Chapter 1

General Introduction

Ubiquitin Proteasome System

Through targeted degradation of both cytoplasmic and nuclear short-lived proteins the 26S proteasome complex regulates the concentration of the large majority of the proteins in the cell (Rock *et al.*, 1994; Collins and Goldberg, 2017). Furthermore, the proteasome functions as a quality control system by degrading potentially harmful damaged or misfolded proteins (Goldberg, 2003; Kostova and Wolf, 2003). Proteins are targeted for proteasome-mediated degradation by degrons or polyubiquitin chains. Ubiquitin is an 8.5kDa modular protein which can be attached with its C-terminus to the epsilon-amino group of lysine residues on target proteins, or more rarely, to the protein N-terminus, or to the side chain of a cysteine residue in the target protein (Komander and Rape, 2012). Conjugation of ubiquitin to target proteins is regulated by the sequential action three enzymes: ubiquitin is first activated by an E1 ubiquitin-activating enzyme through thioester bond formation between the E1's active cysteine residue and ubiquitin's C-terminal carboxyl group in an ATP-dependent manner. The ubiquitin protein is then transferred to a cysteine residue of an E2 ubiquitin-conjugating enzyme. An E3 ubiquitin ligase catalyzes the transfer of ubiquitin from the E2 enzyme to specific target proteins. Ubiquitin conjugates on proteins can in turn also become targets for ubiquitination, which can result in the formation of polyubiquitin chains on target proteins (Figure 1).

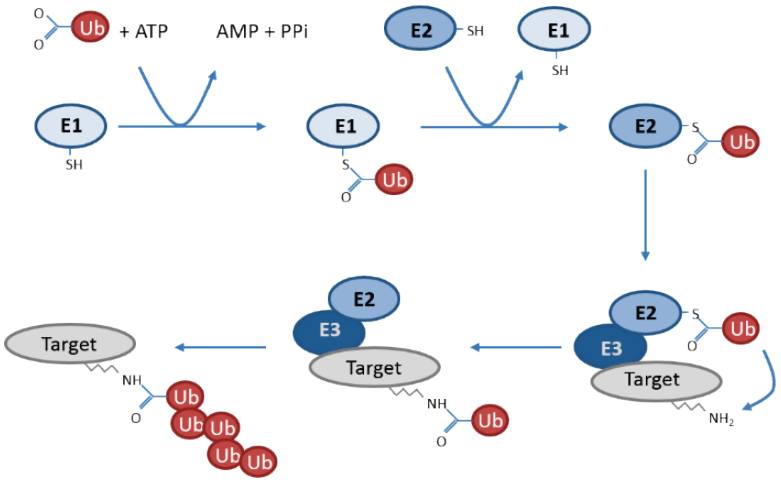

Figure 1. Schematic representation of the ubiquitination pathway. Ubiquitin is activated by an E1 ubiquitin activating enzyme in an ATP dependent manner. Then ubiquitin is transferred from the E1 enzyme to an E2 ubiquitin conjugating enzyme. The ubiquitin-conjugated E2 enzyme interacts with an E3 ubiquitin ligase which can bind specific target proteins. Ubiquitin can be transferred either to substrate proteins or to other ubiquitin molecules to form a polyubiquitin chain.

The concerted action of ubiquitin targeting via E1, E2 and E3 enzymes and proteasome-dependent degradation is also referred to as the ubiquitin-proteasome system, or UPS. As such the UPS plays a central role in the cell by regulating processes such as cell cycle progression, DNA repair, protein quality control, transcription, signal transduction, antigen processing and the maintenance of protein and cellular homeostasis (Kloetzel, 2004; Geng, Wenzel and Tansey, 2012; Tu *et al.*, 2012). Malfunctioning of the Ubiquitin Proteasome System has been implicated in a wide variety of diseases such as cancer and neurodegenerative disorders (Schwartz and Ciechanover, 2009).

Proteasome structure

The 26S proteasome is functionally and structurally divided into two parts *i.e.,* the 20S catalytic core particle (CP) and the 19S regulatory particles (RP) (Hough, Pratt and Rechsteiner, 1986; Chu-Ping *et al.*, 1994; Walz *et al.*, 1998) (Figure 2). The 20S proteolytic core particles can also function independently and have been identified in eukaryotes, archaea, and in bacteria of the Actinomycetes phylum (Gille *et al.*, 2003), whereas the 19S RPs were found only in eukaryotes and archaea. The 19S RP was already identified in the last eukaryotic common ancestor, LECA, and evolved independently through duplications and loss events in specific lineages (Fort *et al.*, 2015). Table 1 gives a collection of different names and abbreviations of 26S proteasome subunits in different eukaryotes. The 20S CP has the shape of a barrel made up of two identical outer α-rings and two identical inner β-rings. Both types of rings consist of 7 subunits (Prosalpha1 – Prosalpha7 and Prosbeta1 – Prosbeta7) (Groll *et al.*, 1997; Unno *et al.*, 2002). The alpha rings are responsible for the regulation of substrate entrance to the inner proteolytic chamber by forming a gate at the center of the ring with their subunits' N-termini. Prosbeta1, Prosbeta2 and Prosbeta5 exhibit respectively caspase-like (cleaving after acidic amino acids), trypsin-like (cleaving after basic amino acids) and chymotrypsin-like (cleaving after neutral amino acids) proteolytic activity which is buried within the barrel (Marques *et al.*, 2009). Typical products of proteasomal degradation are oligopeptides with lengths between 3 and 30 amino acids with an average length of 8 residues (Kisselev, Akopian and Goldberg, 1998). Mutations in genes coding for catalytic subunits of the proteasome, such as prosbeta5i of the immunoproteasome may trigger abnormal inflammation which damages tissues and organs, as observed in several related but different syndromes: CANDLE syndrome, Nakajo-Nishimura syndrome and JMP syndrome. Usually, the 20S proteasome is found in the cell in its inactive state. The 20S core can be activated by docking of regulators (19S, 11S, PA200), unfolded proteins, or proteasomal substrates to the α-ring (Liu *et al.*, 2003; Stadtmueller and Hill, 2011). Damaged proteins can activate the proteasome by binding directly to the α-subunits with their exposed hydrophobic patches, while native and correctly folded proteins have to be targeted (via polyubiquitin) for proteasomal degradation. The most important regulator for the recognition of ubiquitin-conjugated proteins is the 19S regulatory particle (19S RP).

Table 1. Proteasome subunit names across species

Proteasome subunits	*D. melanogaster*	*S. cerevisiae*	Mammals
20S CP			
Prosalpha1	Prosalpha1/CG30382	Scl1/Prc2/Prs2/C7	PSMA6/Pros27/Iota
Prosalpha2	Prosalpha2/Pros25/PROS25/CG5266	Pre8/Prs4/Y7	PSMA2/C3/Lmpc3
Prosalpha3	Prosalpha3/Pros29/PROS-29/CG9327	Pre9/Prs5/Y13	PSMA4/C9
Prosalpha4	Prosalpha4/'Pros28.1/PROS28.1/CG3422	Pre6	PSMA7/C7/XAPC7
Prosalpha5	Prosalpha5/ProsMA5/CG10938	Pup2/Doa5	PSMA5/Zeta
Prosalpha6	Prosalpha6/Pros35/PROS35/CG4904	Pre5	PSMA1/C2/Pros30
Prosalpha7	Prosalpha7/CG1519	Pre10/Prc1/Prs1/C1	PSMA3/C8
Prosbeta1	Prosbeta1/I(2)05070/CG8392	Pre3	PSMB6/Y/delta/LMPY/LMP19
Prosbeta2	Prosbeta2	Pup1	PSMB7/Z/Mmc14
Prosbeta3	Prosbeta3/CG11980	Pup3	PSMB3/C10
Prosbeta4	CG17331	Pre1/C11	PSMB2/C7
Prosbeta5	Prosbeta5/Pros-beta-5/CG12323	Pre2/Doa3/Prg1	PSMB5/X/MB1
Prosbeta6	Prosbeta6/Pros26/PROS26/I(3)37Ai/CG4097	Pre7/Prs3/Pts1/C5	PSMB1/C5
Prosbeta7	Prosbeta7/Prosb4/Prosbeta4/CG12000	Pre4	PSMB4/N3/beta/LMP3
19S RP ATPases			
Rpt1/S7	Rpt1/p48B/CG1341	Rpt1/Cim5/Yta3	PSMC2/Mss1
Rpt2/S4	Rpt2/Pros26.4/p56/p26s4/CG5289	Rpt2/Yhs4/Yta5	PSMC1
Rpt3/S6b	Rpt3/p48A/CG16916	Rpt3/Tnt1/Yta2/Ynt1	PSMC4/Mip224/Tbp7
Rpt4/S10b	Rpt4/p42D	Rpt4/Crl13/Pcs1/Sug2	PSMC6/Sug2/P42
Rpt5/S6a	Tbp-1/p50	Rpt5/Yta1	PSMC3/Tbp1
Rpt6/S8	Rpt6/Pros45/p42C/DUG/Ug/CG1489	Rpt6/Cim3/Crl3/Sug/TbpY /Tby1	PSMC5/p45/Sug1/Trip1
19S RP non-ATPases			
Rpn1/S2	Rpn1/p97	Rpn1/Hrd2/Nas1/Rpd1	PSMD2/p97/Trap2
Rpn2/S1	Rpn2/p110/CG11888	Rpn2/Sen3	PSMD1/p112
Rpn3/S3	Rpn3/p58/Dox-A2/CG42641	Rpn3/Sun2	PSMD3/p58
Rpn4/P27	CG9588/p27	Nas2/Rpn4/Son1/Ufd5	PSMD9/p27/Rpn4
Rpn5/p55	Rpn5/p55/CG1100	Rpn5/Nas5	PSMD12/p55
Rpn6/S9	Rpn6/p42B/CG10149	Rpn6/Nas4	PSMD11/p44.5
Rpn7/S10a/S10	Rpn7/p42A/CG5378	Rpn7	PSMD6/p42a
Rpn8/S12	Mov34/p39B/CG3416	Rpn8/Nas3	PSMD7/p40/Mov34

