the whole cell lysates (figure 1G), indicating that cellular K63 Ub chains have less proteasomal accessibility than K48 chains. Also, we found that a K63 chain-specific binding protein, NEMO, protected K63 or linear Ub chains from deubiquitination by the 26S proteasome and/or deubiquitinating enzymes (figure 6). Thus, some cellular K63 Ub chains could be sequestered by their binding partners. This protecting effect might explain why our mass spectrometric analyses determined that K63 polyUbs have less proteasomal accessibility than K48 polyUbs in cells.

In response to a short time of proteasome inhibition, K63 polyUbs are the only detectable Ub linkage in HeLa cells that did not increase in the cellular level or the 26S proteasome-bound fraction. In contrast, other detectable linkages including the K6, K11, K27, K29 and K48 increased promptly. Under a severe proteasome inhibition condition (10 µM MG132 for 15 hours) we found that the cellular levels of all detectable Ub linkages (K11, K48 and K63) increased (data not shown), consistent with two recent studies in yeast and mammalian cells (37;38). The increase of the cellular level of K63 polyUbs in the later time of proteasome inhibition would suggest that most K63 chains are not used as signals for proteasomal degradation, otherwise they would have promptly increased after proteasome inhibition in a similar manner as the K48 polyUbs. The delayed accumulation of K63 polyUbs in response to proteasome inhibition might come from a secondary effect of an im-
paired Ub-proteasome pathway (37). For instance, K63 polyUbs are likely protected from deubiquitination/degradation by binding to their partners. Therefore, impaired degradation of their binding partners under severe proteasome inhibition could result in the accumulation of more K63 polyUbs in cells. Certainly, a small portion of proteasome-bound K63 Ub conjugates could be degraded (see below), thus severe proteasome inhibition would eventually result in an increase of K63 polyUbs as well.

Interestingly, we found that the 26S proteasome deubiquitinates K48 and K63 Ub chains differently. While both chains were bound similarly by the 26S proteasome, K63-linked Ub4 was deubiquitinated six times more rapidly than K48 Ub4. When conjugated to UbcH10, K48 Ub4 efficiently targeted UbcH10 for degradation, whereas only 12% of K63 Ub4-UbcH10 was degraded. The majority of K63 Ub4-UbcH10 was rapidly deubiquitinated into Ub-UbcH10 without degradation (figure 5), possibly because low proteasomal binding affinity of monomeric Ub causes dissociating Ub-UbcH10 from the 26S proteasome. Rapid deubiquitination of K63 polyUbs compared to K48 polyUbs might result from the difference in topology of these chains. The isopeptide bonds in K63 polyUbs are exposed in an open conformation, while they are buried in K48 polyUbs (39). The open conformation might make K63 polyUbs more accessible to the deubiquitinating enzymes of the 26S proteasome than K48-linked. Certainly, we cannot exclude the possibility that the different proteasomal binding geometries, if any, among the different Ub linkages might also cause varied accessibility to the deubiquitinating enzymes. Accordingly, the topologies of the Ub linkages might determine their rates of deubiquitination by the 26S proteasome, conferring a layer of substrate selectivity for proteasomal degradation.

Degradation of polyubiquitinated proteins requires highly coordinated actions including substrate binding, deubiquitination, unfolding, translocation, peptide hydrolysis and ATP hydrolysis (18). Disturbing this process by disrupting any one of these actions could be detrimental to proteasomal degradation. In the case of K63 polyUb conjugates, rapid deubiquitination could cause the conjugates to be released from the proteasome prior to being unfolded for translocation and degradation. In this regard, we would expect that K63 polyUbs could target degradation of unfolded proteins much more efficiently than that of stably folded ones. Using this same line of reasoning, reducing the deubiquitination activity of the 26S proteasome would facilitate the degradation of K63 polyUb conjugates. We show this to be true using a 26S proteasome preparation which had decreased deubiquitinating activity (figure 5D). Therefore, it is not surprising that K63 polyUbs were found to target several proteins for degradation *in vitro* in cases where the substrates are not well-folded (14), the deubiquitinating activity of the 26S proteasome is partially inhibited by Ubal (15) or deubiquitination activity is reduced by depletion of the deubiquitinating enzymes (32). In addition to targeting proteins to the proteasome, K63 Ub chains play a role in endosomal sorting and could target proteins to lysosomal degradation (40-42), a process that is also regulated by endosome-residing deubiquitinating enzymes (40).

