From the Department of Cell and Molecular Biology,
Medical Nobel Institute,
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF THE UBIQUITIN-PROTEASOME SYSTEM IN NEURODEGENERATIVE DISORDERS

Lisette Gerridina Gezina Catharina Verhoef

Stockholm 2006

Cover picture: Ub^{G76V}-GFP HeLa cells expressing $^{myc}UBB^{+1}$.
(Illustration by L.G.G.C. Verhoef)

Printed by Larserics digital print.

ISBN 91-7140-743-X

To my grandmother

ABSTRACT

Neurodegenerative disorders are a heterogeneous group of clinically and pathologically diverse diseases. The diseases are characterised by selective loss of neurons, in specific regions of the brain. The result is disruption of motor, sensory or cognitive systems, leading to severe disability of the patients. Despite the variability between the diseases, there are some striking similarities. A common feature in many of these diseases is the presence of aggregated proteins that are covalently linked to ubiquitin (Ub). The ubiquitin-proteasome system (UPS) is the main pathway in the cell for the elimination of aberrant or misfolded proteins. Nevertheless, in neurodegenerative diseases these proteins accumulate with disastrous consequences for neurons, eventually leading to cell death. In this thesis, the role of the UPS in neurodegeneration was investigated. These studies focus on the degradation of specific disease related proteins and the general status of the UPS under conditions of an excess of aberrant or misfolded proteins.

To evaluate the capacity of the UPS to degrade disease related proteins, polyglutamine (polyGln) proteins were targeted for proteasomal degradation. These proteins were efficiently degraded independent of the length of the polyGln repeat. However, aggregation of the aggregation-prone polyGln proteins prevented proteasomal degradation. Thus the formation of aggregates renders these toxic proteins resistant to proteasomal degradation and initiates the accumulation of polyGln proteins and polyGln-interacting proteins.

A mutant form of Ub, UBB^{+1} is another protein that can resist proteasomal degradation. UBB^{+1} accumulates in neurons of patients with several neurodegenerative diseases. We show that UBB^{+1} is a substrate of the proteasome but is too short to be efficiently degraded. The lack of UBB^{+1} degradation causes an inhibitory effect on the UPS.

The accumulation of misfolded proteins inside the endoplasmic reticulum (ER) causes ER stress which is found in many neurodegenerative disorders. Since the UPS is also responsible for the degradation of ER proteins we investigated the effect of ER stress on the functionality of the UPS. We found that ER stress compromises the UPS though not fully blocks its function. This suggests that the load of ER proteins and the ER environment may be important parameters for the gradual progressive accumulation of misfolded proteins in neurodegenerative diseases.

In conclusion, the UPS is involved in the degradation of accumulated misfolded or aberrant proteins occurring in neurodegenerative diseases. However, in these diseases the UPS is compromised and some proteins might resist degradation.

LIST OF PUBLICATIONS

This thesis is based on the following papers that will be referred to in the text by their Roman numerals:

I **Lisette G. G. C. Verhoef**, Kristina Lindsten, Maria G. Masucci and Nico P. Dantuma. Aggregate formation inhibits proteasomal degradation of polyglutamine proteins. (2002) *Hum. Mol. Genet.* 11(22):2689-2700.

II Kristina Lindsten, Femke M. S. de Vrij, **Lisette G. G. C. Verhoef**, David F. Fischer, Fred W. van Leeuwen, Elly M. Hol, Maria G. Masucci and Nico P. Dantuma. Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion-degradation substrate that blocks proteasomal degradation. (2002) *J. Cell. Biol.* 157(3):417-427.

III **Lisette G. G. C. Verhoef** and Nico P. Dantuma. Designed ubiquitin fusion degradation substrates reveal minimal substrate length for efficient proteasomal degradation. *Manuscript.*

IV Victoria Ménendez-Benito, **Lisette G. G. C. Verhoef**, Maria G. Masucci and Nico P. Dantuma. (2005) Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. *Hum. Mol. Genet.* 14(19):2787-2799.

Other publications

Florian A. Salomons, **Lisette G. G. C. Verhoef** and Nico P. Dantuma. Fluorescent reporters for the ubiquitin-proteasome system. (2005) *Essays Biochem.* 41(1):113-128. Review article.

ABBREVIATIONS

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
CBP	CREB binding protein
CFTR	cystic fibrosis membrane conductance regulator
CP	core particle
CUE	coupling of Ub conjugation to ER degradation
DALIS	dendritic cell aggresome-like induced structure
DRPLA	dentatorubal pallydolusian atrophy
DUB	deubiquitination enzyme
E1	ubiquitin activation enzyme
E2	ubiquitin conjugation enzyme
E3	ubiquitin ligase
ER	endoplasmic reticulum
ERAD	ER-associated degradation
GFP	green fluorescent protein
Gly-Ala	glycine-alanine
GRR	glycine-rich region
HD	Huntington's disease
Hsp	heat shock protein
Htt	huntingtin
IκBα	inhibitor of NF-κB
IB	inclusion body
IKK	IκB kinase
MHC	major histocompatibility complex
MTOC	microtubule organization centre
NLS	nuclear localisation signal
ODC	ornithine decarboxylase
OTU	ovarian tumour protease
Pael-R	parkin-associated endothelin-receptor-like receptor
PAZ	polyubiquitin-associated zinc finger
PD	Parkinson's disease
PHD	plant homeodomain
PIM	proteasome interacting motif
polyGln	polyglutamine
RP	regulator particle
SBMA	spinobulbar muscular atrophy
SCA	spinocerebellar ataxia
SCF	Skp1/Cul1/F-box protein
TPPII	tripeptidyl peptidase II
Ub	ubiquitin
UBA	ubiquitin associated domain
UBB^{+1}	product of ubiquitin B transcript with +1 frame shift
UBL	ubiquitin-like domain
Ubl	ubiquitin-like protein
U-box	Ufd2 homology
UBP	ubiquitin specific protease
UCH	ubiquitin C-terminal hydrolase
UEV	ubiquitin conjugation enzyme variants
UFD	ubiquitin fusion degradation
UIM	ubiquitin interacting motif
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
VCP	valosin-containing protein
VHL	Von-Hippel Lindau
YFP	yellow fluorescent protein

