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Review

The complexity of recognition of ubiquitinated substrates by the 26S proteasome ☆

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ABSTRACT

The Ubiquitin Proteasome System (UPS) was discovered in two steps. Initially, APF-1 (ATP-dependent proteolytic Factor 1) later identified as ubiquitin (Ub), a hitherto known protein of unknown function, was found to covalently modify proteins. This modification led to degradation of the tagged protein by – at that time – an unknown protease. This was followed later by the identification of the 26S proteasome complex which is composed of a previously identified Multi Catalytic Protease (MCP) and an additional regulatory complex, as the protease that degrades Ub-tagged proteins. While Ub conjugation and proteasomal degradation are viewed as a continued process responsible for most of the regulated proteolysis in the cell, the two processes have also independent roles. In parallel and in the years that followed, the hallmark signal that links the substrate to the proteasome was identified as an internal Lys48-based polyUb chain. However, since these initial findings were described, our understanding of both ends of the process (i.e. Ub-conjugation to proteins, and their recognition and degradation), have advanced significantly. This enabled us to start bridging the ends of this continuous process which suffered until lately from limited structural data regarding the 26S proteasomal architecture and the structure and diversity of the Ub chains. These missing pieces are of great importance because the link between ubiquitination and proteasomal processing is subject to numerous regulatory steps and are found to function improperly in several pathologies. Recently, the molecular architecture of the 26S proteasome was resolved in great detail, enabling us to address mechanistic questions regarding the various molecular events that polyubiquitinated (polyUb) substrates undergo during binding and processing by the 26S proteasome. In addition, advancement in analytical and synthetic methods enables us to better understand the structure and diversity of the degradation signal. The review summarizes these recent findings and addresses the extrapolated meanings in light of previous reports. Finally, it addresses some of the still remaining questions to be solved in order to obtain a continuous mechanistic view of the events that a substrate undergoes from its initial ubiquitination to proteasomal degradation. This article is part of a Special Issue entitled: Ubiquitin-Proteasome System. Guest Editors: Thomas Sommer and Dieter H. Wolf.

The diversity and complexity of the ubiquitin proteasomal degradation signal

Ub, similar to many post-translational modifiers, mediates protein–protein interactions. However, in contrast to many other types of modifications it enables a complex and diverse array of biological processes. As for proteasomal degradation, the prototypic 'canonical' signal is a polyUb chain where the Ub moieties are linked to one another via an isopeptide bond between the C-terminal Gly76 of the distal moiety and internal Lys48 in the proximal one. The most proximal Ub moiety is linked to an ε-NH_2 group of an internal lysine (Lys) residue in the target substrate [1–3]. Furthermore, it was suggested that the shortest chain recognized by the proteasome has to contain at least four Ub moieties [4]. Recent investigations show however that the proteasomal proteolytic signal is far more complex and diverse: chains based on different internal linkages, linear — head-to-tail chains, mixed chains made of Ub-like (UbL) proteins and Ub, and surprisingly also a single Ub moiety, can be recognized by the proteasome. Also, chains conjugated to internal residues other than Lys as well as to the N-terminal residue were described, all challenging the current 'canon'. All these different modifications are depicted in Fig. 1.

1. Single and multiple Ub moieties and structural characteristics of the substrate can be parts of the proteolytic signal

Several recent investigations demonstrate that the proteasome can recognize and degrade protein targets that were conjugated by a single or multiple single Ubs. One of the first findings described was that a single ubiquitination on a specific Lys residue on paired box 3 protein (PAX3) – a regulator of muscle differentiation – targets the protein for degradation [5]. The ubiquitination reaction is catalyzed by the TAF1

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ligase [6]. Similarly, the conjugation of a single Ub in response to Wnt signaling results in proteasomal degradation of the cell adhesion membrane receptor Syndecan 4 (SCD4) that is involved in regulating cell migration during embryonic development [7].

Detailed mechanistic studies of monoubiquitination as a degradation signal revealed that Ub fused linearly to a peptide longer than 20 residues can efficiently target itself for degradation with no need for further chain extension [8]. The hypothesis raised was that peptides shorter than 20 residues that are fused to Ub cannot bridge the distance between the bound Ub and the proteolytic chamber in the 20S subcomplex – where the proteolytically active β-subunits are located – and therefore cannot be degraded. Hence, only peptides longer than 20 residues can target the N-terminally fused Ub for degradation. Since there is a minimal length of a peptide the degradation of which can be driven by a single Ub moiety, the question raised was whether there is a maximal length of a protein the degradation of which can be driven by a single Ub moiety, and whether the length of a protein plays a role in the extent of ubiquitination that leads to its degradation. It was shown that a single Ub moiety can support the degradation of extensions of up to ~150 residues [9]. Of note is that most of the extensions used in these studies appeared to be artificial and raised the concern that they are misfolded/denatured, and do not represent naturally occurring proteins. However, a random and preliminary search for cellular targets demonstrated that Cks2, a 79 residues cell cycle regulator, and Hug1, a yeast protein involved in Mec1p-mediated checkpoint pathway that responds to DNA damage or replication arrest, are degraded both in a cell free system and in cells following monoubiquitination [9]. Similarly, properly folded monoubiquitinated α-Synuclein, a 140 residue protein that was generated by a combination of chemical and biological methods, was degraded by a purified proteasome in a manner that was completely dependent on the presence of the conjugated single Ub moiety [9]. Furthermore, it was shown that that α-globin (142 residues) can be degraded following monoubiquitination [10,11].

The finding that Ub (that is a stably folded protein except for a short C-terminal segment) is a long-lived protein, but a tail longer than 20 residues can target it for rapid degradation, strongly suggests that – besides Ub – the proteasomal degradation signal has to contain a second important characteristic, that of an unstructured tail or an initiation domain. This domain must be sufficiently long to cross the 19S subcomplex and to reach the catalytic sites/proteolytic chamber in the β rings, pulling the entire substrate behind it [12,13]. The question whether this domain is part of the natural structure of the protein, a result of misfolding, or generated by ubiquitination and/or 19S binding/processing, is still elusive.

For other proteins, a more extended modification by multiple single Ub moieties is necessary in order to promote their proteasomal degradation. For example, p105, the precursor of the NF-κB transcription factor p50, undergoes multiubiquitinations in the C-terminal domain of the precursor. These modifications result in processing of the precursor, releasing the N-terminal p50 active subunit of the transcription factor [14]. The cell cycle regulator cyclin B1 is also degraded by the proteasome following multiubiquitination catalyzed by the APC/C E3 Ub ligase [15]. Restricting the number of Lys residues that serve as Ub anchors in the case of p105 reduces the efficiency of processing whereas in the case of cyclin B1 it "forces" the generation of oligo- and/or polyUb chain(s).

