Ubiquitination

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The Lysine48-Based Polyubiquitin Chain Proteasomal Signal: Not a Single Child Anymore

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monoubiquitination · polyubiquitin chains · proteasome · protein degradation · ubiquitin

The conjugation of ubiquitin (Ub) to proteins is involved in the regulation of many processes. The modification serves as a recognition element in trans, in which downstream effectors bind to the modified protein and determine its fate and/or function. A polyUb chain that is linked through internal lysine (Lys)-48 of Ub and anchored to an internal Lys residue of the substrate has become the accepted "canonical" signal for proteasomal targeting and degradation. However, recent studies show that the signal is far more diverse and that chains based on other internal linkages, as well as linear or heterologous chains made of Ub and Ub-like proteins and even monoUb, are recognized by the proteasome. In addition, chains linked to residues other than internal Lys were described, all challenging the current paradigm.

1. Introduction

The covalent conjugation of ubiquitin (Ub) is mediated by a three-step enzymatic reaction. 1) A Ub-activating enzyme (E1) catalyzes an energy-dependent reaction (energy provided by ATP), giving a high-energy thioester bond between the carboxy group of the terminal glycine residue of ubiquitin and a cysteine moiety of the enzyme. 2) One of several Ub carrier proteins (E2; also known as Ub-conjugating enzymes, UBCs) transferres ubiquitin from the high-energy thioester bond on E1 to a high-energy thioester bond on E2. 3) One of numerous substrate-specific Ub-protein ligases (E3s) catalyzes a third reaction. Depending on the type of the ligase, ubiquitin can generate a third high-energy thioester bond on this enzyme (in case of HECT (homologous to the E6-AP C-terminus) domain ligases), or it can be transferred directly to the ligase-bound substrate (in case of RING (really interesting gene) domain ligases). In many cases, the reaction progressively generates a polyUb chain, in which the first Ub moiety is attached to an ε-NH_2 group of a Lys residue in the substrate. The Ub moieties are linked to one another by an isopeptide bond between the C-terminal Gly of one moiety and the ε-NH_2 group of the internal Lys48 of the previous one.[1] Proteins thus modified are targeted for degradation by the 26S proteasome, which is an enzyme with three different proteolytic activities, chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activity. During protein degradation, Ub is recycled through the activity of deubiquitinating enzymes (DUBs), which disassemble ubiquitin chains by hydrolyzing the isopeptide bond between the C-terminal glycine of Ub and the amino group of the lysine residue in either the more proximal Ub moiety in the chain or the substrate (in case it is the first Ub moiety). Proteasomal degradation of Ub-tagged proteins plays an important role in regulating basic cellular processes, among them control of cell cycle and division, maintenance of the cellular quality control, and regulation of the level of transcription factors.[2] Ub has seven internal lysine residues, and the general hypothesis has been that chains based on linkages through lysines other than Lys48 serve non-proteolytic functions. For example, several steps in the activation of NF-κB or regulation of endocytosis are mediated by Lys63-linked Ub chains.[3] In these cases, the modification controls the function of the tagged protein that is not eliminated.

For many years, the accepted notion had been that the chain 1) must contain at least four Ub moieties;[4] 2) is made of Ub moieties that are linked through internal Lys48;[5] and 3) is anchored to an ε-NH_2 group of a lysine residue in the target substrate.[6] However, recent findings show a much broader set of Ub-based proteolytic signals, which will be discussed herein.

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2. Single and multiple monoubiquitinations

Several recent studies show that the proteasome can recognize substrates that were modified through a single or multiple monoubiquitinations (Figure 1). Thus, a peptide with a minimal length of 20 residues is efficiently degraded when linearly fused to Ub without a requirement for further chain extension.[7] For these linear fusions, it has been shown that a single Ub can support the degradation of extensions of up to approximately 150 residues.[8] An example of a naturally occurring substrate that is degraded following a single monoubiquitination on a specific Lys residue is paired box 3 protein (PAX3), a regulator of muscle differentiation. This reaction is catalyzed by the TAF1 ligase.[9] Similarly, the conjugation of a single Ub leads to proteasomal degradation of the cell adhesion receptor syndecan 4, which is involved in cell migration.[10]