TABLE OF CONTENTS

ABSTRACT
LIST OF PUBLICATIONS
ABBREVIATIONS

1. AIMS OF THE STUDY 1

2. GENERAL INTRODUCTION TO THE UBIQUITIN-PROTEASOME SYSTEM AND NEURODEGENERATIVE DISEASES 2

3. UBIQUITIN, TARGETING PROTEINS FOR DEGRADATION 6
- 3.1 Ubiquitin 6
- 3.2 Ubiquitination 7
- 3.3 Ubiquitin modifications 10
- 3.4 Deubiquitination 12
- 3.5 Degradation signals 13
- 3.6 Stabilization signals 15

4. THE PROTEASOME, A LARGE PROTEASE 18
- 4.1 General introduction 18
- 4.2 The 20S core particle 19
- 4.3 The 19S regulatory particle 21
- 4.4 Alternative 20S activators 23
- 4.5 Proteasome associated proteins 24
- 4.6 Proteasome localization and regulation 27

5. GFP-BASED REPORTER SUBSTRATES TO MONITOR FUNCTIONALITY OF THE UPS 29
- 5.1 Fluorescent UPS substrates 29
- 5.2 Fluorescent reporters to monitor the UPS in neurodegeneration 30

6. THE UPS AND NEURODEGENERATIVE DISORDERS 33
- 6.1 Neurodegeneration and polyglutamine diseases 33
- 6.2 Protein aggregation 35
- 6.3 Mechanisms of pathogenesis 37
- 6.4 Involvement of the UPS in neurodegeneration 39
- 6.5 UBB^{+1}, a mutant form of ubiquitin and neurodegeneration 42

7. PROTEIN QUALITY CONTROL, ER STRESS AND NEURODEGENERATION 48

8. CONCLUSIONS AND FUTURE PROSPECTS 53

9. ACKNOWLEDGEMENTS 56
10. REFERENCES 60
APPENDIX (PAPERS I-IV)

1. AIMS OF THE STUDY

The general aim of the work presented in this thesis was to investigate a possible role of the ubiquitin-proteasome system in neurodegenerative disorders. In particular under conditions where an excess of aberrant proteins accumulate.

The specific aims were to:

- Evaluate the effect of expanded polyglutamine repeats on proteasomal degradation.

- Investigate the effect of the mutant form of ubiquitin UBB^{+1} on the ubiquitin-proteasome system.

- Study the underlying mechanism for UBB^{+1} stability and inhibitory effect on the ubiquitin-proteasome system.

- Analyse the effects of ER stress on the ubiquitin-proteasome system.

2. GENERAL INTRODUCTION TO THE UBIQUITIN-PROTEASOME SYSTEM AND NEURODEGENERATIVE DISEASES

All proteins in the cell exist in a dynamic state. Their steady-state levels are maintained by a delicate balance between synthesis and degradation. For a long time, the lysosome was thought to be the organelle in which all protein breakdown took place; extracellular proteins by endocytosis and pinocytosis and intracellular proteins through microautophagy (35). This idea was challenged by Brian Poole in 1978 (36). He carried out experiments that could distinguish between degradation of intra- and extracellular proteins and concluded:

> '*The exogenous proteins will be broken down in the lysosomes, while the endogenous proteins will be broken down wherever it is that endogenous proteins are broken down during protein turnover*'

Further research by amongst others Irwin Rose, Avram Hershko and Aaron Ciechanover, revealed that an extract of rabbit reticulocytes was able to perform ATP-dependent proteolysis (40, 72). A step-by-step identification of crucial components present in reticulocyte lysate fractions led to the discovery of ubiquitin (Ub) as a required factor for proteolysis (39). Ub was found either in a free form or covalently conjugated to other proteins. At that time Ub had already been described in processes unrelated to protein degradation and was called UBIP, for ubiquitous immunopoietic polypeptide (95). After the discovery that Ub conjugation was required for ATP-dependent proteolysis further research revealed three additional factors required for the conjugation of Ub; the Ub activating enzyme (E1), Ub conjugating enzymes (E2) and Ub ligases (E3). Nowadays we know that in eukaryotes only one E1, several E2's, and many E3 enzymes exist, and the list is still growing (91).

The ATP-dependent protease responsible for the degradation of polyubiquitinated proteins was characterized by several laboratories much later (8, 281) and is now known as the 26S proteasome (3). The proteasome is a large, compartmentalized, multisubunit protease

responsible for the proteolytic processing of polypeptides into short peptides. It can be subdivided into a 19S regulatory particle (RP) and a 20S core particle (CP). The catalytic sites are secured inside the barrel shaped core of the proteasome to prevent uncontrolled proteolysis. In order to pass the narrow entrance of the catalytic core, polypeptides have to be unfolded; a function provided by the AAA-ATPases of the 19S RP that covers one or both sides of the 20S CP.

The ubiquitin-proteasome system (UPS) (figure 1) is responsible for protein degradation in the cytosol and nucleus and is also used for the disposal of proteins from the endoplasmic reticulum (ER) through retranslocation of these proteins into the cytosol. This vital proteolytic pathway is involved in many cellular processes, like antigen presentation, transcriptional regulation, apoptosis, cell cycle progression and the turnover of aberrant or misfolded proteins. It is not surprising that many studies suggest that inefficiencies or dysfunction of the UPS are implicated, either as primary cause or as secondary consequence in several diseases, such as cancer, metabolic disorders, inflammation and genetic disorders.

Neurodegenerative disorders represent a clinically and pathologically diverse group of conditions, in which selective loss of neurons in specific areas of the brain underlies the disease symptoms. Most are complex disorders where genetic and environmental factors play a role. However, a common feature seen in many of the neurodegenerative diseases is the accumulation of abnormal protein aggregates in neurons, such as the neurofibrillary tangles in Alzheimer's disease (AD), Lewy bodies in Parkinson's disease (PD), nuclear inclusion bodies (IB) in polyglutamine (polyGln) diseases and Bunina bodies in amyotrophic lateral sclerosis (ALS). The accumulation of misfolded proteins and the presence of components of the UPS in these protein deposits were initial indications for involvement of the UPS in these diseases and indicated an attempt of the cell to degrade the aberrant proteins. Mutations in the UPS can also be a primary cause for neurodegeneration, as genetic evidence clearly demonstrates that disruption of Ub-mediated processes can lead to neurodegeneration. The most common causes of inheritable Parkinsonism are mutations affecting the *parkin* gene. Parkin is a Ub ligase and several substrates of this ligase