Proteasome subunits	*D. melanogaster*	*S. cerevisiae*	Mammals
19S RP non-ATPases			
Rpn9/S11	Rpn9/p39A	Rpn9/Nas7	PSMD13/p40.5
Rpn10/S5a	Rpn10/Pros54/p54/PROS-54/CG7619	Rpn10/Sun1/Mcb1	PSMD4/S5a/Mcb1
S5b	CG12096	-	PSMD5/KIAA0072
Rpn11/S13	Rpn11/p37B/yip5/CG18174	Rpn11/Mpr1	PSMD14/Pad1/Poh1
Rpn12/S14	Rpn12/p30	Rpn12/Nin1	PSMD8/p31
Rpn13	Rpn13/p42E/CG13349	Rpn13/Daq1	ADRM1/-
p28	-	Nas6	PSMD10/p28/Gankyrin
P27	CG9588	Nas2	PSMD9/p27
Rpn15/Sem1	-	Rpn15/Sem1/Dsh1/ DSS1/HOD1	SHFM1/DSS1/SHFDG1
UCHL5	UCHL5/Uch-L3/p37A/CG31639/CG3431	-	UCHL5/UCH37
Sub-stoichiometric proteasome protein			
USP14	USP14/CG5384	UBP6	USP14

The subunits of the 19S RP recognize, deubiquitinate and unfold ubiquitinated proteasome substrate and subsequently translocate it into the 20S CP. Elucidation of the structure of the 19S RP was challenging, partly due to the different conformational states of the 19S RP as well as due to the number of substoichiometric binding partners. Over the last years, advances in the determination of the structure of the 19S RP at high resolution were made with the aid of cryo-electronmicroscopy, crystallography, biochemistry and computer modeling (Bohn *et al.*, 2010; Beck *et al.*, 2012; Lander *et al.*, 2012; Lasker *et al.*, 2012; Sledz *et al.*, 2013). The 19S RP, also called the 19S cap, is composed of two distinct subcomplexes, the base and the lid (Glickman *et al.*, 1998). The base contains four non-ATPase subunits (RPN1, RPN2, RPN10 and RPN13) and six AAA-ATPase subunits *i.e.*, RPT1-RPT6. The Rpt proteins form a heterohexameric ring and dock with the C-termini of their AAA+ domains into the α-ring of the 20S CP. Their N-termini contain an OB-fold domain which assemble into a distinct N-ring above the AAA+ domain ring. The ATPase ring subunits form a channel which runs through approximately two-thirds of the 19S RP, basically extending the channel of the 20S RP. The ATPase ring engages an unstructured initiation region of the substrate and triggers unfolding, pore opening and active translocation of the substrate to the proteolytic sites of the 20S CP. Two large subunits, which serve as interaction platforms, bind with the ATPase channel: RPN1 binds to the outside of the channel and provides binding sites for non-stoichiometric proteasome interactors such as UBL-UBA proteins, but also for deubiquitinating enzyme USP14/Ubp6. RPN2 binds to the top of the ATPase ring and provides a binding site for ubiquitin receptor RPN13. The other intrinsic ubiquitin receptor of the proteasome, RPN10, interacts with RPN1 although this association is stabilized by RPN2. The proteasome lid contains eight subunits (RPN3, RPN5-RPN9, RPN11

and RPN12). RPN8 and RPN11 form dimers near the entrance of the ATPase ring. Both belong to the JAMM or MPN domain metallo-protease family of deubiquitinating (DUB) enzymes, however only RPN11 is an active DUB. RPN11 can cleave entire polyubiquitin chains off the substrates concomitant with translocation into the proteolytic core (Yao and Robert E. Cohen, 2002; M. J. Lee *et al.*, 2011). The other subunits of the lid might function as scaffolding proteins that bind to the outside of the cap, running from the entrance of the ATPase ring where they interact with RPN2 and ubiquitin receptor RPN10 all the way down via the ATPase ring to the α-ring of the 20S CP (Figure 2). They are suggested to stabilize the proteasome particle and to facilitate conformational changes upon substrate binding.

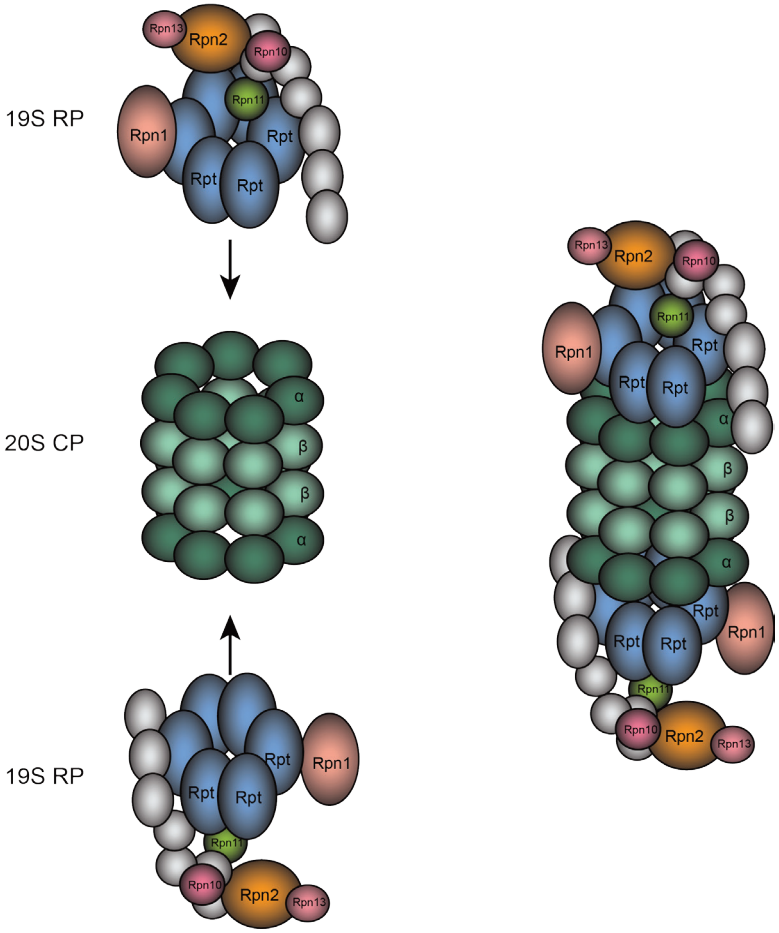

Figure 2. Illustration of the structure of the 26S proteasome consisting of two 19S regulatory particles and one 20S core particle.

Conformational changes of the 26S proteasome are mostly driven by ATP binding and hydrolysis. All RPT subunits of the proteasome are able to bind and hydrolyze ATP (Beckwith *et al.*, 2013; Peth, Nathan and Goldberg, 2013), which may theoretically give rise to a large number of different conformational states. To date three major 26S conformational states are identified: s1 (substrate-free state), s2 (intermediate state) and s3 (substrate engaged state). The s1 and s2 states are observed in the presence of both ATP and the slowly hydrolysable ATP analog ATP-γS. As the relative abundance of s1:s2 was observed to be ~4:1 for both conditions, it might be reasonable to assume that s1, or the substrate-free state, corresponds to the ground state because it is more abundant (Unverdorben *et al.*, 2014). Also in intact neurons it was observed that ~80% of the proteasomes were present in the substrate-accepting ground state (Asano *et al.*, 2015). In the substrate-free state, the AAA+ domains of the RPTs adopt a steep spiral-staircase arrangement that restricts access to the proteolytic core, but may facilitate substrate engagement (Beckwith *et al.*, 2013). Substrate engagement in turn induces conformational changes. The s3 state is only observed with the use of ATP-γS, suggesting that this is a high-energy pre-hydrolysis state. The RPTs adopt a more planar spiral-staircase arrangement in this state and both the N-ring and AAA+ ring of these proteins are coaxially aligned with the 20S pore, thereby creating a continuous central channel for substrate translocation into the proteolytic core (Matyskiela, Lander and Martin, 2013; Sledz *et al.*, 2013). Another characteristic of the s3 state is the placement of RPN11 above the entrance of the 20S pore, which is an ideal location for the removal of ubiquitin chains during polypeptide translocation. The s2 state is considered an intermediate state as the ATPase module remains in essentially the same conformation as in the s1 state, whereas the lid together with RPN2 is in a position and conformation similar to s3 (Unverdorben *et al.*, 2014).