The above findings suggest a new dynamic concept for the Ub signal. It seems that not all substrates require an equally long polyUb chains for targeting them for proteasomal degradation, and that the proteolytic signal can adapt itself to the substrate. Mechanistically, one can envision that in the cell the ubiquitination and degradation machineries are found in a loosely associated complex. As Ub moieties are added to the substrate and the chain is elongated, the avidity of the conjugate to the proteasome increases. Once the avidity reaches a certain threshold, and a stable binding of the adduct to the proteasome is secured, it is detached from the conjugating machinery, bound stably to the proteasome, and degraded processively and efficiently. With larger protein targets that may require a longer processing time, a longer polyUb chain may be necessary to generate the required avidity. Therefore, a single Ub moiety or a short chain is not sufficient to bind stably a long polypeptide for the proteasome to ascertain its processive digestion. For substrates that are multiubiquitinated, the spatial arrangement of a large enough number of single Ub moieties that bind to multiple points in the proteasomal Ub receptors ascertains the strong binding necessary for processive degradation (see Section 7). In the case of cyclin B1, restriction of the number of Ub anchors that "forces" the formation of oligo-/polyUb chains, can substitute for the multiple single moieties that were distributed among a higher number of anchors along the protein substrate.

2. PolyUb chains

2.1. Homogenous Ub chains based on a single internal link

Ub has 76 residues with seven lysines in positions 6, 11, 27, 29, 33, 48 and 63. As noted, the most common Ub polymer involved in targeting a substrate for degradation was thought to be a homogeneous chain where the Ub moieties are linked to one another via an isopeptide bond between the C-terminal Gly76 of the distal moiety and Lys48 of the previously conjugated one [1]. Mass spectrometry analysis has shown however that, in addition, chains based on Lys29, 11, 27 and 6 (in decreasing abundance) can also target proteins for proteasomal degradation, and their formation is dependent on different stress states [16]. Other studies have shown that homogeneous chains based on Lys11, generated by the APC/Cyclosome Ub ligase, can also target certain proteins for degradation during cell division ([17–19] and reviewed recently in [20]). These chains, like the 'canonical' Lys48-based chains, appear also to bind to the Rpn10/S5a subunit of 19S sub-complex [21], probably via a TEK box motif that is found on both the substrate and the Ub moiety [22]. Other studies have shown that Lys33- [23,24] and Lys63-based chains [25], are also recognized by the proteasome. It should be noted that some of these results were obtained in studies using cell free reconstituted systems. The validity of these systems was questioned recently in a study showing that while purified proteasome can degrade proteins conjugated with Lys63-based chains, this does not occur in cells [26]. The reason being that in cells factors such as ESCRT0 (Endosomal Sorting Complex Required for Transport) and its components, STAM and Hrs bind to the Lys63 chains and inhibit their association with the proteasome.

Thus, it appears that chains assembled via almost all Ub internal lysines can target proteins for degradation by the proteasome. It will be interesting to study the mechanistic and physiological conditions that lead to the assembly of such chains on different protein substrates. Some of the interesting questions are whether a single substrate can be modified by several types of chains?, is the synthesis of these chains catalyzed by a single or multiple ligases and under what conditions?, and why at all single or different substrates require different chains for their degradation?

2.2. Heterogeneous Ub chains based on different internal links

The fact that chains are synthesized enzymatically led naturally in the assumption that they must be homogenous, and all internal linkages are mediated via a single specific Lys residue in the Ub moiety. However, mass spectrometry analyses have shown that this is not the case, and heterogeneous/mixed chains – where the linkages involve different internal lysines – are also recognized by the proteasome. One example is cyclin B1 that, as noted, is targeted by multiubiquitinations [15], but was also shown to be targeted by short Ub chains containing K11, K48, and K63 internal linkages [27]. It is possible that in order to generate a proteasomal recognition signal of sufficient Ub avidity, different proteins use mixtures of chains of different lengths and internal linkages. The generation of such chains may be dependent on the