have been discovered, such as α-synuclein (253), a key component of Lewy bodies, synphilin-1, an α-synuclein interacting protein (34), and the misfolded parkin-associated endothelin-receptor-like receptor (Pael-R) (121). It is hypothesized that ubiquitination of parkin substrates targets them for proteasomal degradation. Disease-linked mutations impair the ligase function of parkin, causing accumulation of its substrates, and hence the underlying cause of neurodegeneration might be the alterations in protein turnover leading to compromised cell survival. However, parkin knockout mice do not develop neurodegeneration (94, 123, 210) and a null mutation of parkin in Drosophila did not impair the nervous system (98, 212). Nevertheless, upon overexpression of the parkin substrates α-synuclein or Pael-R, dopaminergic neurons specifically degenerate (74, 296). Surprisingly, none of the parkin substrates was found accumulated in neurons of parkin knockout mice (94, 202). It is possible that alternative pathways exist in mice brain for the degradation of parkin substrates (133). Other examples are mutations in the deubiquitination enzyme (DUB) ataxin-3 as a cause for spinocerebellar ataxia type 3 (SCA-3) (33), mutations in the Ub C-terminal hydrolase (UCH)-L1 causative to PD and a frame shift mutation in the Ub precursor protein leading to a mutant form of Ub that is associated with several neurodegenerative diseases. Moreover, systemic exposure of proteasome inhibitors leads to a progressive model of PD in adult rats (181).

In conclusion, there are several indications that optimal functioning of the nervous system depends on a functional UPS, however a major challenge is to understand the details of Ub-dependent proteasomal degradation in neurodegenerative disorders.

The work described in this thesis deals with a possible involvement of the UPS in neurodegenerative diseases. It covers several proteins that due to mutation or misfolding are destined for degradation but are inefficiently cleared by the UPS and accumulate in cells. Additionally this thesis describes the aberrant form of Ub UBB^{+1} found in neurodegeneration, as the first natural Ub fusion degradation (UFD) substrate and describes some minimal requirements for efficient proteasomal degradation of endogenous or engineered UFD substrates. Finally, the effect of a stress situation associated with neurodegenerative

disorders, endoplasmic reticulum (ER) stress, is examined and reveals a novel link between ER stress and the functionality of the UPS.

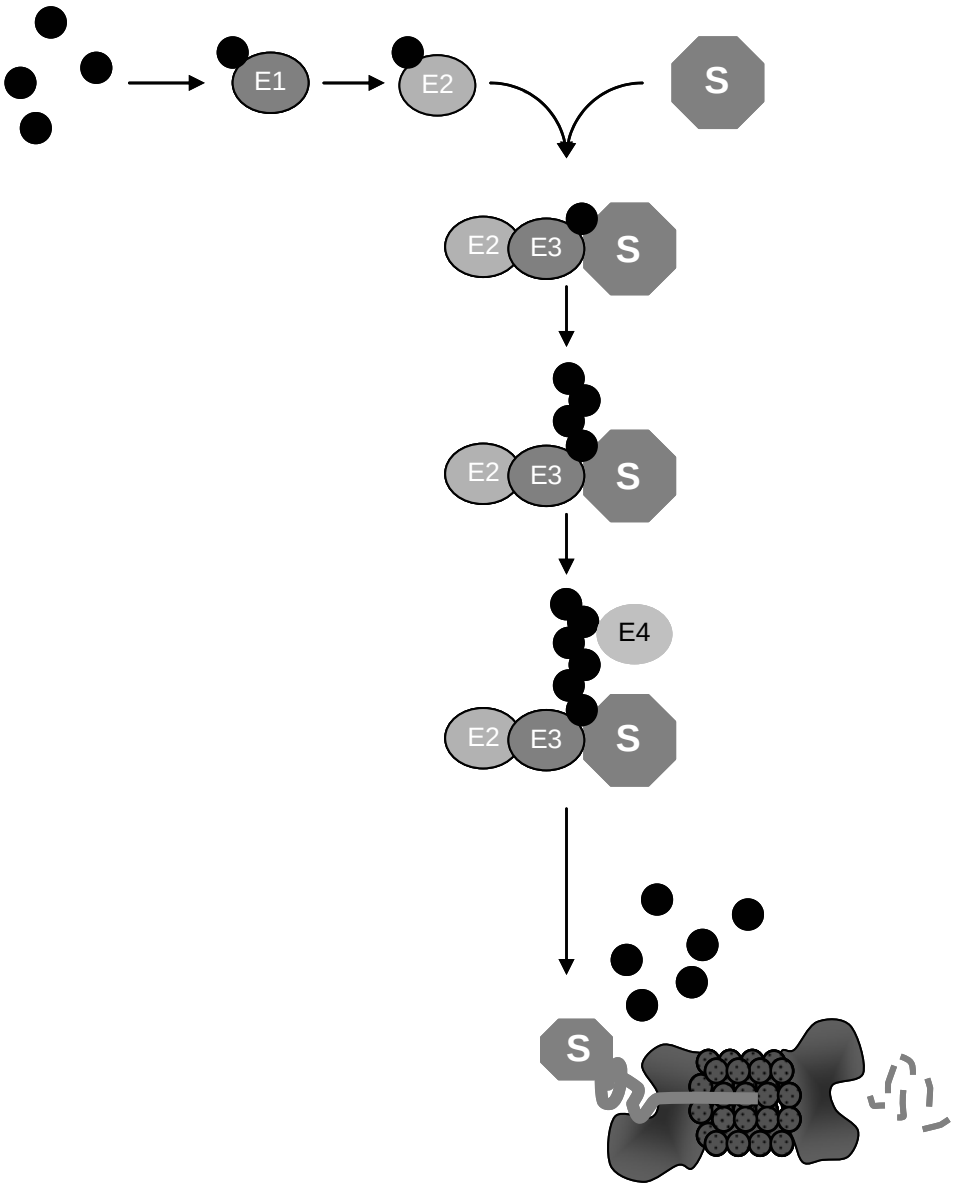

Figure 1. *The ubiquitin-proteasome system*. I) activation of Ub by E1. II) transfer of Ub to E2. III) covalent linkage of Ub to the substrate (S) which is recognized by E3. IV) Multiple rounds of Ub conjugation (I-III) leads to the formation of a polyUb chain. V) In some cases an E4 is involved in the elongation of the polyUb chain. VI) The polyubiquitinated substrate binds to the proteasome where deubiquitination takes place while the substrate is unfolded, translocated into the proteasome and degraded into small peptides.