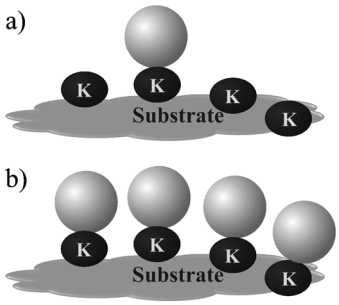
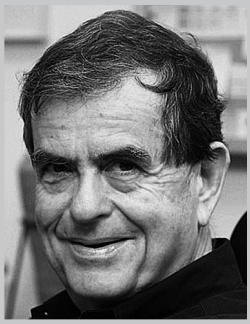


Figure 1. Monoubiquitination. a) Modification of a protein through single monoubiquitination. b) Modification of a protein through multiple monoubiquitinations. Gray sphere: Ub, black sphere: Lys (K).

For some substrates, modification through multiple monoubiquitinations is necessary to promote their proteasomal degradation. For instance, p105, the precursor of the NF-κB transcription factor p50, has to be monoubiquitinated on multiple lysines in the C-terminal domain of the molecule in order to be processed, releasing the N-terminal p50 segment, which is the active subunit of the transcription factor.[11] The cell-cycle regulator cyclin B1 is degraded by the proteasome following multiple monoubiquitinations catalyzed by APC/C Ub ligase.[12] Restricting the number of lysine residues that serve as Ub anchors "forces" the generation of oligoUb and/or polyUb chain(s).[12]

These findings suggest a new dynamic concept of the density/avidity of the Ub signal: the spatial arrangement of a large enough number of single Ub moieties that bind to multiple points of the proteasomal Ub receptors ascertains strong binding of the tagged substrate to the proteasome, leading to its efficient and processive degradation. Restriction of the number of Ub anchors on the substrate forces the formation of a polyUb chain, in which the moieties that are bound to one another substitute for the single moieties that are otherwise distributed among several anchors along the substrate. The concept of a dynamic and adaptive signal can be further extended to include, among other characteristics, the length of the substrate. Whereas a single Ub has the lowest affinity to the proteasome, the progressive elongation of the chain gradually increases the affinity. Thus, for short substrates, a single moiety or a short Ub chain may be sufficient to bind them strongly enough to the proteasome to secure their efficient degradation. Longer substrates may need longer chains. This hypothesis has been corroborated by a recent study, in which it was demonstrated that a single Ub can support the degradation of artificial (HA repeats) or naturally occurring (Hug1, Cks2, and α-synuclein) substrates made of up to approximately 150 residues.[8]



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3. PolyUb chains

Homogenous Ub chains based on identical internal linkages

Ubiquitin is a polypeptide that consists of 76 residues with seven lysine residues in positions 6, 11, 27, 29, 33, 48, and 63. The most common Ub polymer involved in targeting a substrate for degradation is a homogeneous chain in which each Ub moiety is linked through an isopeptide bond to Lys48 of the preceding conjugated Ub moiety.[5,13] It was assumed that only such chains can target substrates for proteasomal degradation. Mass spectrometry analysis[13] has shown that homogeneous chains based on linkages of lysine residues 29,