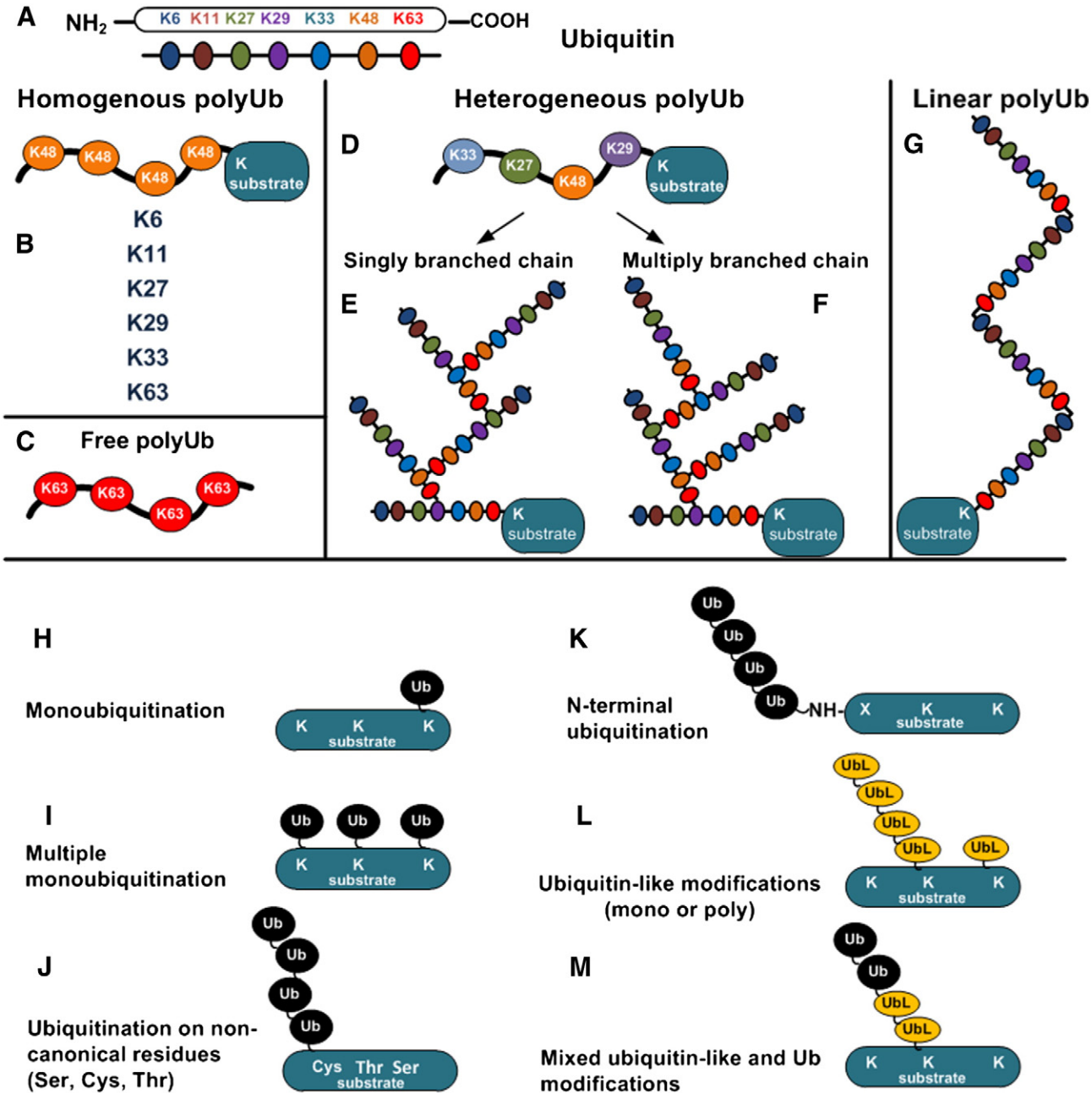

Fig. 1. *The variety of ubiquitin (Ub) and ubiquitin-like proteins (UbL) chains.* A. The seven lysine residues in the Ub molecule that serve as ubiquitination sites for additional distal moieties in the polyUb chain. B. Homogenous chains where each Ub moiety is bound to the previously conjugated one via the same lysine residue (exemplified graphically for Lys48-based chain). C. A free Ub chain (in this case, based on Lys63). D. Heterogeneous Ub chains where each Ub moiety can be linked to a different internal lysine residue in the previously conjugated moiety. The heterogeneous chains can be either singly branched (E.; where each Ub moiety is conjugated by a single Ub) or multiply branched [F.; where each Ub moiety is conjugated by two (or more) moieties, each on a different internal lysine residue]. G. Linear Ub chain where the Ub moieties are conjugated to one another head-to-tail (the C terminus of one Ub moiety is bound in a linear peptide bond to the N terminus of the previous Ub moiety). H. Modification by a single Ub moiety (monoubiquitination). I. Modification by multiple single Ub moieties (multiubiquitination). J. Ub modification of 'non-canonical' internal residues (Ser, Cys, or Thr) in the target substrate. K. Ub modification of the N-terminal residue of the target substrate. L. A single modification or modification by a chain of a UbL. M. Modification by a mixed chain, the proximal part of which is made of a UbL whereas the distal one is made of Ub.

exposure/availability of the Ub anchors within the substrate on one hand, and on the fitting of the chains to the appropriate receptors on the proteasomal subunits on the other hand.

In addition, multiply branched (forked) chains where more than one Ub moiety is conjugated to the previous moiety have also been described [28,29]. However, these chains appear to associate only poorly with the proteasome [29,30] and probably do not play a role in targeting substrates for degradation. Thus, autoubiquitination of the E3 Ub ligase Ring1B generates multiply branched chains on lysines 6, 27, and 48. The role of these chains appears to stimulate the monoubiquitinating ligase activity of Ring1B towards its substrate – the nucleosomal histone H2A, thus serving for self-regulation of the ligase [28].

2.3. Heterologous mixed Ub–UbL chains

Modification of Lys residues of certain proteins by the UbL protein Small Ub MOdifier (SUMO) regulates a variety of processes, including the cellular response to stress and DNA repair, signal transduction, and targeting of proteins to their proper subcellular destinations [31–35]. SUMO is conjugated in most cases as a monomer to an internal Lys

residue in the substrate, yet similar to Ub, it can form also a homogeneous chain. The two modifications – SUMOylation and ubiquitination – have been shown recently to cooperate with one another. Cells incubated with the proteasome inhibitor MG132 were shown to accumulate chains containing SUMO1 [36] and SUMO2 and 3 [37], which suggested that these chains are involved in targeting their conjugated proteins for proteasomal degradation. In a different experiment, purification of Ub conjugates yielded SUMO2 conjugates as well, suggesting that SUMO can be part of a polyUb chain. Kinetic measurements suggested, though did not demonstrate it directly, that SUMOylation is required for priming subsequent polyubiquitination (and degradation), thus linking the two modifications to the same pathway [37]. Heterologous chains composed of both SUMO and Ub can lead to proteasomal degradation. Evidence to this comes from studies on acute promyelocytic leukemia (APL) that is treated with arsenic shown to induce the degradation of the promyelocytic leukemia (PML)-retinoic acid receptor alpha (RARα) fusion protein (reviewed recently in Ref. [38]). Here, the protein first undergoes polySUMOylation that serves as a signal to recruit the RING finger protein 4 (RNF 4) Ub ligase. The subsequent elongation of the SUMO chain by Ub targets PML-RARα to proteasomal degradation [39–41].

Interestingly, RNF4 that synthesizes Lys48-based chains on PML, can synthesize Lys63-based chains during the DNA damage response. In this case, polySUMOylated MDC1 (mediator of DNA damage checkpoint 1) recruits RNF4 which probably ubiquitinates the protein and/or other proteins recruited to the damage point [42]. This ubiquitination is important to secure proper recruitment of the many proteins involved in the damage repair. Involvement of RNF4 in these two different ubiquitination reactions are both dependent on prior SUMOylation (reviewed in Ref. [43]). In another case it was demonstrated that deletion of the specific deSUMOylating protease SENP1 results in suppression of the hypoxic response via targeting the Hypoxia Inducible Factor (HIF)1α for rapid degradation [44]. It was shown that HIF1α is SUMOylated which leads to the recruitment of the Ub ligase protein von Hippel Lindau (pVHL), resulting in its ubiquitination and degradation (reviewed in Ref. [45]). Deletion of the SUMO protease most probably stabilizes the SUMO chain on HIF1α, which renders the elongation of the 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 where specific proline residues in the protein are hydroxylated by proline hydroxylase, a modification that was also reported to recruit pVHL without pre-SUMOylation requirement [46–48].

These examples unravel an additional layer of complexity of the Ub system that broadens the signal beyond the Ub molecule. The cooperation between the two modification systems may contribute to a more accurate control of protein degradation. One possibility is that proteins are first SUMOylated, and the modification serves a non-proteolytic function. They are then ubiquitinated and eliminated in order to turn off the process that the SUMOylation initiated. We still do not know how many protein targets are modified in this manner, and whether this modification occurs on proteins tagged by other UbLs besides SUMO.