3. UBIQUITIN, TARGETING PROTEINS FOR DEGRADATION

3.1 Ubiquitin

Ub is an essential 76 amino acid protein. Human Ub is only at three amino acids different from yeast, making it one of the most conserved proteins in eukaryotes (96, 129). Post-translational modification of a protein with Ub can consist of conjugation of one or several Ub molecules. Ub conjugation is involved in cell cycle regulation, endocytosis, viral budding, transcriptional regulation and DNA repair (109, 111). However, the best known function of Ub conjugation is the covalent attachment of multiple Ub molecules to a substrate targeting it for proteasomal degradation (109).

Ub is transcribed as a precursor protein, either as several head-to-tail fusions or as a fusion with the ribosomal proteins L40 or S27 (129). The precursor proteins are post translational cleaved into single Ub moieties by UCHs (7). The availability of several Ub genes, single or multi-copy, plus recycling of Ub after conjugation to a substrate ensures high levels that are required for a functional UPS. Interestingly, most Ub in the cell is not in a free form but conjugated to substrates, though in a dynamic equilibrium adjustable to environmental stimuli (52).

Several Ub-like proteins (Ubl), like Nedd8, SUMO, HUB1 and FAT10, are closely related to Ub. Despite little sequence homology, their tertiary structure is almost identical to Ub; five β-strands wrapped around an α-helix (246). Ubls can be conjugated to proteins in a way that resembles Ub conjugation (283). By tagging proteins with Ub or Ubls, the cell can create a large diversity of modified proteins that can be identified by downstream effector proteins and used to control many regulatory pathways in the cell (246). Interestingly, while in general conjugation of Ubl proteins does not lead to proteasomal degradation but serves other functions, FAT10 is the first Ubl that can also target for proteasomal degradation (112). Moreover, Ub and Ubl can compete for the same lysine residues in acceptor proteins, for example both SUMO and Ub can modify

the same residues in IκBα (59) and PCNA (113) possible counteracting each others functions.

3.2 Ubiquitination

Covalent linkage of Ub is a multi-step process involving at least three enzymes (109). First, Ub is activated by the Ub activating enzyme (E1), forming a thiolester linkage between the C-terminal carboxyl group of Ub and a specific cysteine (Cys) of the E1. In yeast, only one E1 exists, while in mammals two isoforms are present due to alternative translation initiation sites (43). The Ub moiety of the E1~Ub thiolester is subsequently transferred to one of the Ub conjugating enzymes (E2). The Ub moiety of the E2~Ub thiolester is conjugated via an isopeptide bond to the ε-amino group of a lysine (Lys) residue in a substrate or a preceding Ub molecule conjugated to the substrate resulting in a substrate-linked polyUb chain (figure 2). Several E2 enzymes are known; all sharing the same conserved globular domain of approximately 150 residues, with an active site Cys positioned in the highly conserved sequence (216). Interestingly, a family of Ub conjugation enzyme variants (UEV) that have a striking similarity to E2 enzymes but lack the active site have been shown to bind another E2 enzyme, Ubc13, and function as a cofactor to form an active complex (115).

Most E2 enzymes function in complexes with E3s. The functions of E3s include the initial recognition of degradation signals (degrons) in substrate proteins, with different E3 enzymes recognizing different classes of degrons. At present several hundred E3 enzymes are known with the list still growing. Most E3s are classified into two families: HECT (homology to E6-associated protein C-terminus) and RING (really interesting new gene) E3s, based on their catalytic modules and features of sequence and structure (2). Additionally, the RING finger group of E3s can be subdivided in classic RING fingers and UFD2 homology (U-box) proteins. A HECT-domain E3 can accept a Ub moiety from an associated E2~Ub thiolester, forming an E3~Ub thiolester and acting as a proximal Ub donor to the substrate it selects. In contrast, formation of thiolesters between the RING E3s and Ub has not been detected (91) (figure 2). It is thought that a RING E3s act as an adaptor to optimize the orientation of

the ubiquitination site of a substrate to the active site of the E2, which allows the transfer of the Ub molecule from an E2~Ub thiolester to the substrate. Additionally, RING finger proteins are capable of auto-ubiquitination suggesting a mechanism by which E3s, many of which unstable, might regulate their own stability (125). RING-finger E3s come in different flavours; as single subunit or multi-subunit proteins. Among the multimeric RING E3 are the APC/cyclosome complex involved in degradation of cell cycle regulators, the Von-Hippel Lindau (VHL)-Elongins B and C (VBC)-Cul2-RING finger complex, involved in the degradation of HIF1α, and the Skp1-Cullin/Cdc53-F-box protein (SCF)-RING finger complexes involved in the degradation of signal- and cell cycle-induced phosphorylated proteins. In SCF and VBC, the RING-finger domain component Rbx1/Hrt1/Roc1 is involved in the E2 recruitment and assembly of other components of the complex, but not in substrate recognition. The F-box protein, the variable component of the SCF complex, and most probably the pVHL subunit in VBC are responsible for substrate recognition (2).

Structural analysis of the plant homeodomain (PHD) revealed remarkable similarity with RING-fingers (24). The PHD domain was first recognized in an *Arabidopsis* homeobox protein (243). The discovery of PHD domains in viral proteins revealed the link between PHD domains and RING-finger E3s (45). The murine γ-herpesvirus-68 K3 (MEK3), is a PHD containing protein with Ub ligase activity to target the major histocompatibility complex (MHC) class I for proteasomal degradation (16). Similar activity was found for the PHD containing cytosolic protein MEKK1 to mediate ubiquitination and degradation of ERK1/2 (170).

The Ub chain elongation factor E4, was shown in some cases to be necessary for efficient polyubiquitination (146). E4 defines a novel protein family that shares a modified version of the RING finger, designated as U-box. A number of U-box proteins have been shown to elongate Ub chains dependent on E1 and E2 but independent from E3, suggesting that the E4 represents another E3 ligase activity (106). However, in other cases the U-box E3 ubiquitinates substrates in concert with classic E3s (146). An interesting U-box protein is carboxy terminus of Hsc70 interacting protein (CHIP). CHIP is involved in the degradation of misfolded proteins such as the mutant cystic fibrosis transmembrane conductance regulator

(CFTR)ΔF508 and the unfolded Pael receptor involved in PD. CHIP can interact with Hsc70-Hsp70 and Hsp90 which are involved in refolding of misfolded proteins, while CHIP targets for proteasomal degradation; thus CHIP is probably involved in regulating the cellular balance between folding and degradation (179).

Once a single Ub is attached with its C-terminal glycine (Gly) residue to an internal Lys residue in the substrate, additional rounds of ubiquitination can take place attaching the next Ub molecule with its C-terminal Gly to a Lys residue in the previous Ub molecule, forming a polyUb chain. Interestingly, E1 and E3 binding sites on E2 overlap, and their binding is mutually exclusive (68). Therefore it is not possible for E1 and E3 to bind to E2 at the same time during the formation of polyUb chains. Multiple cycles of E2-E3 binding and release are probably necessary.