Proteasome targeting

Proteins are targeted for proteolysis by the 26S proteasome via the attachment of ubiquitin residues. The amino acid sequence of ubiquitin is shown in Figure 3A. Proteins can undergo conjugation with a single ubiquitin moiety on one or multiple sites, which is referred to as respectively monoubiquitination or multi-monoubiquitination (Figure 3B). Additionally, proteins can become polyubiquitinated when other ubiquitin molecules bind to the conjugated ubiquitin moiety, leading to the formation of a polyubiquitin chain on target proteins (Figure 3C). Ubiquitin harbors 7 internal lysine residues and one N-terminal methionine residue which can function as a target for polyubiquitination: M1, K6, K11, K27, K29, K33, K48 and K63 (Figure 3A). Ubiquitin chains can be either homeotypic, that is when they harbor a single ubiquitin linkage type, or chains can be heterotypic when they contain mixed linkages. Mixed chains can furthermore be non-branched or branched/forked, of which the latter is the result of multi-ubiquitination of one or more ubiquitin moieties in the chain. Branched chains are frequently found on short-lived proteins *in vivo* (Liu *et al.*, 2017). Chains can furthermore consist of both ubiquitin and ubiquitin-like proteins, such as SUMO. Finally, ubiquitin can also be

modified by other post translational modifications, such as acetylation and phosphorylation (reviewed in (Swatek and Komander, 2016)). The tertiary structures of ubiquitin chains differ depending on which linkage types are present. K48 linkages result in rather compact structures (Tenno *et al.*, 2004; Ryabov and Fushman, 2006), however it was also shown to exhibit a predominantly open conformation (Hirano *et al.*, 2011), Met1-linked diubiquitin has been observed both as a compact (Rohaim *et al.*, 2012) and open structure (Komander *et al.*, 2009), while K63-linked chains exhibit a more open conformation (Tenno *et al.*, 2004; Varadan *et al.*, 2004; Komander *et al.*, 2009). Conjugation of different ubiquitin chain types has been shown to regulate the fate and/or function of target proteins in different ways (Kulathu and Komander, 2012) (Figure 3C).

Many aspects of substrate targeting to the proteasome remain unclear. Conventionally, K48-linked polyubiquitin chains of at least 4 ubiquitin moieties and anchored to a ε-NH_2 group of a lysine residue in the target substrate have been established as the canonical signal for targeted 26S proteasome-mediated proteolysis (Thrower *et al.*, 2000). However, a much broader set of ubiquitin-based signals for proteasomal targeting has been identified. For instance, multiple short heterotypic ubiquitin chains were shown to be a more effective signal for Cyclin B degradation compared to a single long chain (Kirkpatrick *et al.*, 2006). Furthermore, homotypic ubiquitin chains of all linkage types, except K63, are able to behave as proteasome targeting signals *in vivo* (Xu *et al.*, 2009; Bedford *et al.*, 2011; Nathan *et al.*, 2013). K11-linked polyubiquitin chains, for example, can target cell cycle proteins for proteasomal degradation (Jin *et al.*, 2008). To date, K63-linked ubiquitin chains were found in complex with the proteasome in cell free systems (Nathan *et al.*, 2013), however the involvement of K63 chains in the cellular UPS is not yet defined. Mixed chains made of both ubiquitin and ubiquitin-like proteins, such as SUMO, can target substrate for proteolysis (Tatham *et al.*, 2008). Ubiquitin chains can also be anchored to residues other than internal lysines in substrates, such as cysteine, serine and threonine residues and become a target for degradation (Tait *et al.*, 2007). However, ubiquitination on non-lysine residues is not common and it might just be a method by which the cell can target abnormal proteins, whose lysine residues are not exposed, masked or not present, for degradation (Wang, Herr and Hansen, 2012). Furthermore, several monoubiquitinated and multi-monoubiquitinated proteins were found to be targeted to the proteasome (Dimova *et al.*, 2012; Braten *et al.*, 2016; Livneh *et al.*, 2017). It is hypothesized that mono-ubiquitination or multi-monoubiquitination is especially a relevant proteasome targeting signal for relatively small proteins and that larger proteins require polyubiquitination in order to be properly docked at the 19S cap. Finally, some proteins can be degraded by the proteasome without prior ubiquitination. All non-canonical ubiquitin signals for proteasomal degradation are elegantly reviewed in (Kravtsova-Ivantsiv and Ciechanover, 2012; Swatek and Komander, 2016). The wide variety of ubiquitination signals suggests that there is a high level of specificity and selectivity in targeting proteins for degradation and/or recognition of ubiquitinated substrate by the proteasome. It is currently not clear what

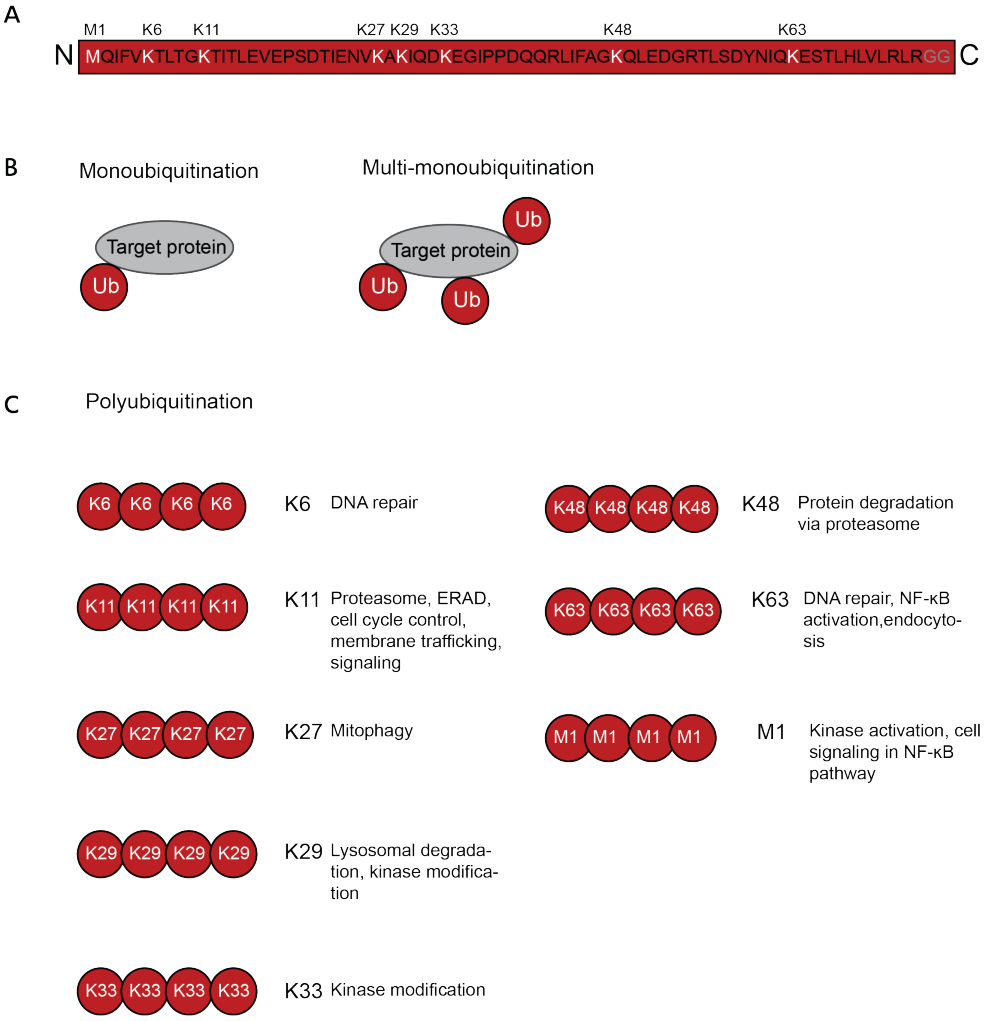

Figure 3. Illustration of multiple forms of ubiquitination. A) Ubiquitin amino acid sequence with methionine and lysine residues highlighted, as these may function as ubiquitination targets in polyubiquitin chains. The glycine residues are highlighted in grey. Gly76 is used for substrate binding, which may be either target substrate proteins as well as other ubiquitin molecules. Gly75 and Gly76 are both important for the recognition and purification of diGly-peptides by α-K-ε-GG antibodies, which greatly propelled the discovery of novel ubiquitination sites by mass spectrometry. B) Variations in mono ubiquitination. C) Selection of variations of polyubiquitination and their effects on the cellular level.

characteristics of the substrate drives these diverse ubiquitination patterns. It is also not clear which ubiquitin receptors or shuttle proteins recognize specific atypical ubiquitin linkage types. On the contrary, an 'ubiquitination threshold' model is proposed, where the amount of polyubiquitin is important as a degradation signal rather than the linkage type (Swatek and Komander, 2016). In another model, a minimal number of short (di)ubiquitin chains is required for tight interaction with the proteasome while a longer chain would promote translocation into the 20S core (Ying *et al.*, 2015). More research is required to better understand the characteristics of effective degradation signals as well as the biological significance of the wide variety of ubiquitin linkages.