2.4. Linear Ub chains

In all the Ub chains described thus far, the moieties are linked to one another via an angular isopeptide bond. Recently, another type of chain was described where the moieties are linked to one another linearly — head-to-tail. Here, the C-terminal Gly76 of the distal moiety is conjugated in a 'classical' linear peptide bond to the N-terminal residue of the more proximal moiety. The linear chain is synthesized by the Ub ligase Linear UB chain Assembly Complex (LUBAC) which is a complex made of three proteins: (i) SHank-Associated Rh domain-interacting ProteIN (SHARPIN); (ii) longer isoform of Heme-Oxidized Iron-regulatory protein 2 Ub Ligase-1 (HOIL-1L); and (iii) HOIL-1L-Interacting Protein (HOIP) [49–51]. While it has been shown that the linear chains are involved in protein–protein interaction in the NF-κB activation pathway (LUBAC polyubiquitinates NEMO which induces IKK activation and subsequent degradation of IκBα; [52]), it was demonstrated that LUBAC can assemble linear Ub chains on GFP to which the first Ub is fused (artificially) to the N-terminal residue. This modification led to proteasomal degradation of the conjugated GFP, suggesting that linear Ub chains can target substrates for degradation [53]. In another example, researchers used the eukaryotic replication clamp PCNA, a natural target of K63-linked polyUb chains, as a model substrate to compare the consequences of modification by different types of Ub chains. While the K63-based chain-tagged PCNA was not degraded by the proteasome, it was shown that a linear tetraUb chain is sufficient to promote its proteasomal degradation mediated by the Cdc48–Npl4–Ufd1 complex without a requirement for additional modification [54]. Though all the experiments where linear Ub chains targeted the conjugated proteins for degradation were carried out using artificial constructs in yeast cells, they nevertheless demonstrate that the proteasome can recognize these chains and degrade the tagged proteins.

3. Ubiquitination of residues other than Lys

3.1. Internal sites of ubiquitination

In most cases described, Ub modifies internal Lys residues of the target protein. Recent studies have shown however that Ub can be conjugated also via an ester bond to the hydroxyl group of Ser or Thr, or via a thiol ester bond to the –SH group of Cys [55–59]. In some of these cases however, the ubiquitination in a non-Lys residue was "forced" by mutating all Lys residues on the target substrate, suggesting that lysines are the preferred targets for Ub, but it can also modify other residues. Thus, a lysineless cytosolic tail of MHC class 1 molecule was ubiquitinated on a Cys residue by MIR1, a Kaposi's sarcoma-associated herpes virus Ub ligase [55]. The finding that Lys residues are preferred targets can stem from the fact that they are more exposed on the surface of the target proteins rather than from preferred chemical reaction conditions. In another similar example, the mouse γ-herpes virus E3 ligase mK3 along with the E2 Ube2j2 conjugated Ub preferentially to Thr and/or Ser residues in the cytosolic tail of the MHC class I heavy chain. The protein was then targeted via the Endoplasmic Reticulum Associated Degradation (ERAD) pathway [60]. Interestingly, ubiquitination on either the Ser or Thr residue was sufficient to destabilize the protein. In another case it has been shown that neurogenin (NGN), a transcription factor that regulates neuronal differentiation, is tagged by Ub on Lys, Cys, Ser, and Thr residues and also on its N-terminal residue. Thus, it appears that Ub chains can serve as proteasomal recognition signals regardless of their anchoring sites [56,59].

3.2. Ubiquitination at the N-terminal residue

In addition to the modification of internal residues, it appears that Ub can modify also the α-NH_2 group of the N-terminal residue of the target protein.

The first substrate that was identified as a target for N-terminal ubiquitination was the myoblast determination protein 1 (MyoD) [61]. Several additional proteins that are similarly ubiquitinated were described later [62–66]. An interesting case is that of SUMO. It was shown that the E2 Ube2W (Ubc16) can conjugate Ub to its own amino terminus, but also to that of SUMO in a manner dependent on the SUMO-targeted Ub ligase (STUBL) RNF4. The N-terminal monoubiquitination of SUMO2 primes it for further polyubiquitination by the Ubc13/UEV1 E2, demonstrating that N-terminal ubiquitination can regulate protein fate [67]. This is the first description of an E2 with N-terminal ubiquitinating activity which highlights the importance of E2s in directing the outcome of E3-mediated ubiquitination [68]. N-terminal ubiquitination raises an interesting hypothesis about another well-known and common

post-translational modification — N-terminal acetylation that may serve to protect proteins from ubiquitination and subsequent destruction [69–71].

The diversity of the amino acids that anchor Ub provides different pKas and other chemical properties. This versatility probably provides the UPS with additional layers of subtle recognition required for fine tuning of the proteolysis of its myriad substrates.

4. Are there consensus sites for ubiquitination?

Ub conjugation is catalyzed by a cascade of enzymatic reactions and intuitively appears to be highly specific. Therefore and in contrast to other post-translational modifications such as phosphorylation, it has been surprising to find that there is no consensus or homologous ubiquitination site(s). It appears that for a few proteins the ubiquitination sites are unique, for some they share vague characteristics, whereas for most others they appear to be promiscuous and can occur on different Lys residues even if inserted in a non-natural position along the polypeptide chain, or even on non-Lys residues. This situation is opposed to SUMOylation that occurs typically on Lys residues within the sequence -ψ–K–X–E- [72].

For one group of proteins, $p19^{INK4d}$, PAX3, IκBα and p53, for example, it appears that specific Lys residues that serve as Ub anchors have been identified, though the neighboring residues do not share any common features among the different proteins. These Lys residues are either single or multiple, and if multiple they appear to be clustered in one region of the target protein. However, the specificity of the lysines in the cluster appears to be loose. This is because other lysines besides those in the cluster can be involved too, and not all lysines in the cluster appear to be required, and their number and specific sites within the cluster can vary. For example, in $p19^{INK4d}$ the major Ub acceptor is Lys62 [73] whereas in PAX3, modification of either Lys437 or 475 is sufficient to target the protein for proteasomal degradation [6]. Interestingly, ubiquitination on one Lys residue inhibits modification of the other. Modification of IκBα occurs on Lys21 and/or 22 [74], whereas for p53, it is a cluster of 6 lysines in the C-terminal domain that was reported to be modified [75].

Tandem mass spectrometry systematic analysis of yeast proteins that enabled semi-quantitative and unbiased mapping of post-translational modification sites revealed that with one exception, all ubiquitinated lysines must reside on the surface of the protein, and even for the single case where the Lys residue is buried, ubiquitination requires prior unfolding of the protein (K370 in glutamate dehydrogenase) [76–79]. There is also a preference for ubiquitination in loops followed by α-helices [80]. Interestingly, both Ub lysines 48 and 63 that are modified most frequently in polyUb chains, reside within loops. Similar findings were also reported in a different study where the researchers demonstrated that preferred ubiquitination sites are surface accessible Lys residues located in ordered secondary region with the following priority: coil > helix > β-sheet > turn. Interestingly, they also showed that these Lys residues are typically surrounded by small positively charged residues [81].

In many cases, the ubiquitination sites are much more difficult to predict. In the case of the p105 precursor of NF-κB, ubiquitination occurs on multiple lysines (~30) that reside in the C-terminal half of the molecule (~500 residue segment). This portion is degraded following (or along with) processing of the molecule. However, the exact location of the modified lysines and even their exact number does not seem to affect the ultimate outcome [14,82]. For most substrates, it appears that the ubiquitination sites are not specific, and for a few it was shown in a clear way. Thus, Cyclin B1, for example, can be ubiquitinated on any single Lys residue within the molecule, even if inserted in non-natural sites [83]. Similar observations were reported also for the ζ chain of the T cell antigen receptor [84].