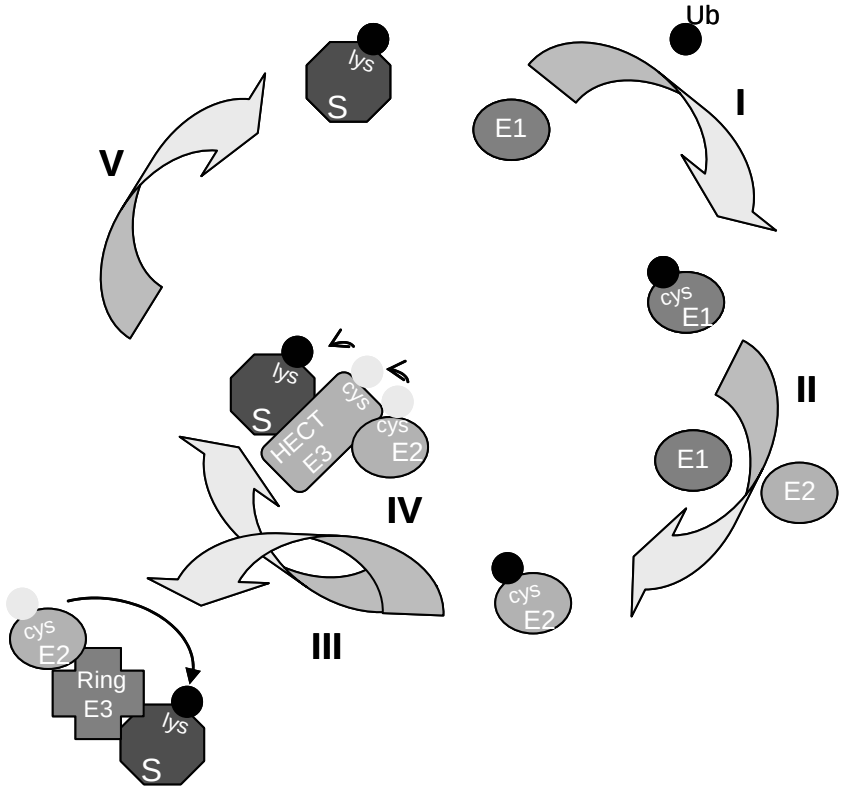

Figure 2. *Ubiquitination*. I) Ub is activated by E1. II) Activated E1 is then transferred to a Cys residue in E2. III) Ub is conjugated to a Lys residue in the substrate (S) with help of a RING E3 that does not bind to Ub itself. IV) Alternatively, Ub can be transferred from E2 to a Cys residue in a HECT E3 prior to conjugation to a Lys residue in the substrate. V) Both III and IV lead to the formation of an isopeptide bond between the Lys residue of the substrate and Ub.

While in the majority of cases polyubiquitination takes place at a Lys residue, other residues might also function as Ub acceptor sites. Linear fusion of the first Ub in a chain to the α-NH2 group of the N-terminal residue referred to as N-terminal ubiquitination might occur for a selected group of substrates, like the myogenic transcriptional switch protein MyoD, the human papillomavirus 16 (HPV16) oncoprotein E7, latent membrane proteins of the Epstein-Barr virus LMP1 and LMP2A, and the cell-cycle-dependent kinase p21 (37). A recent study provided the first indication of an isopeptide bond between Ub and a Cys residue in the substrate that can target for degradation (22).

Degradation independent of Ub has been described for several substrates, some of which can be degraded in a Ub-dependent manner as well. One of the model proteins that have been used for a long time is casein, the breakdown of which can occur in the absence of Ub, even though Ub enhances its degradation. Casein however, lacks a defined tertiary structure. In a similar way, denaturation of ovalbumin is sufficient for proteasomal degradation (10). Nevertheless, there are substrates with a defined tertiary structure that can be degraded in a Ub-independent manner: ornithine decarboxylase (ODC) is degraded upon non-covalent association with its cofactor antizyme (42). Interestingly, like Ub, antizyme is recycled after targeting its substrate to the proteasome (42). In the case of ODC, the interaction with antizyme provides a tag for degradation, but how other folded substrates are targeted to the proteasome remains unknown. In the case of calmodulin it is a special tertiary structure that is the recognition signal for the proteasome (10). In the absence of calcium, calmodulin undergoes spontaneous chemical modifications and thereby looses its ability to bind calcium. Consequently, the helixes of the calcium binding loops become less tightly bound. This higher flexibility possibly leads to recognition of calmodulin by the RP of the proteasome and subsequent degradation.

3.3 Ubiquitin modifications

Ub contains seven Lys residues at position 6, 11, 27, 29, 33, 48 and 63. In theory each of the Lys residues can be a target for attachment of another Ub molecule to form a polyUb chain (209). Most of the chains

have indeed been found *in vitro* (209). Predominantly the Lys^{48} linked chains target proteins for proteasomal degradation, and overexpression of a Ub mutated at Lys^{48} (Ub^{K48R}) in yeast is lethal (77). However, Lys^{48} linked Ub chains are not always a signal for degradation as shown for the hepatocyte growth factor/scatter factor Met. Polyubiquitinated Met through Lys^{48} linkage leads to Met endosomal trafficking (26, 79). For proteasomal degradation, a chain of at least four Ub molecules is required, suggesting that the surface provided by the four-monomer structure of a polyUb chain is recognized by the proteasome, rather then a single Ub molecule (269).

Polyubiquitination through Lys^{29} has also been shown *in vivo*, though it is not common. The only known substrates which are tagged with a Lys^{29} chain are non-cleavable Ub fusion degradation (UFD) substrates. In **paper II**, we show that UBB^{+1} is a UFD substrate that is targeted for proteasomal degradation through the linkage of Ub to both Lys^{29} and Lys^{48} (167). On the other hand, *in vitro* studies propose that polyubiquitination of UFD substrates by the E4 is only through Lys^{48} linked Ub chains (233). It remains possible that the first Ubs are conjugated to Lys^{29} but that chain elongation proceeds through Lys^{48} ubiquitination (146).