Both the Ubal- and the 1,10-phenanthroline-sensitive deubiquitinating activities of the 26S proteasome contribute to K48- and K63-linkage deubiquitination

We found that the Ubal- and the 1,10-phenanthroline-sensitive deubiquitinating activities of the 26s proteasome have different inhibitory effects on K48 and K63 polyUbs. Simultaneous inhibition of both the Ubal- and the 1,10-phenanthroline-sensitive activities were required for complete inhibition of K63 chain deubiquitination. In contrast, Ubal alone exhibited nearly complete inhibition of K48 chain deubiquitination, while 1,10-phenanthroline had a more mild inhibitory effect. To distinguish the role of the two Ubal-sensitive enzymes (Uch37 and Usp14), we found that add-back of Uch37/Adrm1 to the 26S (SW) proteasome was able to stimulate
deubiquitination. In contrast, add-back of Usp14 to the 26S (SW) proteasome or PA700 did not stimulate deubiquitination of any tested substrates (figure 4 and supplementary figure 5). This may imply that Usp14 is not a major deubiquitinating enzyme of the mammalian proteasome. However we can not exclude possibilities that Usp14 may have specific activity against other untested Ub linkages; Usp14 is activated by an unknown protein which is not present in our in vitro system; and/or Usp14 is redundant when coexisted with Uch37. Additionally, Usp14 did stimulate a modest deISGylation activity at the proteasome and may very well be an authentic deISGylating enzyme, but further investigation is required.

A recent study reported that neither N-ethyl maleimide (NEM, a cysteine modifier that inhibits thiol proteases) nor Ubal block bovine PA700 or 26S (unspecified source)-catalyzed deubiquitination of K63 Ub2, while 1,10-phenanthroline does (43). Thus, the authors proposed that S13 is responsible for K63 chain deubiquitination of the 26S proteasome. It seems unlikely that the different conclusions originate from different proteasome sources because we purified PA700 and the 26S proteasome from bovine red blood cells as well. Different experimental setup might explain why the previous study did not observe the activity of Uch37 in catalyzing deubiquitination of K63 polyUbs. First, we used 2.5 µM Ubal to inhibit Uch37 activity in this study, while the other study used 0.5 µM Ubal (43). A concentration of 0.5 µM Ubal cannot efficiently block the chain-trimming activity of the 26S proteasome (supplementary figure 4). Second, we used K63 Ub4 to evaluate proteasomal deubiquitination activities, while K63 Ub2 was used in the other study (43). It is possible that deubiquitination of K63 polyUbs by Uch37 depends on efficient proteasomal binding, which requires a minimal chain length of four Ubs (44). Unfortunately, we were not able to evaluate the effect of NEM-treatment on deubiquitination because our purified 26S proteasome was disassembled when incubated with 2 mM NEM (data not shown). Presumably, NEM modifies cysteine residues in some subunits that are essential for maintaining proteasome integrity.

Uch37 and S13 belong to two different deubiquitinating enzyme families: Uch37 is a thiol protease; S13 is a Zn\(^{2+}\)-dependent metalloprotease and a member of the JAMM/MPN+ deubiquitinating family. Interestingly, the deubiquitinating activities of both Uch37 and S13 are activated when integrated into the 26S complex. The JAMM/MPN+ family members, including AMSH and Brcc36, have been shown to have specificity for K63 polyUbs (43;45). Not surprisingly, S13 catalyzes deubiquitination of K63 polyUbs (43). In contrast to the specificity of the JAMM/MPN+ deubiquitinating enzymes for K63-linked chains, the thiol-utilizing deubiquitinating enzymes have diverse deubiquitination specificities. For example, Usp2, Usp5 and Usp15 can deubiquitinate both the K48- and K63-linkages (46); whereas CYLD only cleaves K63 polyUbs (46). The data from our study reveals that Uch37 belongs to a thiol-dependent deubiquitinating enzyme group that cleaves both the K48- and K63-linkages.

1Abbreviation: APC/C, anaphase promoting complex/cyclosome; polyUb, polyubiquitin; Suc-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; Ub, ubiquitin.

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Figure Legends

Figure 1 Neither the cellular level nor the 26S proteasome-bound fraction of K63 Ub conjugates increases in response to short-time proteasome inhibition in HeLa cells.