The low evolutionary conservation and promiscuity of ubiquitination sites probably attests to the vitality and adaptability of the UPS that one of its most important roles is removal of foreign, mutated and otherwise denatured/misfolded proteins. The "chemical" roots of this loose or even lack of specificity reside in the high reactivity of the activated Ub that can be attacked nucleophilically by different groups with various characteristics.

5. Ubiquitin-independent proteasomal degradation

As a rule, the 26S proteasome recognizes only ubiquitinated proteins. The single well-established and well-studied case where the proteasome degrades a protein without prior ubiquitination is that of Ornithine Decarboxylase (ODC). Interestingly however, the recognition of ODC by the 26S proteasome is mediated by its "own Ub/chaperone" – antizyme (Az) – which is a polyamine-induced protein. While the degradation of ODC is clearly Ub-independent, the degradation of its regulator Az, and of a specific antizyme-inhibitor (AzI) – an ODC homologous protein that regulates Az availability – are Ub-dependent (Reviewed in Ref. [85]). Importantly, Ub-tagged substrates or even free polyUb chains compete with AZ-stimulated proteasomal degradation of ODC, strongly suggesting that the two processes — AZ-ODC degradation and degradation of ubiquitinated proteins, are mediated by the same element(s) in the proteasome[86]. Interestingly, it was reported that ODC is degraded also by the 20S proteasome, a process that is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1) [85,87]. NQO1 is a cytosolic enzyme that catalyzes the reduction of different quinones using flavin adenine dinucleotide (FAD) as a cofactor. It was shown to rescue proteins containing intrinsically unstructured domains, such as p53 and p73, from degradation by the 20S proteasome and to be regulated – via mutually inhibitory loops – by the 20S proteasome [88].

Other researchers support the notion that an important role of the 20S proteasome is to degrade damaged proteins/proteins that are intrinsically disordered [89–91], though the discussion whether the 20S proteasome has at all a role(s) in intracellular protein degradation has not been settled yet.

An interesting discussion evolved around the degradation of p21, a cyclin-dependent kinase (CDK) inhibitor, where it was suggested that its regulated, cell cycle-dependent degradation is mediated by the 26S proteasome in a process that requires prior ubiquitination, whereas its unregulated, basal degradation is mediated by the 26S proteasome in a process that does not require modification by Ub [92].

6. Summary and outlook for the ubiquitin signal

As we can see, the Ub signal code is rather complex and its decoding by current proteomic methods is difficult if not impossible. That is because the analysis requires proteolytic destruction of the signaling chain. Nevertheless, even in the absence of analytic tools, it is important to attempt to understand the evolutionary reasons behind this complexity. It appears that the myriad target substrates of the Ub system, the need to set a 'priority' for their funneling into the proteasome, and not less importantly, the numerous roles of non-proteolytic modifications by Ub and UbL proteins, required the evolution of numerous distinct signals recognized by the proteasome, the shuttling proteins, and other downstream effectors. The different structures and lengths of the Ub chains, and the various Ub-anchoring sites can provide the subtle alterations to the strength and/or conformation of the interaction between the proteasome and the substrates, thus regulating degradation rates. It should be noted that the most common chain targeting proteins for degradation is based on Lys48 internal linkages, but even for those, the finding that they contain also other linkages may provide them with different characteristics required for fine tuning of the proteolytic process. Yet, it should be emphasized that the multiplicity of linkages within a predominant Lys48-based chain can also reflect the promiscuity/lack of accuracy of the conjugation machinery rather than any specific biological requirement/characteristic, and all that the proteasome needs "to see" is a "critical mass" of a certain length of Ub moieties

linked via Lys48. Also, aberrations in the Ub system underlie the pathogenesis of numerous diseases — certain malignancies, neurodegenerative diseases, and immune and inflammatory disorders among them. The added level of complexity of the signal is probably mirrored also in these pathologies, and its understanding is necessary for future development of novel therapeutic modalities to affect these aberrations.

7. Proteasomal recognition and processing of the proteolytic signal

7.1. Spatial organization of the 19S regulatory particle subunits

Targeting signals for ATP-dependent protease degradation is a feature that is well conserved in evolution, and the various proteases share numerous characteristics. All protease complexes in this category consist of a compartmentalized cylinder-shaped complex attached to an AAA type ATPase that harbor an 'unfoldase' activity required for substrate processing by the protease (for reviews see [93–97]). However, unlike other ATP-dependent proteases, the 26S proteasome is the only known Ub ATP-dependent protease. Subunit orientation and detailed structure of the different subunits of the 20S catalytic particle was resolved 15 years ago [98]. The 20S particle is barrel-shaped, composed of four stacked heptagonal rings consisting of the two inner β catalytic rings (β 1–7) and two outer α rings (α 1–7) of which β 1, 2, and 5 are active subunits with distinct catalytic preferences. The outer α rings serve as an interface for regulatory particle (RP) binding through a pocket created between two adjacent α subunits. Upon RP engagement, the α subunit N-termini that obstruct entry into the catalytic cavity [98,99] are displaced and the entrance into the chamber's interior is dilated. One of the functions of the regulatory particle is therefore to open the narrow entrance of the latent 20S catalytic particle (CP).

Recent studies have resolved the 19S particle structure at a subatomic level [100–102]. The results have revised our view regarding the RP architecture and require a revision of our model of how polyubiquitinated substrates are recognized and processed by the 19S RP.

The initial view of the 19S suggested that it is composed of a subcomplex proximal to the 20S catalytic particle termed 'base', and a distal sub-complex termed 'lid' [103]. The base is composed of six ATPases — Rpt1-6, Rpn1, Rpn2 and the two Ub receptors Rpn10/S5a and Rpn13. The 'lid' contains the remaining Rpn subunits, of which only Rpn11 (PSMD14) has a known catalytic DUB activity [104,105]. In light of the new structural information, the 'base' and 'lid' sub-complexes should be viewed as assembly module intermediates, consistent with our current view of proteasomal assembly mechanisms and kinetics [106].