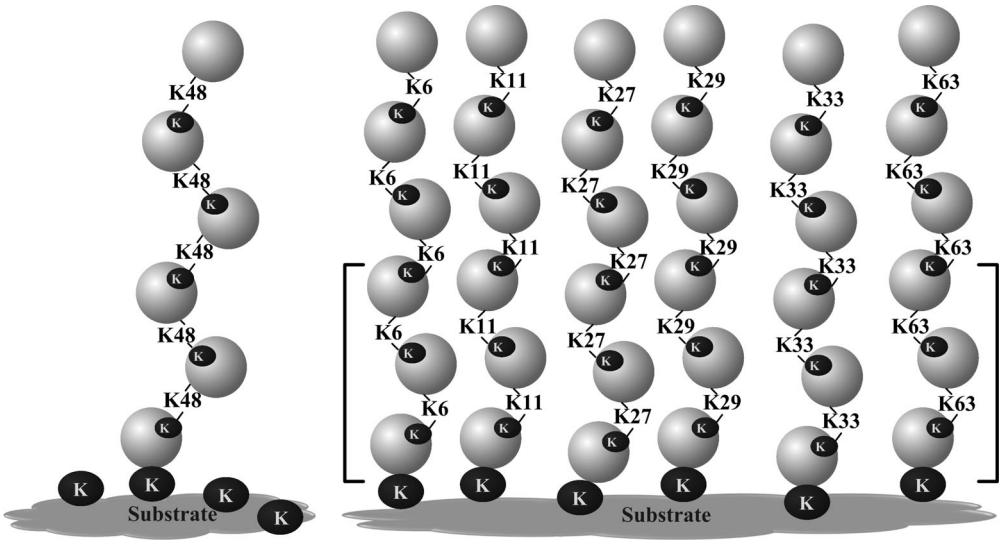

Figure 2. Homogeneous ubiquitin chains. Chains based on linkages involving lysines 6, 11, 27, 29, 33, 48, or 63.

11, 27, and 6 (in decreasing abundance) can also mediate proteasomal degradation. For example, several studies have shown that homogeneous chains based on Lys11 linkages and synthesized by APC/C Ub ligase[14] are important mediators of protein degradation during cell division.[15] The assembly of such chains, which (as Lys48-linked chains) also bind to the S5 subunit of the proteasome,[16] possibly depends on a TEK box motif found both on Ub and on the substrate.[15b]

Other studies have shown that Lys33-linked[17] and Lys63-linked chains[18] are also recognized by the proteasome. Thus, it appears that homogeneous chains based on linkages of all Ub internal lysine residues (Figure 2) can target proteins for proteasomal degradation. However, it should be noted that some of the studies were carried out using cell-free systems and may thus not reproduce the corresponding cellular events realistically.

Heterogenous Ub chains based on distinct internal linkages

Because Ub chains are synthesized enzymatically, it was natural to assume that they are homogenous, that is, linked by the same internal Lys residue. However, mass spectrometry analyses showed that mixed chains, in which linkages of different internal lysine residues are involved (Figure 3 a), can also target proteins for proteasomal degradation. An example for such a protein is cyclin B1, which, besides being targeted by multiple monoubiquitinations,[12] was also shown to be targeted by short ubiquitin chains containing internal linkages of lysines 11, 48, and 63.[15c] In order to generate a signal of sufficient Ub density, it is possible that different proteins use mixtures of chains of different lengths and internal linkages, depending on the availability of Ub anchors.

Additionally, multiply branched (forked) chains have been described,[19] in which more than one Ub moiety is attached to the previously conjugated molecule (Figure 3 b). However, these chains bind poorly to the proteasome,[19a,20] suggesting that they do not target proteins for degradation.

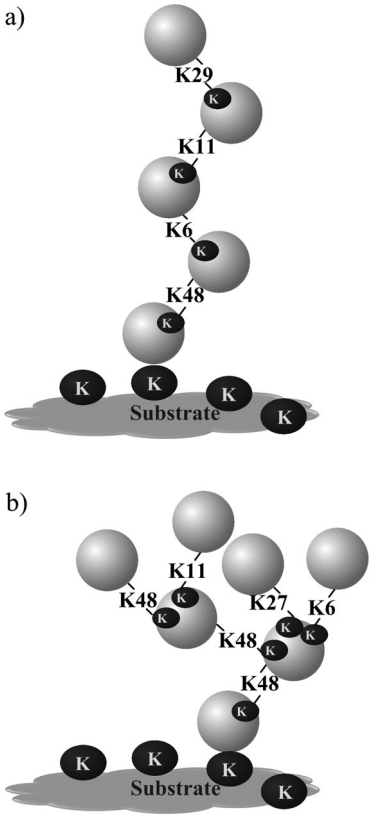

Figure 3. Heterogeneous ubiquitin chains. a) Mixed chains based on linkages involving different lysines. b) Multiply branched (forked) chains, in which several ubiquitin moieties are anchored to distinct Lys residues in a single ubiquitin moiety.