Proteasome substrate recruitment

Proper regulation of the interaction between polyubiquitinated substrate and the 26S proteasome is essential for a functional UPS. Malfunctioning of this regulation may result in proteasome dysfunction and protein accumulation, features which are observed in a variety of malignancies (Ciechanover and Brundin, 2003). The central dogma is that 26S proteasome substrate is recognized by their polyubiquitin tags. The two intrinsic ubiquitin receptors of the proteasome are RPN10 (S5a in human) (Deverauxf *et al.*, 1994) and RPN13 (Husnjak *et al.*, 2008). Proteasome subunits RPN1 (Archer *et al.*, 2008), RPT5 (Lam *et al.*, 2002) and RPT1 (Archer *et al.*, 2008) may provide additional ubiquitin docking sites near the 20S CP. Recently DSS1/SEM1/RPN15 was identified as additional ubiquitin receptor of the proteasome in *Saccharomyces pombe* (Paraskevopoulos *et al.*, 2014). Furthermore, RPN1 was identified as a receptor for both ubiquitin and UBL proteins (Shi *et al.*, 2016). The recognition pathways for ubiquitinated substrates appear to have diverged in different species. For instance, RPN10 and RPN13 are non-essential for the yeast 26S proteasome complex (Fu *et al.*, 1998; Husnjak *et al.*, 2008), while RPN10 is essential in mice and *Drosophila melanogaster* (Szlanka *et al.*, 2003; Hamazaki *et al.*, 2007). RPN10-null mice die at embryonic day 6.5 (Hamazaki *et al.*, 2007), while RPN13-null mice die soon after birth (Hamazaki, Hirayama and Murata, 2015). RPN10 recognizes ubiquitin via a C-terminal ubiquitin interacting motif (UIM). *S. cerevisiae* RPN10 has a single UIM that preferentially interacts with K48-linked ubiquitin chains (Fatimababy *et al.*, 2010), whereas human RPN10 harbors two UIMs (UIM1 and UIM2) which are located towards its C-terminus and joined by a flexible linker region (Wang, Young and Walters, 2005; Kang *et al.*, 2007). UIM1 of human RPN10 is comparable with the yeast UIM. The human UIM2 has about a 5-fold higher affinity for ubiquitin than the UIM1 of RPN10 (Wang, Young and Walters, 2005). RPN13 has an N-terminal pleckstrin-like receptor of ubiquitin (PRU) domain, which shows a preference for the proximal ubiquitin of K48-linked chains (Schreiner *et al.*, 2008). An NMR study showed the concurrent interaction of RPN10 and RPN13 with a diubiquitin molecule whereby RPN13 and RPN10 preferably associated with respectively the proximal and distal ubiquitin moiety (Zhang *et al.*, 2009). It is however unclear whether this also occurs *in vivo*. In addition, proteasome structures obtained by electron microscopy revealed that RPN10 and RPN13 are located in the proteasome in such a way that

they could both interact simultaneously with the same ubiquitin chain, thereby positioning it to facilitate deubiquitination (Lasker *et al.*, 2012; Sakata *et al.*, 2012). Ubiquitination of RPN13 through 26S proteasome-bound Ub ligase Ube3c inhibits association with ubiquitinated substrates and inactivates proteasomes in response to proteotoxic stress (Besche *et al.*, 2014).

Instead of direct interaction with the intrinsic ubiquitin receptors of the proteasome, ubiquitinated substrates can also be shuttled to the proteasome by UBL-UBA proteins, such as RAD23 (hHR23a/b in human) (Chen and Madura, 2002; Elsasser *et al.*, 2004), Dsk2 (hPLIC-1/2 in humans) (Kleijnen *et al.*, 2000), or Ddi1 (Saeki *et al.*, 2002; Kaplun *et al.*, 2005). UBL-UBA proteins bind ubiquitin via their ubiquitin-associating (UBA) domain (Bertolaet *et al.*, 2001; Wilkinson *et al.*, 2001; Wang *et al.*, 2003) and the proteasome via their ubiquitin-like (UBL) domain (Hiyama *et al.*, 1999; Elsasser *et al.*, 2002; Walters *et al.*, 2002). The UBL/UBA proteins are called "shuttle" proteins because they may bind substrates remotely from the proteasome, and subsequently bring them to this complex. The UBL-UBA proteins interact only weakly with proteasomes and are usually substoichiometric components of purified proteasomes. They dock generally at proteasome subunit RPN1 (Elsasser *et al.*, 2002). Additionally, yeast Dsk2 and human RAD23 can interact with RPN10 and RPN13 (Hiyama *et al.*, 1999; Walters *et al.*, 2002; Husnjak *et al.*, 2008), and yeast Rad23 may also bind to Rpt1 and Rpt6 (Schauber *et al.*, 1998). *Drosophila* DSK2 interacts only with proteasomes which harbor the RPN10 ubiquitin receptor, as the interaction is lost in Δp54/RPN10 proteasomes, whereas yeast Dsk2 interacts only with ΔRPN10 proteasomes (Lipinszki *et al.*, 2011). RPN10 and RPN13 are also major acceptors of mHR23B and ubiquilin-1/DSK2 and ubiquilin-4/ataxin-1 in mice (Hamazaki, Hirayama and Murata, 2015). Human RAD23 preferably recruits substrate with K48-linked ubiquitin chains to the proteasome (Raasi and Pickart, 2003; Nathan *et al.*, 2013), whereas the UBA domains of Dsk2- and Ddi1 do not show linkage specificity. The UBA domain of DSK2 has a significantly higher affinity to monoubiquitin as compared to the UBA domain of human Rad23 (Zhang, Raasi and Fushman, 2008). UBL-UBA proteins share redundant functions (Medicherla *et al.*, 2004; Díaz-Martínez *et al.*, 2006; Kang *et al.*, 2006), but they also have distinct roles, such as Rad23 in DNA repair (Schauber *et al.*, 1998) and Dsk2 in neuropathology (Mah *et al.*, 2000).

While it is clear that substrates can use two different pathways to bind to the proteasome, we still do not understand how substrates are assigned to one targeting pathway or the other. Additionally, the extent of crosstalk between both pathways is also yet unclear. Data obtained by electron microscopy and quantitative mass spectrometry suggest that there is a pool of proteasome complexes which do not contain the intrinsic receptors (Nickell *et al.*, 2009; Sakata *et al.*, 2012), which may suggest that the intrinsic ubiquitin receptors are not essential for all proteasomal substrates. Another factor which increases the complexity of this targeting system is the fact that RPN10 also exists as an extra-proteasomal protein (Matiuhin *et al.*, 2008; Piterman *et al.*, 2014). The function of the free pool of RPN10 is not yet clear. Matiuhin and colleagues showed that the pool of free RPN10 can inhibit the interaction of ubiquitin shuttle protein Dsk

with the proteasome complex in yeast (Matiuhin *et al.*, 2008). In contrast, Piterman and colleagues showed that mammalian RPN10 is only able to interact with ubiquitin and ubiquitin-like harboring proteins such as hPLIC-2 (the mammalian homologue of yeast Dsk2) when it is incorporated in the proteasome complex (Piterman *et al.*, 2014). Also, ubiquitin shuttling proteins can, instead of facilitating, oppose substrate degradation by the UPS (Ortolan *et al.*, 2000; Raasi and Pickart, 2003). Thus, further research is required to reveal the complex mechanisms of substrate recruitment to the 26S proteasome, which would provide useful information for elucidating physiological functions and specificities of each ubiquitin receptor.

Substrate processing

Ubiquitinated substrate proteins are bound to the proteasome via interactions with the intrinsic receptors RPN10 and RPN13 or with transiently bound shuttle receptors. The ATPase ring of the proteasome base then engages an unstructured initiation region in the substrate protein which tightens the interaction with the proteasome and uses ATP hydrolysis to unfold and translocate the polypeptide into the proteolytic chamber (Peth, Uchiki and Goldberg, 2010). Ubiquitin is concurrently removed from the substrate by deubiquitinating enzymes. Substrate degradation requires several consecutive conformational changes of the proteasome regulatory particle (Lander *et al.*, 2012). For every substrate turnover, the proteasome transitions from a substrate-free to a substrate-engaged state in which the latter facilitates translocation, unfolding and deubiquitination. The engaged state facilitates translocation of the substrate since the channel axis of the 20S core particle is better aligned with the ATPase ring, as compared with the substrate-free state. Finally, the proteasome must switch back to the substrate-free conformation for the engagement of a new substrate.

Three proteins are known to be involved in the deubiquitination of substrate of the mammalian proteasome: RPN11 (POH1 or PSMD14 in human), UCHL5 (also UCH37) and USP14 (Ubp6 in yeast). This set of DUBs is well conserved evolutionary with the exception of the lack of a recognizable UCHL5 ortholog in *S. cerevisiae*. Consequently, the most intensively studied proteasomal DUBs are RPN11 and USP14. Each of the proteasomal DUBs belongs to a different DUB family and are thus anciently diverged in evolution: RPN11, UCHL5 and USP14 belong to the JAMM, UCH and USP families, respectively. There are evident differences between these DUBs. RPN11, a constituent stoichiometric subunit of the proteasome, is the only essential DUB of the proteasome (Gallery *et al.*, 2007; Finley, 2009) and is critical for both the stability of the 26S proteasome complex and for the promotion of substrate degradation (Verma et al., 2002; Yao & Cohen, 2002, chapter 4 of this thesis). Cross-linking studies on *Schizosaccharomyces pombe* co-localize the RPN11 C-terminal domain with the N-terminal end of RPT3 (Bohn *et al.*, 2010), potentially linking its activity to this ATPase. Insertion sequence insert-2 of RPN11 contributes to proteasome binding (Pathare *et al.*, 2014; Worden, Padovani and Martin, 2014), whereas insertion sequence insert-1 is involved in ubiquitin binding (Worden, Padovani and Martin, 2014). It was found that insert-1 exhibits a closed conformation stabilized