The various structural data obtained from different sources [100–102] combined with chemical cross-linking data to validate and map proximal interactions [107,108], suggest the following scenario: The hexameric Rpt ATPases are layered on top of the heptameric 20S α subunit outer ring with a defined tilt in respect to the coaxial plane of the 20S catalytic particle. The ATPases are organized as a trimer of dimers in their assembly order [109], and are positioned towards the 20S catalytic particle in a 6-fold rotational symmetry. For each RPT, the ATPase domain is facing the α ring, whereas a second oligosaccharide binding (OB) domain faces upwards. All the six OB domains are thus generating an additional ring (see Fig. 2A). The AAA sub-domains appear to be arranged in a spiral staircase with Rpt3 and Rpt2 at the highest and lowest positions in the ring, respectively, bridged by Rpt6 [100,102]. The three C-termini of Rpts 2, 3, and 5 are found docked to the 20S α pockets [102]. This is consistent with the findings that Rpt2 and Rpt5 were able to activate the 20S with their C-termini [110,111], and that Rpt3 and Rpt5 C-termini are essential for proteasomal assembly [112]. In light of this arrangement, one can envision a substrate moving through the 'staircase' generated by the ATPases encountering the different conformational stages of the various ATPases in their various nucleotide-bound states. The final outcome of this transition is unfolding of the substrate polypeptide chain allowing it to enter the 20S catalytic chamber. Structural information of the Rpt conformation during the various nucleotide binding states will progress our understanding in respect to substrate unfolding and threading into the 20S catalytic particle, and is therefore one of the future challenges waiting ahead of us.

The two homologous and largest components of the regulatory particle are Rpn1 and Rpn2 that are required for docking of various proteasomal interacting proteins and Ub receptors [113–115]. Rpn2 localization was assigned to the far ends of the regulatory particle (in respect to the 20S catalytic particle), whereas Rpn1 localization was assigned to the sides of the regulatory particle [100]. In line with assembly data [109,116], Rpn1 was observed to interact with Rpt1 and Rpt2, whereas Rpn2 with Rpt3 and Rpt6 [100,101]. These surface-exposed locations of Rpn1 and Rpn2 would explain their ability to serve as platforms for binding various proteasome adaptors, shuttling factors and Ub receptors [113–115]. The structural data on the hexameric Rpts and on Rpn1 and Rpn2 were obtained from either purified [100,101] or in vitro assembled 26S particles [102]. These data differ from those obtained using in vitro assembled hexameric Rpts–Rpn1–Rpn2 complexes. Here, the Rpn1 and Rpn2 were reported to form an inner stacked channel embedded within the hexameric Rpt ring, where Rpn2 associates with the 20S catalytic particle [117]. This inner channel was suggested to enable substrate entry into the catalytic particle [117]. These discrepancies highlight the importance of structural data obtained from intact complexes and change our view of looking at how substrates are processed by the 26S proteasome (see below).

The remaining Rpn subunits – Rpn3, Rpn5, Rpn6, Rpn7, Rpn9 and Rpn12 – all contain a proteasome–cyclosome initiation factor (PCI) domain. They are found in a horseshoe scaffold structure with solenoid projecting away from the central horseshoe hub (Fig. 2B; [100,101,108]). From the PCI structure it was clear that there is a direct binding between Rpn5 and Rpn6 and the 20S α1 and α2 subunits. This surprising finding was confirmed by cross-linking experiments [102]. The PCI cluster extends all along the 19S RP, all the way from the catalytic particle interface to the distal end of the RP, and adjacent to the Rpt hexamer in the opposing side of the assigned Rpn1 [101]. This finding places for the first time a 'lid' component (Rpn5 and Rpn6) with the catalytic particle.

The remaining regulatory subunit assignments (with exception of the proteasomal Ub receptors Rpn10/S5a and Rpn13) are the Mpr1, Pad1 N-terminal (MPN) domain-containing metalloproteases Rpn8 and Rpn11. Based on the structural data obtained [100–102,108], the catalytic inactive Rpn8 was suggested to form a dimer with the Rpn11, a finding that would fit well with the observed dimerization of soluble Rpn8 [118] and the extensive cross-linking observed between the two subunits [107]. A central density in the regulatory particle flanked by the PCI domain subunits on one side and Rpn2 on the opposing side was assigned to the two MPN domain subunits, thereby positioning Rpn11 in proximity to Rpt3, the highest ATPase in the RPT spiral staircase. This position fits well with several cross-linking findings of Rpn11 with Rpt3 [108,119]. Rpn8 and Rpn11 probably undergo dynamic movements during the 'lid' integration into the RP [102], possibly explaining the activity of Rpn11 observed only in context of the assembled 26S proteasome [104,105].

7.2. Polyubiquitin chain recognition by the 26S proteasome

The two well-characterized regulatory subunits that bind Ub are Rpn10/S5a and Rpn13 [120,121]. The binding of Ub to Rpn10/S5a is mediated by two Ub-interacting motifs (UIMs), each forming an amphipathic helix to create a low affinity hydrophobic interaction with the Ile44 centered hydrophobic patch of Ub [122,123]. Rpn13 mediates proteasomal interaction with Ub through a Pleckstrin-like receptor for Ub (PRU) domain that enables binding to the same hydrophobic surface of Ub as Rpn10/S5a [114]. In addition to Ub, Ub-like (UBL) domain also mediates polyUb substrate delivery to the proteasome via shuttle factors that consist of UBL–UBA domains. These shuttles bind to

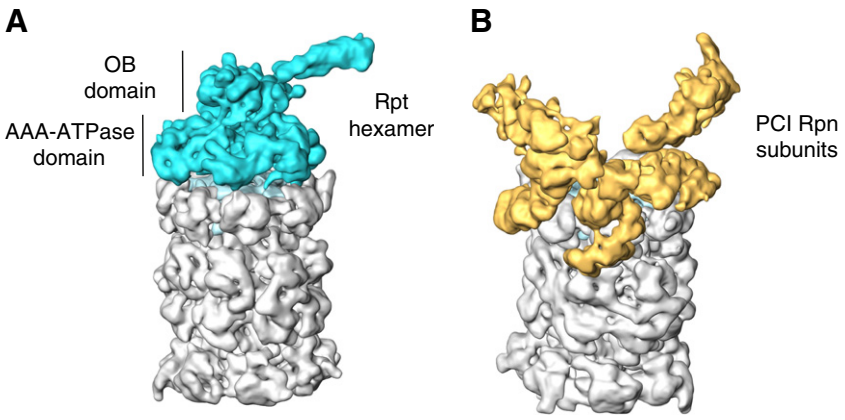

Fig. 2. A. Subunit architecture of the hexameric Rpts (blue) assembled on top of the 20S particle (gray). Depicted are the AAA–ATPase and OB domains of the hexameric Rpt ring. B. Subunit architecture of the PCI-winged helix domain Rpn subunits (gold) resembles a horseshoe-shaped arrangement with close proximity to the 20S particle. Structures were generated based on published data [102].

proteasomes via their UBL domains and the polyUb substrates via their UBA domain [124]. However, UBL-proteasomal interaction is not restricted to the Rpn10/S5a and Rpn13 Ub receptors, [121,125] and is also mediated by Rpn1 [113,126]. Yet, exact location and high resolution structure of the binding sites of the shuttle factors on the proteasome are still missing. In addition to the above mentioned Ub/UBL binding factors, cross-linking experiments identified Rpt5 in close proximity to the proximal Ub moiety in a polyUb chain [127].