For example, autoubiquitination of Ring1B generates mixed multiply branched chains based on linkages of lysines 6, 27, and 48; these chains stimulate the monoubiquitinating ligase activity of the protein toward its substrate histone H2A.[19b]

Heterologous mixed chains of ubiquitin and ubiquitin-like proteins

The Lys residues of certain proteins can be modified by the small ubiquitin modifier (SUMO), a Ub-like (UbL) protein. This modification regulates a variety of processes, including stress response and DNA repair, signal transduction, and targeting of proteins to their proper subcellular compartments.[21] SUMO can be conjugated to a Lys residue of the substrate as a monomer or, similar to Ub, it can form a homogeneous chain. Recently, SUMOylation and ubiquitination have been shown to cooperate with one another. Initially, it was shown that cells treated with the proteasome inhibitor MG132 accumulate chains of SUMO1,[22] and SUMO2 and SUMO3,[23] thus suggesting that such chains are involved in targeting proteins for degradation. In a different experiment, purification of Ub adducts also gave SUMO2 conjugates,[23] therefore suggesting that this UbL can be part of a polyUb chain (Figure 4). Furthermore, it was suggested, though not shown directly, that (mono- or poly-)SUMOylation is a prerequisite for subsequent polyubiquitination and degradation, thus linking the two modifications to the same pathway.[23]

Direct evidence that heterologous chains consisting of both SUMO and Ub can lead to proteasomal degradation came from studies on treatment of acute promyelocytic leukemia (APL) with arsenic trioxide, showing that the drug induces the degradation of the promyelocytic leukemia/retinoic acid receptor alpha (PML-RARα) fusion protein. The protein first undergoes polySUMOylation, recruiting the RING finger protein 4 (RNF 4) Ub ligase.[24] The subsequent elongation by polyubiquitination of the SUMO chains targets PML-RARα for proteasomal degradation.[24b] In another example, it was shown that removal of the SUMO-specific protease SENP1 (a deSUMOylating enzyme) leads to suppression of the hypoxic response by targeting the hypoxia inducible factor (HIF) 1α for rapid degradation. It was further shown that HIF1α is SUMOylated, recruiting the ubiquitin ligase protein von Hippel Lindau (pVHL) to HIF1α and thus resulting in its ubiquitination and degradation.[25] Deletion of SENP1 probably stabilizes the SUMO chain on HIF1α, thus rendering the elongation of the SUMO chain by Ub and consequently HIF1α degradation more efficient. It is still not clear what stimulates SUMOylation of HIF1α in the first place and how this modification is related to normoxia, in which specific proline residues in the protein are hydroxylated, a process that was also reported to recruit the pVHL ligase.[26]

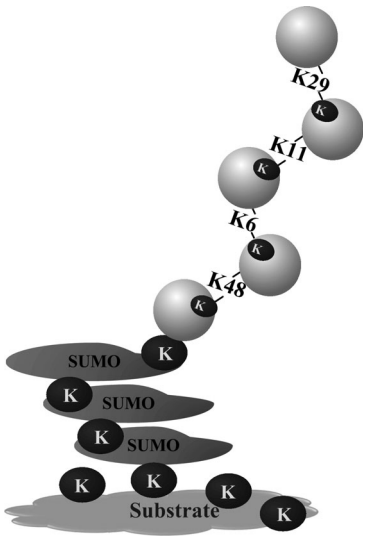

Figure 4. Heterologous ubiquitin chains. Chains made of Ub and a Ub-like protein.

These examples highlight another layer of complexity of the Ub system that broadens the signal beyond the homogenous Lys48 based polyubiquitin chains. The partnership between the two different modification systems may contribute to a more precise regulation of protein degradation. However, it should be noted that the formation of heterogeneous chains may be of limited biological role, and their involvement in proteasomal recognition is yet to be expanded to additional targets. It is possible that proteins are primarily SUMOylated for non-proteolytic functions and are ubiquitinated later in order to be directed to the proteasome.

Linear Ub chains

In the Ub chains described above, the moieties are conjugated to one another through isopeptide bonds between the C-terminal Gly76 of the distal Ub molecule, and different internal lysines of the proximal one. Recently, another type of chain was described in which the linkages are conjugated to one another head-to-tail. Here, the C-terminal residue of one moiety is linked in a peptide bond to the N-terminal residue of the more proximal one (Figure 5). The linear chain is assembled by the linear ubiquitin chain assembly complex (LUBAC, a Ub ligase), which consists of three proteins, the SHANK-associated RH domain-interacting protein (SHARPIN), a longer isoform of heme-oxidized iron-regulatory protein 2 (IRP2) Ub ligase-1 (HOIL-1L), and the HOIL-1L-interacting protein (HOIP).[27] It was shown that LUBAC can assemble linear Ub chains on GFP, to which the first Ub is fused at the N-terminus. This modification results in proteasomal degradation of the tagged GFP, suggesting a potential involvement of linear Ub chains in the recognition of substrates by proteasomal receptors.[28] Supporting these data is the finding that a linear tetraUb chain was shown to promote the degradation of the eukaryotic replication clamp protein PCNA. This reaction is mediated by the complex of AAA ATPase cell division protein 48 (CDC48), nuclear protein localization 4 (NPL4), and Ub fusion degradation 1 (UFD1) proteins.[29]