by RPN5 in the 19S cap prior to incorporation in a 26S complex, which inhibits the access of ubiquitin to free 19S caps (Dambacher *et al.*, 2016). In contrast, the insert-1 closed conformation is only weakly stabilized in the 26S proteasome substrate-free s1 ground state (Worden, Dong and Martin, 2017). Substrate engagement induces a conformational change of the entire 19S particle from the s1 state via s2 to finally the s3 substrate engaged state (Unverdorben *et al.*, 2014), in which RPN11 is repositioned directly above the translocation channel of the 20S complex (Matyskiela, Lander and Martin, 2013). Ubiquitin binding then induces a conformational change of the RPN11 insert-1 loop from an inactive closed state to an active open state, which is further accelerated by mechanical translocation of ubiquitinated substrate into the proteolytic core (Worden, Dong and Martin, 2017). Due to this acceleration, RPN11-dependent deubiquitination of engaged substrates was found to be about ~40 times faster as compared to pre-engaged substrates (Worden, Dong and Martin, 2017). RPN11 cleaves entire polyubiquitin chains at the proximal ubiquitin (Yao and Robert E. Cohen, 2002; M. J. Lee *et al.*, 2011) and does not confer ubiquitin linkage type specificity. Furthermore, deubiquitination by RPN11 is dependent on ATP hydrolysis by the proteasome base (Verma *et al.*, 2002; Yao and Robert E. Cohen, 2002). The timing of RPN11 deubiquitination activity is relatively late in the degradation process, *i.e.,* during substrate translocation, thereby probably avoiding premature substrate deubiquitination and release. On the other hand, UCHL5 and USP14 act already before the commitment step. UCHL5 is not essential for the structure and the activity of the proteasome (Elena Koulich, Xiaohua Li, 2008, chapter 4 of this thesis). UCHL5 is activated and recruited to the proteasome by ubiquitin receptor RPN13 (Hamazaki *et al.*, 2006; Qiu *et al.*, 2006; Yao *et al.*, 2006). Isolated UCHL5 displays an 8-fold increased catalytic activity when complexed with RPN13 compared to UCHL5 alone (VanderLinden *et al.*, 2015). The N-terminal catalytic UCH domain of UCHL5 contains active-site residues which can interact with ubiquitin (Burgie *et al.*, 2012). UCHL5 can only deubiquitinate proteins when it is in complex with the proteasome, in its free form it can only remove small peptides from the C-terminus of ubiquitin (Yao *et al.*, 2006). The role of UCHL5 in the proteasome has not yet been clearly defined. One suggestion is that UCHL5 performs an editing function by removing single ubiquitin moieties from the distal end of polyubiquitin chains which results in the release of substrate from the proteasome prior to degradation (Lam *et al.*, 1997). Another suggestion is that UCHL5 regulates proteasome activity via deubiquitination of proteasome subunits that can undergo regulatory ubiquitination (Jacobson *et al.*, 2014). Yet another suggestion is that UCHL5 clears unanchored polyubiquitin chains from proteasome-associated ubiquitin receptors (Zhang *et al.*, 2011). Lastly, deubiquitinating enzyme USP14 is a substoichiometric interactor of the proteasome and RNAi of USP14 does not affect proteasome stability (Elena Koulich, Xiaohua Li, 2008, chapter 4 of this thesis). USP14 interacts reversibly with the proteasome and is the most abundant proteasome interacting protein (PIP). Dependent on the study, Ubp6/USP14 interacts with about 10-40% of the proteasome 19S caps (Aufderheide *et al.*, 2015; Kim and Goldberg, 2017; Kuo and Goldberg, 2017). The DUB interacts via its N-terminal UBL domain with proteasome

subunit RPN1 (David S Leggett *et al.*, 2002; Rosenzweig *et al.*, 2012) whereby its catalytic USP domain is rather mobile (Aufderheide *et al.*, 2015). A substantial fraction of USP14 is not in complex with proteasomes (Elena Koulich, Xiaohua Li, 2008). Free USP14 is relatively inactive whereas its activity is enormously enhanced upon interaction with the purified proteasome base complex or the entire proteasome complex (David S Leggett *et al.*, 2002; Lee *et al.*, 2010). Hu *et al.,* found that the active site of free USP14 is present in a productive conformation, but the interaction of the active site with ubiquitin is inhibited by two loops, BL1 and BL2 (Hu *et al.*, 2005). Activation of USP14 can also (or further) be mediated via phosphorylation by the Akt protein kinase (Xu *et al.*, 2015). USP14 shows a preference for K11, K33 and K48 ubiquitin linkages (Flierman *et al.*, 2016). Recently it was found that the deubiquitinating activity of USP14 is enhanced when the proteasome switches from the substrate-free state towards the substrate engagement conformation (Bashore *et al.*, 2015). Ubiquitin-bound Ubp6/USP14 stabilizes this conformation to prevent a return to the substrate-free conformation while substrate is engaged for degradation. Polyubiquitin-bound USP14 also stimulates the ATPase rate of the proteasome and regulates channel opening of the 20S CP (Peth, Besche and Goldberg, 2009). Ubp6 is furthermore involved in RP assembly (Sakata *et al.*, 2011).

The current model couples the activity of RPN11 with the promotion of substrate degradation whereas the activity of UCHL5 and USP14 could counteract this process by trimming ubiquitin moieties from the distal end of the chains thereby detaching substrate from the proteasome prior to degradation (Lam *et al.*, 1997; Hu *et al.*, 2005; Lee *et al.*, 2010). However, recent findings of the lab of Andreas Martin reveal that Ubp6/USP14 is involved in the regulation of the 19S RP conformational changes and inhibition of RPN11 DUB activity. Their results suggest a degradation-facilitating role for Ubp6, rather than an inhibitory role, as USP14 is mainly active on already engaged substrate (Bashore *et al.*, 2015). For instance, they show with Ub-AMC substrates that Ubp6 is activated when its USP domain interacts with the proteasome ATPase base, probably as the result of conformational changes of two surface loops, BL1 and BL2, in the USP domain. The deubiquitinating activity of Ubp6 was then further increased when the proteasome switched to the ATP-γS-induced substrate-engaged conformation, even in combination with RPN11 catalytic mutants, which suggests that Ubp6 is responsible for the enhanced deubiquitinating activity of the proteasome in the engaged state. Furthermore, by EM they showed that ubiquitin binding of Ubp6 stabilized the engaged state via interactions with both the N-ring and the AAA+ ring of the RPT subunits in the proteasome base, thereby preserving the coaxial alignment of both rings with the 20S core channel. In the engaged state ubiquitin-bound Ubp6 is placed in close proximity with RPN11. Biochemical assays showed that ubiquitin-bound Ubp6 inhibits the degradation-coupled DUB activity of RPN11 (Bashore *et al.*, 2015). Lastly, stabilization of the engaged state by ubiquitin-bound Ubp6 also prevented the engagement of subsequent substrates prior to ubiquitin dissociation. These results suggest a model in which substrate engagement induces conformational changes in the 19S RP which in

turn facilitate substrate unfolding, deubiquitination, translocation and degradation. Ubp6 plays two important roles in this process: Ubp6 can inhibit the deubiquitination activity of RPN11 and it can prevent the return to the substrate-free conformation. Inhibition of RPN11 might be a way to extend the time window in which Ubp6 can deubiquitinate the engaged substrate. This may be important to process complex substrates with multiple long and/or branched polyubiquitin chains that need to be co-translocationally trimmed (Bashore *et al.*, 2015). There are more studies which show contrasting USP14 functionalities compared to the ubiquitin editing model. It was for instance found that instead of removing single ubiquitin moieties, USP14 removes polyubiquitin chains en bloc until a single polyubiquitin chain remains on the substrate. Substrates might be spared from degradation when the remaining polyubiquitin chain is relatively short whereas a relatively long chain would be a target for RPN11-dependent deubiquitination followed by proteasomal degradation. After en bloc removal of polyubiquitin chains by RPN11 and subsequent translocation and degradation of the substrate, USP14/Ubp6 may trap the substrate-engaged state until it trimmed all remaining polyubiquitin chains of the just processed substrate, thereby maintaining the high levels of free ubiquitin. When all ubiquitin is removed, the proteasome can switch back to the substrate-free state. In this model Ubp6 thus facilitates protein degradation and confers clearance of proteasome-bound polyubiquitin chains during translocation (Lee *et al.*, 2016).

Ubiquitin proteasome system and disease

Cancer

Proteasome inhibitors have effective anti-tumor activity in cell culture, inducing apoptosis by disrupting the regulated degradation of pro-growth cell cycle proteins (Adams *et al.*, 1999). This approach of selectively inducing apoptosis in tumor cells has proven effective in animal models and human trials, although the development of drug resistance in relapsed patients is a problem (Tew, 2016). Lactacystin, a natural product synthesized by *Streptomyces* bacteria, was the first non-peptidic proteasome inhibitor discovered (Omura *et al.*, 1991) and is widely used as a research tool in biochemistry and cell biology. Lactacystin covalently modifies the amino-terminal threonine of catalytic β-subunits of the proteasome, particularly the β5 subunit of the proteasome's chymotrypsin-like activity (Fenteany *et al.*, 1995). The discovery helped to establish the proteasome as a mechanistically novel class of protease: an amino-terminal threonine protease. Another commonly used proteasome inhibitor in laboratories is the peptide aldehyde MG132. MG132 binds to all beta subunits of the proteasome, thereby effectively blocking its proteolytic activity. MG132 inhibits the growth of tumor cells by inducing the cell cycle arrest as well as triggering apoptosis (Han and Park, 2010). Different mechanisms of apoptosis induction by MG132 are nicely reviewed (Guo and Peng, 2013). Bortezomib is the first proteasome inhibitor to reach clinical use as a chemotherapy agent and was brought to the market for the treatment of multiple myeloma (Adams and Kauffman, 2004; Richardson *et al.*, 2005). It reversibly inhibits the chymotrypsin-like activity of Prosbeta5 (Crawford *et al.*, 2006). This results

in a dysregulation of the ER-associated degradation (ERAD) pathway and induces the terminal Unfolded Protein Response (UPR), leading to apoptosis (Obeng *et al.*, 2006). Initially, Bortezomib may improve the outcome for myeloma patients, however relapses are frequent and patients often develop resistance against the therapy. Advances and challenges of the application of proteasome inhibitors in the clinic are nicely reviewed (Manasanch and Orlowski, 2017).