After assigning the densities of the various regulatory particle subunits, a small remaining density in the Rpn12–Rpn9 interface was assigned to Rpn10/S5a [100–102,108] based on the crystal structure of the Rpn10 VWA domain [128]. This assignment is consistent with the Rpn10–Rpn12 observed interaction [128], and positions Rpn10 in close proximity to Rpn8 [100], explaining the cross-linking of the two subunits [107]. Rpn13 was observed to interact only with Rpn2 [102,108], consistent again with previous reports showing Rpn13 proteasomal docking via the Rpn2 subunit [129–131]. Similar to Rpn11, proteasomal interaction of Rpn13 is necessary in order to enable its Ub binding ability. Its binding to Rpn2 weakens an intra-molecular association between the UCH37 and the Ub-binding domain within Rpn13 [129], thereby ensuring that only proteasomal incorporated Rpn13 will be able to bind Ub. In this respect it is worthy to note that the additional Ub receptor Rpn10/S5a can bind Ub also as an isolated subunit [132]. These observations are consistent with the suggested role for the Rpn10/S5a receptor serving also as a proteasome-free shuttling factor carrying polyubiquitinated substrates to the proteasome [132]. Furthermore, it was reported that Rpn10/S5a undergoes multiubiquitination [133], which may also regulate non-proteasomal association between the receptor and polyubiquitinated substrates. On the other hand, Rpn13 is found only associated with the proteasome [130] and therefore may not require any such regulation.

The subunit assignments on the 19S regulatory particle place the two Ub receptors at a distance that varies between 70 and 100 Å [102,134], a distance that sets a minimal length for the polyubiquitin chain that is required in order to ascertain an avid binding between the polyubiquitinated substrate and both Ub receptors (Fig. 3). In addition, Rpn11 is positioned between the two Ub receptors, which is an ideal location to ensure timely (rather than premature) deubiquitination, thus committing the polyUb substrate for degradation [135].

NMR studies from in vitro assembled Rpn10/S5a, Rpn13 and Lys48-linked diUb suggested a preferential binding of Rpn13 to the proximal Ub moiety [136]. Based on these and additional observations, it was suggested that Rpn13 and Rpn10/S5a can bind to a single K48 triUb chain [136]. As a triUb chain cannot span the distance between Rpn10/S5a and Rpn13, previous conclusions [136] obtained from structures of individual subunits appear to be limited, and the in vivo scenario in the intact 26S complex is probably different. Considering the open conformation of Lys48-based polyUb chains [137], the minimal length required for a polyUb chain to bind simultaneously to one Ub receptor (Rpn13 or Rpn10/S5a) while being deubiquitinated by Rpn11, would be four Ub [102]. This is consistent with previous reports regarding the minimal lengths of four K48-based polyUb chains required in order to support proteasomal degradation [4]. How these findings relate to observations on the degradation of shorter Ub chains or even monoubiquitinated proteins, or chains based on different internal linkages (see Section 2) remains to be elucidated.

The Ubp6/Usp14 sub-stoichiometric proteasomal DUB was assigned to the 26S particle by various maps using WT and *ubp6Δ* strains [102]. Its position was assigned to a variable location between the peripheral Rpn1 and Rpn2 subunits in line with known data regarding Ubp6–Rpn1 interaction [113,126,138] and also with Rpn1-independent interactions [126]. The shuttling factors Rad23, Dsk10, and Ddi1 bind the proteasome via Rpn1 [113,126,138]. This location places Ubp6 in close proximity to Rpn1, and therefore also to polyubiquitinated substrates that are brought by these shuttling factors. This proximity may enable Ubp6 to process the polyUb chains on such substrates. The polyUb substrates delivered to the proteasome by this mechanism may therefore require an extended polyUb signal in order to bridge the longer distance to Rpn11 and the hexameric Rpt ATPases.

The only proteasomal catalytic subunit absent from the yeast *Saccharomyces cerevisiae* is UCH37 (UCHL5). While no data regarding UCH37 proteasomal positioning is available from the recently published data, proteasomal binding of UCH37 was found to be Rpn13-dependent [130,131], thus placing UCH37 in close proximity to substrates that are bound to the proteasome via this Ub receptor. However, as the Rpn13 Ub receptor is conserved between mammals and *S. cerevisiae*, it is unlikely that the specificity of UCH37 is aimed towards Rpn13-dependent polyubiquitinated substrates. This is also inferred from the fact that UCH37 deficiency results in prenatal lethality in mice, while Rpn13-deficient mice survive to adulthood, suggesting Rpn13-independent role(s) for UCH37 [139].

8. Pathologies associated with aberrations in recognition/processing of the proteasomal degradation signal

Most of the pathologies described that are related to the Ub signal stem from aberrations in the deubiquitinating enzymes. In most cases, the mechanistic linkage between the phenotypic defect and the underlying biochemical defect has not been identified. Furthermore, even the culprit ubiquitinated substrates involved in the pathogenesis of most of

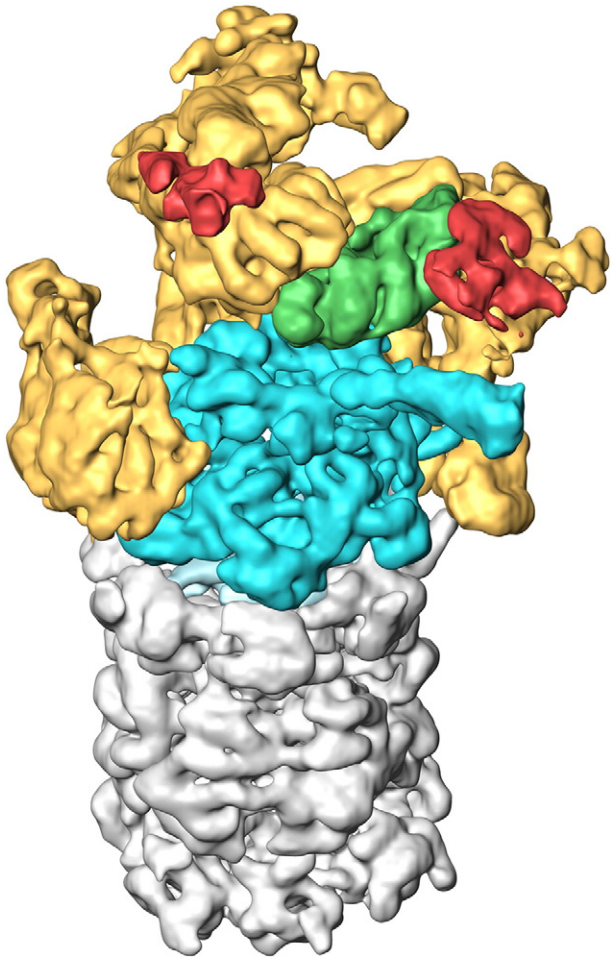

Fig. 3. Intact 26S subunit architecture is presented, where the 20S particle is positioned underneath the Rpt hexameric ring (blue). The Rpn11 DUB (green) is located on top of the Rpt ring, positioned between the two Ub receptors Rpn10/S5a and Rpn13 (red). The 80 Å distance between the two Ub receptors is predicted to enable binding of a tetraUb K48-linked polyUb chain. Structures were generated based on published data [102].

these syndromes has remained elusive. It is not always clear that the abnormal processing involves a proteolytic substrate or a protein, the ubiquitination of which serves a non-proteolytic function. We shall review several pathologies involved in processing of the Ub signal, some have been described only in model organisms.