Conjugation of other Ub chains has not been reported to lead to proteasomal degradation. Yeast cells expressing the K63R Ub mutant (Ub^{K63R}) are defective in DNA repair but proteolytically competent, indicating a role for Lys^{63} linked chains in DNA repair (259). Lys^{63} linked chains also signal activation of the IκBα kinase (IKK) in inflammatory signalling pathways (263). Another target for Lys^{63} chains is the Ub ligase TRAF6, which leads to the activation of IKK (136).

The breast and ovarian cancer specific tumour suppressor BRCA1, when in complex with BARD1, functions as a Ub ligase and has the capacity to form Lys^{6}-linked chains (199). Moreover, Ub mutated at Lys^{6} is shown to inhibit Ub-dependent degradation (249).

Lys^{11} Ub chains have only been shown *in vitro* to target for proteasomal degradation however, their biological function remains unknown (5).

Based on the localization of the different Lys residues in the tertiary structure of Ub, it is possible that the different linkages form a different

structure of the polyUb chain, representing distinct functions (217). Furthermore, different charges on the surfaces of Ub might play a role in interactions with other proteins. Ub contains for example a hydrophobic patch formed by leucine at position 8, isoleucine at 44 and valine at position 70. These hydrophobic residues together with the electrostatic potential caused by the positive charges around the hydrophobic patch play a possible role in interaction with other proteins that might influence the function of the polyubiquitinated protein.

Besides polyubiquitination leading to proteasomal degradation, covalent attachment of only a single Ub to an internal Lys in a protein, i.e. monoubiquitination regulates several other processes, for example histone regulation, endocytosis and the budding of retroviruses from the plasma membrane (111). Monoubiquitination is involved in membrane trafficking and sorting of internalized proteins that are degraded in the lysosome, linking the Ub not only to proteasomal but also lysosomal degradation.

3.4 Deubiquitination

While an array of enzymes is involved in the conjugation of Ub to substrates, there are also several deubiquitination enzymes (DUBs) known. DUBs can be subdivided into five different classes based on their sequence similarity and mechanisms of action; Ub specific proteases (UBP), UCH, ovarian tumour-related proteases (OTU), ataxin-3/Josephin domain and Jab1/Pad1/MPN domain metallo-enzyme (JAMM or MNP+) (1, 290). Four of the subclasses are cys proteases while the fifth class (JAMM) is a novel type of metalloprotease (15). UBPs and UCHs comprise the two larges families. UCH are generally small enzymes (20-30kDa) that remove short peptides from the C-terminus of Ub. UBPs are larger enzymes (~100kDa) that can cleave isopeptide bond linkage between Ub-Ub and Ub-protein. In addition they can also cleave linear fusions of Ub.

DUBs play an important role in maintaining the steady state levels of free Ub and in affecting the stability of Ub conjugated proteins (1). This includes the generation of Ub, recycling of Ub, editing polyUb chains and assisting in proteasomal degradation. Ub is expressed as a precursor protein either as head-to-tail fusions or as fusions to ribosomal subunits

that can be cleaved into single Ub molecules through the action of UCHs. Ub is also recycled by DUBs that remove the whole Ub chain from a substrate or that disassemble chains. DUBs that disassemble polyUb chains such as IsoT, may act as negative regulators of Ub-dependent degradation since deubiquitination of these chains counteracts Ub-dependent degradation (218).

Deubiquitination activity has also been reported to be connected to the 19S RP (102). Rpn11, a subunit of the lid of the 19S RP was found responsible for a fraction of proteasome-associated deubiquitination activity (287, 297). Interestingly, Rpn11 is also the most conserved non-ATPase subunit of the proteasome (102). The lid and the base have been found independently to contribute to deubiquitination at the proteasome, suggesting that additional DUBs associate with the proteasome. Indeed, several proteasome-associated DUBs were identified. Ubp6 is a ~60kDa DUB that in addition to the UBP domain at its C-terminus also contains a Ub-like domain (UBL) at its N-terminus which interacts with Rpn1, a subunit of the 19S base (18). Another proteasome associated DUB is UCH37, involved in trimming of polyUb chains from their distal end (153, 156). UCH37 is located near the polyUb binding subunit Rpn10 (116). In budding yeast, Doa4, a DUB of the UBP family, interacts weakly with the proteasome and is involved in the release of Ub by trimming short residual polyUb chains from proteasome bound substrates (266).

3.5 Degradation signals

Degradation of a protein by the proteasome is initiated by the recognition of a degradation signal, also known as degron, in the substrate by a Ub ligase. Among the degradation signals, the N-end rule is probably the best characterized. The N-end rule relates the half-life of a protein to its N-terminal residue. The discovery came from a study on Ub genes, which encodes fusions of Ub itself or other proteins (6). When an engineered Ub-β-galactosidase fusion was expressed in *Saccharomyces cerevisiae*, it was efficiently deubiquitinated by Ub specific proteases. Moreover, the deubiquitination of Ub-X-β-galactosidase occurred irrespectively of the identity of the X amino acid, with the exception of proline. Surprisingly, depending on the N-terminal amino acid of β-galactosidase, it was either

rapidly degraded or a long-lived protein. In addition to a destabilizing N-terminal amino acid, the protein needs a lysine residue in close proximity to the N-terminus that can serve as a Ub acceptor (6, 265). Several substrates have been discovered since then linking the N-end rule pathway to i.e. chromosome stability (197), regulation of peptide import (21), apoptosis (63) and muscle wasting (23).

Ub fused to the N-terminus of a protein can serve as a 'primary' degradation signal itself (130). Ub fusions are normally efficiently removed in the cell by DUBs which require the di-glycine motif at the C-terminus of Ub. Mutating this glycine to an alanine or a valine leaves a 'non-removable' Ub moiety. The N-terminal Ub moiety functions as the anchor for polyUb chains. UFD substrates are rapidly degraded by the proteasome in a Ub-dependent fashion. Interestingly, in contrast to most proteasomal substrates that are targeted for degradation through the attachment of a Lys^{48} Ub chain, UFD substrates are polyubiquitinated at Lys^{29}, Lys^{48} or both lysines of the Ub moiety (131). The reason for ubiquitination at both Lys^{29} and Lys^{48} remains unknown, but the double Ub tree might have an effect on the strength of the binding to the proteasome. Even though the UFD pathway is very well characterized, the only known natural occurring UFD substrate is UBB^{+1} (identified in **paper II**).

Degradation of many cyclins and other cell cycle related proteins is mediated by the destruction box. It consists of a conserved, nine amino acids sequence motif usually located at 40-50 amino acids from the N-termini of the cyclins (201) . Another polypepide stretch targeting proteins for proteasomal degradation has been described for the 67 residue-long Deg1 region of Matα2, a yeast transcriptional regulator (31).