Neurodegenerative diseases

A characteristic of many neurodegenerative disorders, including Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), is the accumulation of toxic protein species which results in the formation of protein inclusions and/or plaques in degenerating brains. This implies that protein homeostasis is poorly regulated in this type of diseases. Therefore, both autophagy and the UPS are topics of intense research in this field. There is currently no effective treatment that could cure or considerably delay the onset or progression of the neurodegenerative diseases described above. Drug development strategies aim to increase the proteolytic activity in the cell. For instance, the upregulation of proteasomal gene expression, upregulation of proteasome activators such as PA28 or PA200 or the identification of small molecules that can activate the CP. Another strategy is to enhance the targeting of disease-associated proteins to the proteasome by altering activities of relevant ubiquitin ligases or deubiquitinating enzymes.

References

Adams, J. *et al.* (1999) 'Proteasome Inhibitors : A Novel Class of Potent and Effective Antitumor Agents', *Cancer Research*, 59, pp. 2615–2622.

Adams, J. and Kauffman, M. (2004) 'Development of the Proteasome Inhibitor Velcade™ (Bortezomib)', *Cancer Investigation*, 22(2), pp. 304–311.

Archer, C. T. *et al.* (2008) 'Physical and functional interactions of monoubiquitylated transactivators with the proteasome', *Journal of Biological Chemistry*. 283(31), pp. 21789–21798.

Asano, S. *et al.* (2015) 'Proteasomes. A molecular census of 26S proteasomes in intact neurons', *Science*, 347(6220). pp. 439-443

Aufderheide, A. *et al.* (2015) 'Structural characterization of the interaction of Ubp6 with the 26S proteasome.', *Proceedings of the National Academy of Sciences of the United States of America*, 112(28), pp. 8626–31.

Bashore, C. *et al.* (2015) 'Ubp6 deubiquitinase controls conformational dynamics and substrate degradation of the 26S proteasome', *Nature Structural & Molecular Biology*. Nature Publishing Group, 22(9), pp. 712–719.

Beck, F. *et al.* (2012) 'Near-atomic resolution structural model of the yeast 26S proteasome.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(37), pp. 14870–5.

Beckwith, R. *et al.* (2013) 'Reconstitution of the 26S proteasome reveals functional asymmetries in its AAA+ unfoldase.', *Nature structural molecular biology*, 20(10), pp. 1164–1172.

Bedford, L. *et al.* (2011) 'Ubiquitin-like protein conjugation and the ubiquitin–proteasome system as drug targets', *Drug Discovery*, 10, pp. 29–46.

Bertolaet, B. L. *et al.* (2001) 'UBA domains of DNA damage-inducible proteins interact with ubiquitin.', *Nature structural biology*, 8(5), pp. 417–422.

Besche, H. C. *et al.* (2014) 'Autoubiquitination of the 26S Proteasome on Rpn13 Regulates Breakdown of Ubiquitin Conjugates', *The EMBO Journal*, 33, pp. 1159–1176.

Bohn, S. *et al.* (2010) 'Structure of the 26S proteasome from Schizosaccharomyces pombe at subnanometer resolution', *Proceedings of the National Academy of Sciences of the United States of America*, 107(49), pp. 20992–20997.

Braten, O. *et al.* (2016) 'Numerous proteins with unique characteristics are degraded by the 26S proteasome following monoubiquitination.', *Proceedings of the National Academy of Sciences of the United States of America*, 113(32), pp. E4639-47.

Burgie, S. E. *et al.* (2012) 'Structural characterization of human Uch37', *Proteins: Structure, Function and Bioinformatics*, 80(2), pp. 649–654.

Chen, L. and Madura, K. (2002) 'Rad23 Promotes the Targeting of Proteolytic Substrates to the Proteasome', *molecular and cellular biology*, 22(13), pp. 4902–4913.

Chu-Ping, M. *et al.* (1994) 'Identification, purification, and characterization of a high molecular weight, ATP-dependent activator (PA700) of the 20 S proteasome.', *The Journal of biological chemistry*, 269(5), pp. 3539–3547.

Ciechanover, A. and Brundin, P. (2003) 'Review The Ubiquitin Proteasome System in Neurodegenerative Diseases: Sometimes the Chicken, Sometimes the Egg', *Neuron*, 40, pp. 427–446.

Collins, G. A. and Goldberg, A. L. (2017) 'The Logic of the 26S Proteasome', *Cell*, 169(5), pp. 792–806.

Crawford, L. J. A. *et al.* (2006) 'Comparative Selectivity and Specificity of the Proteasome Inhibitors BzLLLCOCHO, PS-341, and MG-132', *Cancer Res*, 66(12), pp. 6379–86.

Dambacher, C. M. *et al.* (2016) 'Atomic structure of the 26S proteasome lid reveals the mechanism of deubiquitinase inhibition', *eLife*, 5(January), pp. 1–17.

Deverauxf, Q. *et al.* (1994) 'A 26 S Protease Subunit That Binds Ubiquitin Conjugates*', *Journal of Biological Chemistry* 269(10), pp. 7059–7061.

Díaz-Martínez, L. A. *et al.* (2006) 'Yeast UBL-UBA proteins have partially redundant functions in cell cycle control.', *Cell division*, 1(28), pp. 1–11.

Dimova, N. V *et al.* (2012) 'APC/C-mediated multiple monoubiquitylation provides an alternative degradation signal for cyclin B1', *Nature Cell Biology*, 14(2), pp. 168–176.

Elena Koulich, Xiaohua Li, and G. N. D. (2008) 'Relative Structural and Functional Roles of Multiple Deubiquitylating Proteins Associated with Mammalian 26S Proteasome', *Molecular biology of the cell*, 19, pp. 1072–1082.

Elsasser, S. *et al.* (2002) 'Proteasome subunit Rpn1 binds ubiquitin-like protein domains', *Nature Cell Biology*, 4(9), pp. 725–730.

Elsasser, S. *et al.* (2004) 'Rad23 and Rpn10 serve as alternate ubiquitin receptors for the proteasome', *Journal of Biological Chemistry*, 279(26), pp. 26817–26822.

Fatimababy, A. S. *et al.* (2010) 'Cross-species divergence of the major recognition pathways of ubiquitylated substrates for ubiquitin/26S proteasome-mediated proteolysis', *FEBS Journal*. Blackwell Publishing Ltd, 277(3), pp. 796–816.

Fenteany, G. *et al.* (1995) 'Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin', *Science*, 268(5211), pp. 726–731.

Finley, D. (2009) 'Recognition and Processing of Ubiquitin-Protein Conjugates by the Proteasome', *Annual review of biochemistry,* pp. 477–513.

Flierman, D. *et al.* (2016) 'Non-hydrolyzable Diubiquitin Probes Reveal Linkage-Specific

Reactivity of Deubiquitylating Enzymes Mediated by S2 Pockets', *Cell Chemical Biology*, 23(4), pp. 472–482.

Fort, P. *et al.* (2015) 'Evolution of proteasome regulators in Eukaryotes', *Genome Biology and Evolution*, 7(5), pp. 1363–1379.

Fu, H. *et al.* (1998) 'Multiubiquitin chain binding and protein degradation are mediated by distinct domains within the 26 S proteasome subunit Mcb1', *Journal of Biological Chemistry*, 273(4), pp. 1970–1981.

Gallery, M. *et al.* (2007) 'The JAMM motif of human deubiquitinase Poh1 is essential for cell viability', *Molecular Cancer Therapeutics*, 6(1), pp. 262–268.

Geng, F., Wenzel, S. and Tansey, W. P. (2012) 'Ubiquitin and Proteasomes in Transcription.', *Annual review of biochemistry*, 81, pp. 177–201.

Gille, C. *et al.* (2003) 'A comprehensive view on proteasomal sequences: Implications for the evolution of the proteasome', *Journal of Molecular Biology*, 326(5), pp. 1437–1448.

Glickman, M. H. *et al.* (1998) 'A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3', *Cell*, 94(5), pp. 615–623.

Goldberg, A. L. (2003) 'Protein degradation and protection against misfolded or damaged proteins', *Nature*, 426, pp. 895–899.

Groll, M. *et al.* (1997) 'Structure of 20S proteasome from yeast at 2.4 A resolution.', *Nature*, pp. 463–471.

Guo, N. and Peng, Z. (2013) 'MG132, a proteasome inhibitor, induces apoptosis in tumor cells', *Asia-Pacific Journal of Clinical Oncology*, 9(1), pp. 6–11.

Hamazaki, J. *et al.* (2006) 'A novel proteasome interacting protein recruits the deubiquitinating enzyme UCH37 to 26S proteasomes', *The EMBO Journal*, 25(19), pp. 4524–4536.

Hamazaki, J. *et al.* (2007) 'Rpn10-Mediated Degradation of Ubiquitinated Proteins Is Essential for Mouse Development', *molecular and cellular biology*, 27(19), pp. 6629–6638.