The Ub C-terminal Hydrolase UCH-L1 (PGP 9.5) is an abundant brain protein (comprising > 1% of the total protein of the tissue), that is almost absent from all other organs [140]. Members of a German family with Parkinson's disease were identified as having missense mutation in the UCH-L1 gene [141]. The mutation – Ile93Met – results in a partial loss of the catalytic activity of this thiol protease, which could lead to aberrations in the UPS, resulting in aggregation of proteins. Interestingly, the enzyme was found in inclusion bodies immunostained for ubiquitinated proteins which are characteristic to neurodegenerative diseases [142]. A recent study implicates Aβ oligomers in impairing BDNF retrograde trafficking by down-regulating UCH-L1, relating mechanistically a reduced activity of the enzyme to a defect observed also in Alzheimer's Disease [143]. A simple hypothesis linking the defective enzyme and the brain pathology is that an inactive or haploinsufficient enzyme will not release Ub from small molecules to which it is conjugated via their amino, thiol, or hydroxyl groups, leading to a deficiency in free Ub. Additionally, binding of the Ub moiety of these short adducts to the proteasome can inhibit the protease, leading to accumulation of other ubiquitinated proteins that are normally degraded by the proteasome which can result in general impairment of the UPS. In mice, an inactivating mutation (intragenic deletion) of the homologous gene leads to gracile axonal dystrophy (GAD), an autosomal recessive disorder that is presented as sensory ataxia at an early stage, followed by motor ataxia later on. Pathologically, the syndrome is characterized by a 'dying-back' type of axon degeneration and formation of spheroid bodies in nerve terminals [144]. However, this simple model, where the activity of an enzyme is related to a clear defect, was complicated by a report that a polymorphic variation of serine to tyrosine at codon 18 in the UCH-L1 gene is associated with a reduced risk of sporadic Parkinson's disease in certain populations [145]. As a matter of fact, it was shown that the S18Y UCH-L1 polymorph, but not the WT protein, protects dopaminergic nigral cells against MPTP toxicity, suggesting that it has an antioxidant and neuroprotective effect [146]. Further complication came when the initial population genetics data could not be confirmed in later studies on other populations [147]. Currently, following numerous genetic, biochemical and neuropathological studies, the association between UCH-L1, the dopaminergic system, and the pathogenesis of Parkinson's disease, are still obscure.

Another defect that was described in the USP14 DUB (the mammalian homolog of the yeast Ubp6; see below) is associated with synaptic defects and aberration in neurotransmitter release [148]. The mice develop severe tremors by 2–3 weeks of age, followed paralysis of the hind limbs that leads to their death at 6–8 weeks of age. Detailed studies revealed that the neuromuscular junctions are unable to mobilize a sufficient number of vesicles during times of intense activity to keep pace with physiological rates of transmitter release. Importantly, unlike UCH-L1 which is a soluble DUB, USP14 is a proteasome-associated DUB, which probably relates the defect in a more direct manner to the inability of the proteasome to degrade a target proteins, the accumulation of which may underlie the pathogenesis.

Another interesting finding regarding Ubp6 is related to the control of euploidy. Aneuploidy is associated with death and severe developmental abnormalities in all organisms studied. It is the leading cause of miscarriages and mental retardation in humans, is found in the vast majority of human malignancies and may even support tumorigenesis. The mechanisms that enable malignant cells to survive the adverse effects of aneuploidy have yet to be unraveled. To understand the underlying mechanisms that allow certain cells to survive aneuploidy, Torres and colleagues [149] identified aneuploid yeast strains with improved proliferative abilities. Their analysis revealed several mutations, among them an inactivating mutation in the DUB Ubp6. The lack of the enzyme was found to improve growth rate in aneuploid yeast strains, probably by attenuating changes in cellular protein composition, though the precise mechanism(s) have not been elucidated. It is clear however that the effect of Ubp6 inactivation is not mediated via a deficiency in free Ub.

An additional defect relates to RPN11, the proteasomal integral DUB. It confers multidrug resistance to several chemotherapeutic agents such as vinblastine, cisplatin, and doxorubicin [150]. It is not clear how overexpression of a single stoichiometric subunit can affect the activity of the entire proteasome. One hypothesis is that it can act also independently, though the isolated subunit is not known to have catalytic activity [104,105].

Other defects are not related to signal processing and involve subunits of the proteasome and the signal itself. Ectopic expression of RPN6 was found to confer protection from proteotoxic stress and to increase lifespan in *Canaerabditis elegans* [151] which may suggest that RPN6 is a candidate to correct age-related decreased proteasomal activity that may be involved in protein homeostasis disorders.

An interesting case is that of UBB+1, a Ub molecule with an extended C-terminal tail of 19 residues. It is generated as a result of a frameshift which is caused by dinucleotide deletions in GAGAG motifs in the respective mRNAs of the coded proteins, and is thought to be the result of 'unfaithful' transcription of the normal DNA by a mechanism called "molecular misreading". This pathological Ub was found in the brains of early onset Alzheimer's Disease patients and also in brains of Down

Syndrome patients [152]. Overexpression of this mutant Ub causes neuronal cell death [153]. The possible mechanism of the deleterious effects of UBB+1 was elucidated only recently. The protein cannot ubiquitinate other targets but can be ubiquitinated. Being Ub by itself and having a polyUb chain attached to it, results in its strong binding to the proteasome. Yet, having a tail of 19 residues does not allow it to penetrate into the proteolytic chamber of the 20S proteasome (see Section 1). The strong binding and its inability to be degraded make it a strong proteasomal inhibitor [8]. Importantly, adding a single residue to the 19 residue tail, alleviates the inhibition [8].

Last but not least, Ig secreting B lymphocytes are sensitive to proteasomal inhibition. The reason being abnormal/misfolded Ig molecules that would have otherwise been degraded by the proteasome via the ERAD pathway, are accumulated in the ER following proteasomal inhibition and induce apoptosis via the Unfolded Protein Response (UPR) [154]. This observation served as the base for the development of efficient proteasome inhibitors (Velcade®/Bortezomib; Kyprolis®/Carfizomib) that are being used successfully for the treatment of Multiple Myeloma [155].

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