Several post-translational modifications have been described to target proteins for degradation. Fbx2, the F-box protein of the SCF E3 was found to recognize N-linked high-mannose oligosaccharides (301). Another post-translational modification that functions as a degradation signal for several proteins is phosphorylation. Phosphorylation at two specific lysine residues of the inhibitor of the transcriptional activator NF-κB, IκBα, results in recognition by a specific Ub ligase and degradation of IκBα. Similarly, phosphorylation of β-catenin, a protein playing an

essential role in embryogenesis and oncogenesis leads to ubiquitination and degradation.

IκBα has, in addition to its inducible degradation signal (phosphorylation) also a PEST sequence that can target IκBα for degradation. PEST sequences are characterized by enrichment in proline (P), glutamic acid (E), serine (S) and threonine (T). They range in length from 12 to 60 residues, and are often flanked by positive charged amino acids. Interestingly, it was pointed out that several, though not all PEST sequences contain phosphorylation sites (109). All known PEST containing proteins appear to be important regulatory molecules, the degradation of which is coupled to environmental changes or cell cycle stage (223).

Protein misfolding is another signal for destruction by the proteasome. While normally located inside globular protein molecules or buried in membranes, exposure of a hydrophobic stretch can be recognized by the UPS. Moreover, it has been suggested that non-native states of proteins caused by mutations or denaturation lead to their accelerated degradation (204). Mutant polyGln proteins, responsible for several neurodegenerative polyGln diseases (see chapter 6.1) contain an expanded glutamine repeat that possible causes protein misfolding and renders the protein aggregation prone. In **paper I**, an expanded polyGln repeat was insufficient to target green fluorescent protein (GFP) for degradation, indicating that just the presence of this aggregation-prone domain was not sufficient for recognition by the UPS.

3.6 Stabilization signals

Besides degradation signals, several proteins resist proteasomal degradation possible due to an intrinsic stabilizing domain (55). Stabilization signals include repetitive sequences and small protein domains. There are additional factors that can contribute to the stability of a protein; for example deubiquitination activity might counteract proteasomal degradation and thereby extend the half-life of a protein.

The glycine-alanine (Gly-Ala) repeat of the Epstein-Barr virus nuclear antigen 1 (EBNA1) can protect against proteasomal degradation (162), which possible aids the virus to escape immune recognition in latent infections (161, 162). How the Gly-Ala repeat resists proteasomal

degradation remains unclear, however it has been shown to be sequence and length dependent (53, 250). The Gly-Ala repeat has furthermore been shown to be a transferable element with an *cis* acting inhibitory effect (161). It has been suggested that the ATPases of the 19S RP slip over the Gly-Ala repeat thereby hindering translocation of the protein into the 20S CP (305). Interestingly, introduction of a strong degradation signal can partially overcome the stabilizing effect of the Gly-Ala repeat (53).

Another example is the partial processing of the NF-κB precursor p105 (73, 203). Ubiquitination of the C-terminus of p105 signals degradation, however, degradation stops when a Gly rich region (GRR) is reached leaving the N-terminal p50 intact (164). Interestingly, the GRR contains a Gly-Ala-Gly-Ala-Gly amino acid sequence, a motif similar to the Gly-Ala repeat of EBNA1 though much shorter. The homologous yeast transcription factors Spt23 and Mga2 are related to NF-κB and control unsaturated fatty acid levels. Like p105, Spt23 and Mga2 are partially processed by the UPS, releasing an active transcription factor (117).

Another repeat containing protein, the polyGln protein, can also resist proteasomal degradation (**paper I**). Expansion of the polyGln repeat over a certain threshold is causing neurodegeneration (311). PolyGln repeats can be found in several unrelated proteins but their function remains unknown. Several transcription factors contain polyGln repeats suggesting the repeat might play a role in protein-protein interactions. We show that expanded polyGln proteins resist proteasomal degradation through the formation of inclusion bodies (IBs) (286).

The Ub associated (UBA) domain has recently been shown to act as another stabilization signal (107). Ub like domain (UBL)-UBA containing proteins are thought to function as shuttle factors for polyubiquitinated proteins sorting them to the proteasome (30). Interaction with polyubiquitinated substrates is facilitated by the UBA domain while the UBL domain interacts with the proteasome forming a physical bridge between substrate and proteasome. How exactly these repeats or the UBA domain prevent proteins from degradation remains unknown.

Several proteins have been described that resist proteasomal degradation by different mechanisms. Despite the presence of intrinsic degradation signals, they all carry an additional 'signal' that stabilizes the

protein. The question remains why proteins would need a stabilization signal. It might provide the opportunity for ubiquitination without degradation. Alternatively a stabilization signal might provide a new level of regulating proteasomal degradation. If the different classes of stabilization signals exist remains to be seen.

4. THE PROTEASOME, A LARGE PROTEASE

4.1 General introduction

Proteins in the cell have very different half-lives, from less then a minute to several days. For many years, cellular protein degradation was thought to take place in lysosomes only. However, it slowly became clear that there was another site for intracellular protein degradation that was depending on ATP. The 26S protease was discovered by its ability to degrade Ub-lysozyme conjugates (118). Nowadays we know that the 26S proteasome is part of the main pathway for non-lysosomal degradation. It is for example responsible for the elimination of aberrant or misfolded proteins (**paper I & II**), the maintenance of a free amino acid pool (102, 273), generation of fragments that act like hormones, antigens (145) or other effectors (61) and for the regulation of the half-life of proteins that have to vary in concentration over time in the cell (109). However, some proteolytic independent processes of the proteasome have more recently also been revealed (75, 194, 231, 282). The 26S proteasome is an approximately 2.5 MDa multisubunit, ATP-dependent complex. It consists of two subcomplexes (figure 3); the 20S core particle (CP), containing the three distinct proteolytic activities and the 19S regulatory particle (RP) involved in binding, unfolding and translocation of the substrate and opening of the 20S CP.