(A) Ub conjugates in the whole cell lysates increase in response to a short time of MG132 treatment. HeLa cells stably expressing the HTBH tag or S13-HTBH were treated with 30 µM MG132 for 45 min. The whole cell lysates were immunoblotted against Ub and β-actin.

(B) Quantitation of K11-, K48- and K63-linked polyUbS in the whole cell lysates by quantitative mass spectroscopy. The data from MG132-treated samples were normalized to DMSO-treated ones. Data are represented as mean ± SEM.

(C) SDS-PAGE of the purified 26S proteasome from HeLa cells using the HTBH-tag.

(D) Immunoblotting the HTBH-tag purified 26S proteasome with antibodies against the proteasome subunits including S6a, S7 and the whole 20S proteasome.

(E) Immunoblotting the HTBH-tag purified 26S proteasome with an anti-Ub antibody.

(F) Quantitation of the 26S proteasome-bound polyUb linkages as shown in (E) by quantitative mass spectroscopy. The data from MG132-treated samples were normalized to DMSO-treated ones. Data are represented as mean ± SEM.

(G) The 26S proteasome-bound fractions have higher K48/K63 ratios than the whole cell lysates (WCL) in both DMSO- and MG132-treated preparations. The percentage of K48 or K63-linked Ub in the total detectable forms was calculated based on mean values from quantitative mass spectroscopy and the percentage ratios of K48 vs. K63 are shown.

Figure 2 K48- and K63-Ub4 bind the 26S proteasome comparably, but K63-Ub4 is deubiquitinated much more rapidly.

(A) K48- and K63-Ub4 have comparable binding efficiency to the 26S proteasome. Binding was assayed by using size-exclusion spin columns. Proteins in the flowthrough after centrifugation were immunoblotted with either an anti-Ub or anti-S7 antibody. The Ub immunoblots were densitometrically quantitated and the corresponding values were shown under the blots.

(B) K63-Ub4 is deubiquitinated much faster than K48-Ub4 by the 26S proteasome. Reactions contained 13.5 nM of purified 26S proteasome and 400 nM of either K48- or K63-Ub4. The right panel shows the densitometric quantitation of the immunoblots. Deubiquitination of 50% of K48- and K63-Ub4 took approximately 19.5 and 3.6 minutes, respectively.

(C) The rapid deubiquitination of K63-Ub4 is due to its higher Vmax in comparison to K48-Ub4. Reactions contained 4 nM 26S proteasome and various concentrations of Ub4 chains (supplementary figure 3). Plots of velocity versus Ub4 concentration were shown and velocity mean values of two independent experiments were fit into the Michaelis-Menten equation for parameters KM and Vmax.

Figure 3 Both the Ubal- and the 1,10-phenanthroline-sensitive deubiquitinating activities of the 26S proteasome contribute to K48- and K63-linkage deubiquitination.

(A) Deubiquitination of 2 µg of K48- or K63-Ub4 by 13.5 nM purified 26S proteasome in the absence or in the presence of 2.5 µM Ubal, 5 mM 1,10-phenanthroline or both. The reaction times for K48- and K63-Ub4 were 120 min and 20 min, respectively.

(B) The reactions were similar to (A) except that 60 mM PA700 was used. The reaction times for K48- and K63-Ub4 were 180 min and 20 min, respectively.

Figure 4 Adding-back Usp14 to the 26S proteasome stimulates proteasome’s deISGylation activity but not deubiquitination activity.

(A) The 26S (SW) proteasome has 9% Usp14 and 66% Uch37 as compared to the regularly purified 26S proteasome. The 26S or 26S (SW) proteasome was immunoblotted with different...
antibodies. Immunoblots were densitometrically quantitated to compare the quantities of Usp14, Uch37 and other subunits in these two preparations. 

(B) The 26S (SW) proteasome has similar structural integrity and peptidase activity as the regularly purified 26S proteasome. 3 µg of the 26S or 26S (SW) proteasome was separated in a 4% native PAGE and the in-gel activity was assayed by using Suc-LLVY-Amc as the fluorogenic substrate. The chymotrypsin-like activity against Suc-LLVY-Amc (the values shown under the gel) was quantitated by a solution assay. D-26S and S-26S indicate the doubly-capped and the singly-capped 26S proteasome, respectively.

(C) Recombinant Usp14 binds the 26S (SW) proteasome and PA700. The binding was assayed with Sephadex G-100 spin columns, similar to that in figure 2A.