Hamazaki, J., Hirayama, S. and Murata, S. (2015) 'Redundant Roles of Rpn10 and Rpn13 in Recognition of Ubiquitinated Proteins and Cellular Homeostasis', *PLoS Genetics*, 11(7), pp. 1–20.

Han, Y. H. and Park, W. H. (2010) 'MG132, a proteasome inhibitor decreased the growth of Calu-6 lung cancer cells via apoptosis and GSH depletion', *Toxicology in Vitro*, 24(4), pp. 1237–1242.

Hirano, T. *et al.* (2011) 'Conformational Dynamics of Wild-type Lys-48-linked Diubiquitin in Solution'. *Journal of Biological Chemistry,* 286(43), pp. 37496-37502

Hiyama, H. *et al.* (1999) 'Interaction of hHR23 with S5a - the ubiquitin-like domain of hhr23 mediates interaction with s5a subunit of 26 s proteasome*', *The Journal of biological chemistry*, 274(39), pp. 28019–28025.

Hough, R., Pratt, G. and Rechsteiner, M. (1986) 'Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates', *Journal of Biological Chemistry*, 261(5), pp. 2400–2408.

Hu, M. *et al.* (2005) 'Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14', *The EMBO Journal*, 24(21), pp. 3747–3756.

Husnjak, K. *et al.* (2008) 'Proteasome subunit Rpn13 is a novel ubiquitin receptor', *Nature*, 453(7194), pp. 481–488.

Jacobson, A. D. *et al.* (2014) 'Autoregulation of the 26S proteasome by in situ ubiquitination.', *Molecular biology of the cell*, 25(12), pp. 1824–35.

Jin, L. *et al.* (2008) 'Mechanism of Ubiquitin-Chain Formation by the Human Anaphase-Promoting Complex', *Cell*, 133(4), pp. 653–665.

Kang, Y. *et al.* (2006) 'UBL/UBA ubiquitin receptor proteins bind a common tetraubiquitin chain', *Journal of Molecular Biology*, 356(4), pp. 1027–1035.

Kang, Y. *et al.* (2007) 'Defining how Ubiquitin Receptors hHR23a and S5a Bind Polyubiquitin', *Journal of Molecular Biology*, 369(1), pp. 168–176.

Kaplun, L. *et al.* (2005) 'The DNA Damage-Inducible UbL-UbA Protein Ddi1 Participates in Mec1-Mediated Degradation of Ho Endonuclease', *molecular and cellular biology*, 25(13), pp. 5355–5362.

Kim, H. T. and Goldberg, A. L. (2017) 'The Deubiquitinating Enzyme Usp14 Allosterically Inhibits Multiple Proteasomal Activities and Ubiquitin-Independent Proteolysis.', *The Journal of biological chemistry*, (3), p. jbc.M116.763128.

Kirkpatrick, D. S. *et al.* (2006) 'Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology', *Nature Cell Biology*, 8(7), pp. 700–710.

Kisselev, A. F., Akopian, T. N. and Goldberg, A. L. (1998) 'Range of Sizes of Peptide Products Generated during Degradation of Different Proteins by Archaeal Proteasomes*', *The Journal of biological chemistry*, 273(4), pp. 1982–1989.

Kleijnen, M. F. *et al.* (2000) 'The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome.', *Molecular cell*, 6(2), pp. 409–419.

Kloetzel, P.-M. (2004) 'The proteasome and MHC class I antigen processing'. *Biochimica et Biophysica Acta,* 1695 pp.225-233

Komander, D. *et al.* (2009) 'Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains', *EMBO reports*, 10, pp. 466–473.

Komander, D. and Rape, M. (2012) 'The Ubiquitin Code', *Annual Review of Biochemistry*, 81(1), pp. 203–229.

Kostova, Z. and Wolf, D. H. (2003) 'For whom the bell tolls: Protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection', *EMBO Journal*, pp. 2309–2317.

Kravtsova-Ivantsiv, Y. and Ciechanover, a. (2012) 'Non-canonical ubiquitin-based signals for proteasomal degradation', *Journal of Cell Science*, 125(3), pp. 539–548.

Kulathu, Y. and Komander, D. (2012) 'Atypical ubiquitylation — the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages', *Nature Reviews Molecular Cell Biology*, 13, pp. 508-523.

Kuo, C.-L. and Goldberg, A. L. (2017) 'Ubiquitinated proteins promote the association of proteasomes with the deubiquitinating enzyme Usp14 and the ubiquitin ligase Ube3c', *Proceedings of the National Academy of Sciences*, 114(17), pp. E3404–E3413.

Lam, Y. A. *et al.* (1997) 'Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome.', *Nature*, pp. 737–740.

Lam, Y. A. *et al.* (2002) 'A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal', *Nature*, 416(6882), pp. 763–767.

Lander, G. C. *et al.* (2012) 'Complete subunit architecture of the proteasome regulatory particle', *Nature*, 482(7384), pp. 186–191.

Lasker, K. *et al.* (2012) 'Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(5), pp. 1380–1387.

Lee, B.-H. *et al.* (2010) 'Enhancement of proteasome activity by a small-molecule inhibitor of USP14', *Nature*. Nature Publishing Group, 467(7312), pp. 179–184.

Lee, B.-H. *et al.* (2016) 'USP14 deubiquitinates proteasome-bound substrates that are ubiquitinated at multiple sites', *Nature*. Nature Publishing Group, pp. 1–16.

Lee, M. J. *et al.* (2011) 'Trimming of ubiquitin chains by proteasome-associated deubiquitinating enzymes.', *Molecular & cellular proteomics : MCP*, 10(5), p. R110.003871.

Leggett, D. S. *et al.* (2002) 'Multiple associated proteins regulate proteasome structure and function', *Molecular Cell*, 10(3), pp. 495–507.

Lipinszki, Z. *et al.* (2011) 'Overexpression of Dsk2/dUbqln results in severe developmental defects and lethality in Drosophila melanogaster that can be rescued by overexpression of the p54/Rpn10/S5a proteasomal subunit', *FEBS Journal*. Blackwell Publishing Ltd, 278(24), pp. 4833–4844.

Liu, C.-W. *et al.* (2003) 'Endoproteolytic activity of the proteasome.', *Science*, 299(5605), pp. 408–11.

Liu, C. *et al.* (2017) 'Ufd2p synthesizes branched ubiquitin chains to promote the degradation of substrates modified with atypical chains', *Nature Communications*, 8.

Livneh, I. *et al.* (2017) 'Monoubiquitination joins polyubiquitination as an esteemed proteasomal targeting signal', *BioEssays*, 39(6), p. 1700027.

Mah, A. L. *et al.* (2000) 'Identification of Ubiquilin, a Novel Presenilin Interactor That Increases Presenilin Protein Accumulation', *The Journal of Cell Biology*, 151(4), pp. 847–862.

Manasanch, E. E. and Orlowski, R. Z. (2017) 'Proteasome inhibitors in cancer therapy', *Nature Reviews Clinical Oncology*, 14(7), pp. 417–433.

Marques, A. J. *et al.* (2009) 'Catalytic mechanism and assembly of the proteasome', *Chemical Reviews*, 109(4), pp. 1509–1536.

Matiuhin, Y. *et al.* (2008) 'Extraproteasomal Rpn10 Restricts Access of the Polyubiquitin-Binding Protein Dsk2 to Proteasome', *Molecular Cell*. Elsevier Inc., 32(3), pp. 415–425.

Matyskiela, M. E., Lander, G. C. and Martin, A. (2013) 'Conformational switching of the 26S proteasome enables substrate degradation.', *Nature structural & molecular biology*. Nature Publishing Group, 20(7), pp. 781–8.

Medicherla, B. *et al.* (2004) 'A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation', *EMBO reports*, 5(7), pp. 692–697.

Nathan, J. A. *et al.* (2013) 'Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes?' *The EMBO Journal*, 32, pp. 552–565.

Nickell, S. *et al.* (2009) 'Insights into the molecular architecture of the 26S proteasome.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 106(29), pp. 11943–7.

Obeng, E. A. *et al.* (2006) 'Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells.', *Blood*, 107(12), pp. 4907–4916.

Omura, S. *et al.* (1991) 'Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells.', *The Journal of antibiotics*, 44(1), pp. 113–6.

Ortolan, T. G. *et al.* (2000) 'The DNA repair protein rad23 is a negative regulator of multi-ubiquitin chain assembly.', *Nature cell biology*, 2(9), pp. 601–8.

Paraskevopoulos, K. *et al.* (2014) 'Dss1 is a 26S proteasome ubiquitin receptor', *Molecular Cell*, 56(3), pp. 453–461.

Pathare, G. R. *et al.* (2014) 'Crystal structure of the proteasomal deubiquitylation module Rpn8-Rpn11', *Proceedings of the National Academy of Sciences*, 111(8), pp. 2984–2989.

Peth, A., Besche, H. C. and Goldberg, A. L. (2009) 'Ubiquitinated Proteins Activate the Proteasome by Binding to Usp14/Ubp6, which Causes 20S Gate Opening', *Molecular Cell*, 36(5), pp. 794–804.

Peth, A., Nathan, J. A. and Goldberg, A. L. (2013) 'The ATP Costs and Time Required to Degrade Ubiquitinated Proteins by the 26 S Proteasome *', *Journal of Biological Chemistry,* 288(40), pp. 29215-29222

Peth, A., Uchiki, T. and Goldberg, A. L. (2010) 'ATP-Dependent Steps in the Binding of Ubiquitin Conjugates to the 26S Proteasome that Commit to Degradation', *Molecular Cell*, 40, pp. 671–681.