4. Non-lysine sites of ubiquitination

Internal sites of ubiquitination

In the majority of cases, ubiquitination occurs on internal Lys residues of the target substrates. However, recent studies showed that, in some cases, Ub can be conjugated through an ester bond to Ser or Thr, or through a thioester bond to Cys

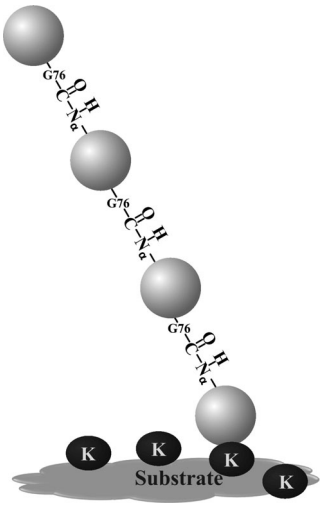

Figure 5. Linear ubiquitin chains. Chains in which the Ub moieties are linked head-to-tail.

(Figure 6).[30] For example, the mouse γ-herpes virus E3 ligase mK3 along with Ube2j2, the cognate E2 enzyme ubiquitinates the cytoplasmic tail of the major histocompatibility complex I heavy chain (MHC) preferentially on Thr and Ser. The protein is then degraded through the endoplasmic reticulum associated degradation (ERAD) pathway.[31] In a different study, it was shown that neurogenin (NGN), a transcription factor that regulates neuronal differentiation, is ubiquitinated both on Lys and non-Lys residues (Cys, Ser, and Thr, and the N-terminal residue). Importantly, Ub chains that are anchored to different residues can serve as proteasomal degradation signals.[30b,c]

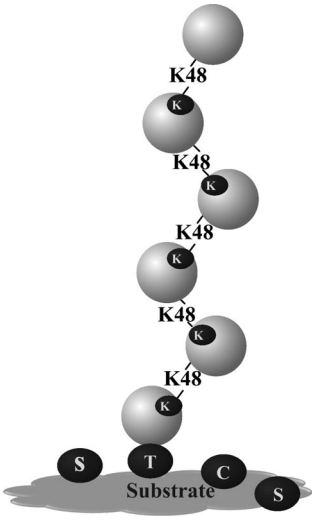

Figure 6. Ubiquitination on residues other than Lys (Cys (C), Ser (S), and Thr (T)).

N-terminal ubiquitination

It appears that, besides modifying internal residues, a Ub chain can be generated on the α-NH_2 group of the N-terminal residue of the substrate (Figure 7).[32]

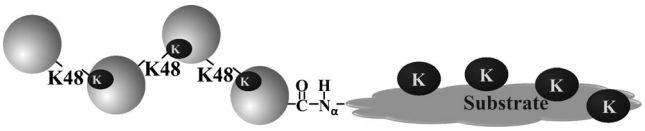

Figure 7. Ubiquitination on an N-terminal residue.

The first protein that was shown to be targeted through N-terminal ubiquitination was myoblast determination protein 1 (MyoD).[32] This study was followed by several additional reports on other proteins that are similarly ubiquitinated.[33] N-terminal ubiquitination raises the interesting hypothesis that N-terminal acetylation, which is a common post-translational modification, serves to protect proteins from ubiquitination and subsequent destruction.[34]

The utilization of additional Ub anchors most probably provides the system with additional layers of flexibility, which is required for fine tuning of the proteolysis of its myriad substrates.