(D) Proteasome-bound Usp14 is partially activated. 26S proteasome (15 nM), 26S (SW) proteasome (15 nM), PA700 (15 nM), Usp14 (45 nM) or their combinations were incubated with UbVS. Covalent conjugation of UbVS on Usp14 was assayed by immunoblotting with an anti-Usp14 antibody.

(E) Binding of Usp14 does not stimulate the deubiquitinating activity of PA700 and the 26S (SW) proteasome. The reactions contained 15 nM PA700 or the 26S (SW) proteasome in the absence or in the presence of 45 nM Usp14. The deubiquitinating activity was assayed with Ub-Amc as the substrate.

(F) Usp14 catalyzes the 26S proteasome’s deISGylation activity. Reactions were similar to (E) except using ISG15-Amc as the substrate.

Figure 5 K63 Ub conjugates are rapidly deubiquitinated by the 26S proteasome without efficient degradation.
(A) Both K48 and K63 Ub4-UbcH10 efficiently bind the 26S proteasome as determined by the spin column assay.
(B) The degradation reaction contained 100 nM K48 Ub4-UbcH10 and 13.5 nM 26S proteasome. The lane denoted epox indicates that the 26S proteasome was preinhibited with epoxomicin.
(C) K63 Ub4-UbcH10 is rapidly deubiquitinated by 26S proteasome without efficient degradation. Reactions were analogous to (b). Lanes denoted with Ubal or phen indicate that the deubiquitinating activities of the 26S proteasome were preinhibited with 2.5 µM Ubal or 5 mM 1, 10-phenanthroline or both.
(D) The 26S (SW) proteasome degrades K63 Ub4-UbcH10. The reactions were analogous to (C) except the 26S (SW) proteasome was used.

Figure 6 K63 polyUbs are protected from deubiquitination by their binding partners.
(A) NEMO prefers binding to long K63 polyUbs. The K63 polyUb mixtures contained 400 nM Ub6 and 400 nM K63 Ub4.
(B) NEMO protects K63 Ub6 from proteasomal deubiquitination. Appropriate amounts of NEMO were preincubated with 300 nM Ub6 for 5 min prior to the supplementation of 13.5 nM purified 26S proteasome to initiate deubiquitination.
(C) NEMO protects K63 Ub6-UbcH10 from proteasomal deubiquitination. Reactions were analogous to (B) except that 100 nM K63 Ub6-UbcH10 was used.
(D) NEMO protects HA-Ub6 from deubiquitination in cells. HEK293T cells were transfected with HA-Ub6, Flag-NEMO, Flag-NEMO (L329P) or a combination. The whole cell lysates were immunoblotted with an anti-HA or anti-Flag antibody.
(E) The whole cell lysates in (D) were immunoprecipitated with an anti-Flag antibody and the precipitates were immunoblotted with an anti-HA or anti-Flag antibody.
Figure 1
Figure 2
Figure 3
Figure 4
**Figure 5**

### A

|        | K48 Ub₄⁻UbcH10 | K63 Ub₄⁻UbcH10 |
|--------|----------------|----------------|
| 20% input | - 26S | + 26S | - 26S | + 26S |

|        | Ub₄⁻UbcH10 | UbcH10 |
|--------|------------|--------|
| α-UbcH10 |            |        |
| α-S7 |            |        |

### B

| 10 + epox | 0 | 10 (min) |
|-----------|---|----------|
| kDa | 55 | 43 | 34 | 26 |

|            | Ub₄(K48)⁻UbcH10 | UbcH10 |
|------------|-----------------|--------|
| α-UbcH10 |                |        |

### C

|        | 2 + Ubal + phen | 2 + Ubal | 2 + phen | 2 + epox |
|--------|-----------------|----------|----------|----------|
| 0 (min) | Ub₄(K63)⁻UbcH10 | Ub-UbcH10| Ub-UbcH10| Ub₄⁻UbcH10|
| kDa | 55 | 43 | 34 | 26 |

### D

|        | 4 + epox | 4 + phen | 4 + Ubal |
|--------|----------|----------|----------|
| 0 (min) | Ub₄(K63)⁻UbcH10 | Ub-UbcH10| Ub₄⁻UbcH10|
| kDa | 55 | 43 | 34 | 26 |

α-UbcH10
Figure 6
The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26S proteasome
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