Piterman, R. *et al.* (2014) 'VWA domain of S5a restricts the ability to bind ubiquitin and Ubl to the 26S proteasome.', *Molecular biology of the cell*, 25(25), pp. 3988–98.

Qiu, X. *et al.* (2006) 'hRpn13/ADRM1/GP110 is a novel proteasome subunit that binds the

deubiquitinating enzyme, UCH37', *The EMBO Journal*, 25(24), pp. 5742–5753.

Raasi, S. and Pickart, C. M. (2003) 'Rad23 ubiquitin-associated domains (UBA) inhibit 26 S proteasome-catalyzed proteolysis by sequestering lysine 48-linked polyubiquitin chains', *Journal of Biological Chemistry*. 278(11), pp. 8951–8959.

Richardson, P. G. *et al.* (2005) 'Bortezomib or High-Dose Dexamethasone for Relapsed Multiple Myeloma', *New England Journal of Medicine*, 352(24), pp. 2487–2498.

Rock, K. L. *et al.* (1994) 'Inhibitors of the Proteasome Block the Degradation of Most Cell Proteins and the Generation of Peptides Presented on MHC Class I Molecules', *Cell*, 78, pp. 761–771.

Rohaim, A. *et al.* (2012) 'Structure of a compact conformation of linear diubiquitin', *Acta Cryst*, 68, pp. 102–108.

Rosenzweig, R. *et al.* (2012) 'Rpn1 and Rpn2 coordinate ubiquitin processing factors at proteasome', *Journal of Biological Chemistry*, 287(18), pp. 14659–14671.

Ryabov, Y. and Fushman, D. (2006) 'Interdomain mobility in di-ubiquitin revealed by NMR', *Proteins: Structure, Function, and Bioinformatics*. Wiley Subscription Services, Inc., A Wiley Company, 63(4), pp. 787–796.

Saeki, Y. *et al.* (2002) 'Ubiquitin-like proteins and Rpn10 play cooperative roles in ubiquitin-dependent proteolysis', *Biochemical and Biophysical Research Communications*, 293(3), pp. 986–992.

Sakata, E. *et al.* (2011) 'The Catalytic Activity of Ubp6 Enhances Maturation of the Proteasomal Regulatory Particle', *Molecular Cell*, 42(5), pp. 637–649.

Sakata, E. *et al.* (2012) 'Localization of the proteasomal ubiquitin receptors Rpn10 and Rpn13 by electron cryomicroscopy.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 109(5), pp. 1479–84.

Schauber, C. *et al.* (1998) 'Rad23 links DNA repair to the ubiquitin/proteasome pathway.', *Nature*, 391(6668), pp. 715–718.

Schreiner, P. *et al.* (2008) 'Ubiquitin docking at the proteasome through a novel pleckstrin-homology domain interaction', *Nature*, 453(7194), pp. 548–552.

Schwartz, A. L. and Ciechanover, A. (2009) 'Targeting Proteins for Destruction by the Ubiquitin System: Implications for Human Pathobiology', *Annu. Rev. Pharmacol. Toxicol*, 49, pp. 73–96.

Shi, Y. *et al.* (2016) 'Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome.', *Science*, 351(6275), p. aad9421.

Sledz, P. *et al.* (2013) 'Structure of the 26S proteasome with ATP-gammaS bound provides insights into the mechanism of nucleotide-dependent substrate translocation', *Proceedings of the National Academy of Sciences of the United States of America*, 110(18), pp. 7264–7269.

Stadtmueller, B. and Hill, C. (2011) 'Proteasome Activators', *Mol Cell.*, 41(1), pp. 8–19.

Swatek, K. N. and Komander, D. (2016) 'Ubiquitin modifications.', *Cell research*. Nature Publishing Group, 26(4), pp. 399–422.

Szlanka, T. *et al.* (2003) 'Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in Drosophila melanogaster', *Journal of Cell Science*, 116(6), pp. 1023–1033.

Tait, S. W. G. *et al.* (2007) 'Apoptosis induction by Bid requires unconventional ubiquitination and degradation of its N-terminal fragment', *Journal of Cell Biology*, 179, pp. 1453–1466.

Tatham, M. H. *et al.* (2008) 'RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation', *Nature Cell Biology*. Nature Publishing Group, 10(5), pp. 538–546.

Tenno, T. *et al.* (2004) 'Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin

chains', *Genes to Cells*. Blackwell Science Ltd, 9(10), pp. 865–875.

Tew, K. D. (2016) 'Commentary on Proteasome Inhibitors: A Novel Class of Potent and Effective Antitumor Agents', *Cancer research*, 76(17), pp. 4916–7.

Thrower, J. S. *et al.* (2000) 'Recognition of the polyubiquitin proteolytic signal', *The EMBO Journal*, 19(1), pp. 94–102.

Tu, Y. *et al.* (2012) 'The Ubiquitin Proteasome Pathway (UPP) in the regulation of cell cycle control and DNA damage repair and its implication in tumorigenesis.', *International journal of clinical and experimental pathology*, 5(8), pp. 726–38.

Unno, M. *et al.* (2002) 'The structure of the mammalian 20S proteasome at 2.75 A resolution', *Structure*, 10(5), pp. 609–618.

Unverdorben, P. *et al.* (2014) 'Deep classification of a large cryo-EM dataset defines the conformational landscape of the 26S proteasome.', *Proceedings of the National Academy of Sciences of the United States of America*, 111(15), pp. 5544–9.

VanderLinden, R. T. *et al.* (2015) 'Structural Basis for the Activation and Inhibition of the UCH37 Deubiquitylase', *Molecular Cell*. Elsevier, 57(5), pp. 901–911.

Varadan, R. *et al.* (2004) 'Solution Conformation of Lys63-linked Di-ubiquitin Chain Provides Clues to Functional Diversity of Polyubiquitin Signaling', *Journal of Biological Chemistry*, 279(8), pp. 7055–7063.

Verma, R. *et al.* (2002) 'Role of Rpn11 Metalloprotease in Deubiquitination and Degradation by the 26S Proteasome', *Science*, 298(5593), pp. 611–615.

Walters, K. J. *et al.* (2002) 'Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a', *Biochemistry*, 41(6), pp. 1767–1777.

Walz, J. *et al.* (1998) '26S proteasome structure revealed by three-dimensional electron microscopy.', *Journal of structural biology*, 121(1), pp. 19–29.

Wang, Q. *et al.* (2003) 'Ubiquitin Recognition by the DNA Repair Protein hHR23a', *Biochemistry*, 42(46), pp. 13529–13535.

Wang, Q., Young, P. and Walters, K. J. (2005) 'Structure of S5a bound to monoubiquitin provides a model for polyubiquitin recognition', *Journal of Molecular Biology*, 348(3), pp. 727–739.

Wang, X., Herr, R. A. and Hansen, T. H. (2012) 'Ubiquitination of substrates by esterification', *Traffic*, pp. 19–24.

Wilkinson, C. R. *et al.* (2001) 'Proteins containing the UBA domain are able to bind to multi-ubiquitin chains.', *Nature Cell Biology*, 3(10), pp. 939–943.

Worden, E. J., Dong, K. C. and Martin, A. (2017) 'An AAA Motor-Driven Mechanical Switch in Rpn11 Controls Deubiquitination at the 26S Proteasome', *Molecular Cell*. Elsevier Inc., pp. 1–13.

Worden, E. J., Padovani, C. and Martin, A. (2014) 'Structure of the Rpn11-Rpn8 dimer reveals mechanisms of substrate deubiquitination during proteasomal degradation.', *Nature structural & molecular biology*. Nature Publishing Group, 21(3), pp. 220–7.

Xu, D. *et al.* (2015) 'Phosphorylation and activation of ubiquitin-specific protease-14 by Akt regulates the ubiquitin-proteasome system.', *eLife*, 4, p. e10510.

Xu, P. *et al.* (2009) 'Quantitative Proteomics Reveals the Function of Unconventional Ubiquitin Chains in Proteasomal Degradation', *Cell*, 137(1), pp. 133–145.

Yao, T. *et al.* (2006) 'Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1', *Nature Cell Biology*, 8(9), pp. 994–1002.

Yao, T. and Cohen, R. E. (2002) 'A cryptic protease couples deubiquitination and degradation by the proteasome.', *Nature*, 419(6905), pp. 403–407.

Ying Lu, Byung-hoon Lee, Randall W King, Daniel Finley, and M. W. K. (2015) 'Substrate degradation by the proteasome: a single-molecule kinetic analysis', *Science*, 348(6231), pp. 1–20.

Zhang, D., Raasi, S. and Fushman, D. (2008) 'Affinity Makes the Difference: Nonselective Interaction of the UBA Domain of Ubiquilin-1 with Monomeric Ubiquitin and Polyubiquitin Chains', *Journal of Molecular Biology*, 377(1), pp. 162–180.

Zhang, N. *et al.* (2009) 'Structure of the S5a:K48-Linked Diubiquitin Complex and Its Interactions with Rpn13', *Molecular Cell*, 35(3), pp. 280–290.

Zhang, N. Y. *et al.* (2011) 'Ubiquitin chain trimming recycles the substrate binding sites of the 26 S proteasome and promotes degradation of lysine 48-linked polyubiquitin conjugates', *Journal of Biological Chemistry*, 286(29), pp. 25540–25546.