5. Non-consensus sites of ubiquitination

Ubiquitin conjugation is catalyzed by a complex series of enzymatic reactions and intuitively appears to be highly specific. Therefore, it was surprising to discover that there are no known consensus or homologous ubiquitination sites. It appears that the ubiquitination sites are unique for a few proteins, for some they share vague characteristics, whereas for many others they appear to be promiscuous.

For one group of proteins, for example, $p19^{INK4d}$, PAX3, IκBα, and p53, specific anchoring lysines have been identified, though the surrounding residues do not share obvious common features. There can be a single or multiple anchors, and if there are multiple anchors, they are typically clustered in one region of the target protein. However, the degree of specificity (i.e., the involvement of other lysine residues besides those at the site) and the requirement for all lysine residues in the cluster to target the specific substrate vary. For example, in $p19^{INK4d}$, the major Ub acceptor is Lys62,[35] whereas in PAX3, modification of either Lys437 or Lys475 is sufficient to target the protein for degradation (and ubiquitination of one residue even prevents the modification of the second one).[9b] Ubiquitination of IκBα occurs on Lys21 and/or Lys22,[36] whereas for p53, it is a cluster of 6 lysine residues in the C-terminal domain that were reported to be modified.[37]

A more systematic analysis of ubiquitination sites was carried out by tandem mass spectrometry of yeast proteins,[38] enabling semi-quantitative and unbiased mapping of post-translational modification sites.[39] By analyzing the datasets generated in these studies, Catic and co-workers found that, with one exception, all lysines modified by Ub must reside on the surface of the protein, and even for the single exception, in which the lysine residue is buried, ubiquitination requires prior unfolding of the protein (Lys370 in glutamate dehydrogenase 1).[40] There is also a clear preference for ubiquitination in loops, followed by ubiquitination in α helices (a spiral structure of a protein, in which the backbone N–H group of

one amino acid is bound to the backbone C=O group of the amino acid four residues away).[40] Interestingly, Ub lysine residues 48 and 63, which are modified most frequently in polyUb chains, reside within loops. Attempts to identify unique neighboring residues adjacent to ubiquitinated lysines did not give clear results.[40] Similar findings were also reported in an independent study, thus demonstrating that preferred sites of ubiquitination are surface-accessible lysine residues that are located in the ordered secondary region (coil > helix > β-sheet > turn) and surrounded by small, positively charged residues.[41]

In many cases, ubiquitination sites are difficult to identify. For example, in the case of the p105 precursor of NF-κB, ubiquitination must occur on multiple lysine residues (≈ 30) that are localized in the C-terminal part of the molecule (≈ 500 residues segment). However, the exact location of the modified residues does not seem to affect the outcome: apparently, the number of modified residues determines the extent and rate of processing of p105.[11,42]

For many other substrates, the ubiquitination sites are not specific. Thus, cyclin B1 can be ubiquitinated on any single lysine residue within the molecule, even if inserted at artificial sites.[43] Similar findings were reported for the ζ chain of the T-cell antigen receptor.[44]

The low evolutionary conservation of ubiquitination sites probably attests to the vitality and adaptability of the ubiquitin–proteasome system (UPS), one of the most important roles of which is the removal of foreign, mutated, and otherwise denatured/misfolded proteins.

6. Summary and Outlook

The countless number of proteolytic substrates and the need to set a "hierarchy" for their funneling into the degradation machinery probably required the evolvement of many different signals that are recognized by the proteasome and the shuttling proteins. Different structures and lengths of Ub chains can provide subtle alterations to the extent of interaction between the proteasome and the substrates, thus regulating affinity and degradation rates. It should be noted that the most common chains targeting the majority of substrates for degradation are still Lys48-linked chains, but even for those, the finding that they also contain other linkages may provide them with different characteristics required for fine tuning of the binding characteristics to the proteasome, and hence of the proteolytic process. It should be emphasized that the multiplicity of linkages within a predominantly Lys48-linked chain can also reflect the promiscuity of the conjugation system rather than a biological requirement, and all the proteasome needs is a "critical mass" of Ub moieties linked through Lys48. Also, aberrations in the Ub system underlie the pathogenesis of numerous pathologies, including malignancies and neurodegenerative disorders. The added level of complexity and specificity of the signal is probably also mirrored in these aberrations. Therefore, a thorough understanding of the mechanism of proteasomal recognition will allow the development of highly specific drugs that have limited side effects.

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