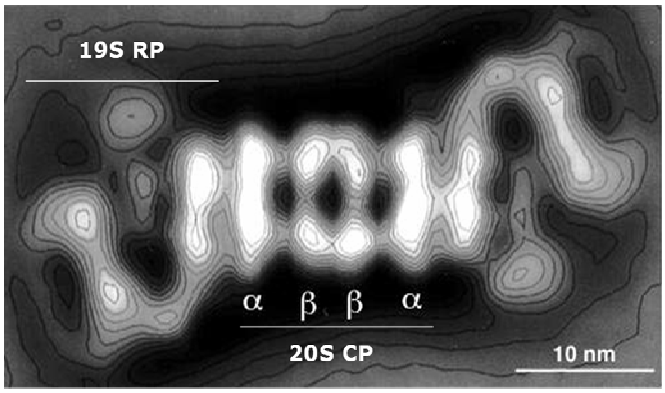

Figure 3. *The 26S proteasome*. The 26S proteasome consists of a 19S RP and a 20S CP. The 20S CP consists of two outer α rings and two inner β rings that contain the catalytic active subunits.

4.2 The 20S core particle

The structure of the 20S CP was first determined by X-ray crystallography (100). It revealed a cylindrical structure composed of four stacked heptagonal rings. Each of the two outer rings is composed of seven structurally similar α subunits, and each of the two inner rings is composed of similarly conserved β subunits. In eukaryotes, three of the β subunits have proteolytic activity. Thus each proteasome has six (three different) proteolytic sites. The proteolytic sites are faced to the inner cavity of the proteasome to ensure controlled proteolysis in a 'closed' environment. The combined action of the three catalytic sites allow proteasomes to cleave virtually after any amino acid but the three catalytic activities have each their own specificity. The β1 subunit has post-glutamyl peptide hydrolysing (PGPH) activity, cleaving preferably after acidic amino acids. The β2 subunit has trypsin-like activity, cleaving preferably after basic amino acids and the β5 subunit displays chymotrypsin-like activity, responsible for cleavage after hydrophobic amino acids (62).

The three catalytic subunits of the 20S CP can be exchanged upon stimulation with interferon γ (IFNγ). The β1, β2 and β5 are replaced by LMP2, MECL1 and LMP7, respectively, upon IFNγ induction (82). Cells with an antigen presenting function constitutively express LMP2, MECL1 and LMP7 (145). Change of the catalytic subunits leads to an increased generation of peptides with a hydrophobic C-terminus, which is preferred by MHC class I molecules (227). The N-terminus of the peptide is thought to be generated by an ER aminopeptidase associated with antigen processing (ERAAP1) (237, 247).

In order to be hydrolysed, a protein has to enter the 20S CP. However, the N-termini of several α-subunits cover the entrance of the CP obstructing the entry of unfolded polypeptides (99). The importance of the N-termini is revealed by the fact that they are highly conserved across eukaryotes even though the tail sequences of each of the α-subunits are different from each other (99). Opening of the catalytic core can be performed by deleting the N-terminal tail of the α-3 subunit or by mild chemical treatments such as addition of sodium dodecylsulfate. Naturally, binding of the 19S RP to the 20S CP results in a rearrangement of the N-

terminal tails of the α-subunits and opening of the narrow gate (100). Interestingly, a recent paper by Liu et al suggested that some unfolded substrates can open the entrance of the 20S CP in the absence of the 19S RP (169). Their study revealed endoproteolytic activity of the proteasome suggesting that substrates can also be degraded starting from the middle of the protein. Endoproteolytic cleavage of the proteasome provided a model for the cleavage of the NF-κB precursor p105 that is rapidly processed by the proteasome to release the N-terminal p50 protein (164). Entry of substrates into the 20S CP is thought to be the rate limiting step in protein degradation as an 'open' 20S yeast mutant, in which the α3 N-terminal tail has been deleted, provides much faster peptide hydrolysis (100). Although free purified 20S CP can hydrolyse small peptides and some unfolded polypeptides, it cannot degrade polyUb proteins.

The average length of the peptides generated is 7 to 9 amino acids, even though it varies between 3 and 23 amino acids (141, 142). A small portion of the generated peptides is used for MHC class I antigen presentation while the majority are further processed into amino acids by cytosolic peptidases such as amino peptidases (12) or tripeptidyl peptidase II (TPPII) (241). Interestingly, TPPII can compensate for low proteasome levels (88, 90). Proteasomes inactivated by treatment with covalently binding inhibitors allow outgrow of inhibitor-resistant cells. Similar to the inhibitor-resistant cells, Burkitt's lymphoma cells are less sensitive to proteasome inhibitor and TPPII is upregulated in these cells (87).

In addition to polypeptide hydrolysis, the 20S proteasome might function as a storage place for substrates. A recent study by Sharon and co-workers suggested that several substrates can be stored in the antechambers of the proteasome which might be of particular importance if degradation is slower then translocation into the 20S CP (251). The storage capacity might be to enhance degradation by providing a constant flow of substrates and thereby prevent accumulation.

Several small peptides that can reversible or irreversible inhibit the proteolytic activity of the proteasome have been identified (195). Some of these inhibitors are specific to one of the catalytic sites while others are more general proteasome inhibitors. In either case, the small molecules are 'suicide substrates', binding irreversible to the catalytic sites. Since

cells die upon addition of chemical proteasome inhibitors, it was surprising that the proteasome inhibitor bortezomib (also called PS-341 and Velcade) is successfully used in the treatment against cancer (188). This drug has mainly been successful in treatment against multiple myeloma. Multiple myeloma is a secretory lymphoma; it secretes or generates large amounts of immunoglobulines. Secretory cells have a continuous unfolded protein response (chapter 7), and proteasome inhibition in such cells would lead to ER-induced apoptosis which would explain the success of proteasome inhibition in these cancers.

4.3 The 19S regulatory particle

The 19S RP, also called the 19S cap serves many functions in regulating proteasomal activity. It activates the 20S proteasome, serves as a docking site for polyubiquitinated proteins and unfolds and translocates polypeptides into the 20S catalytic core. The RP consists of two subcomplexes, the lid and the base (figure 4). The base is composed of two large non-ATPase subunits, Rpn1/S2 and Rpn2/S1 (the yeast/human nomenclature is used) that contain multiple leucine rich repeats (LRR), a domain for protein-protein interactions, possibly functioning as a scaffold for interacting proteins (172). The base further contains six smaller ATPase subunits, Rpt1/S7, Rpt2/S4, Rpt3/S6, Rpt4/S10b, Rpt5/S6', Rpt6/S8, that are members of the AAA-ATPases family (208). The six AAA-ATPases form a ring at the base of the 20S proteasome. Interestingly, this has also been found in several other compartmentalized proteases (103).

The ATPases are also thought to be able to interact with the proteasome ATPase-associated factor-1 that prevents binding of the ATPases to the 20S core and thereby negatively regulate proteasome activity (206). Binding of the 19S RP to the 20S CP opens a narrow hole into the catalytic core of the 20S proteasome.

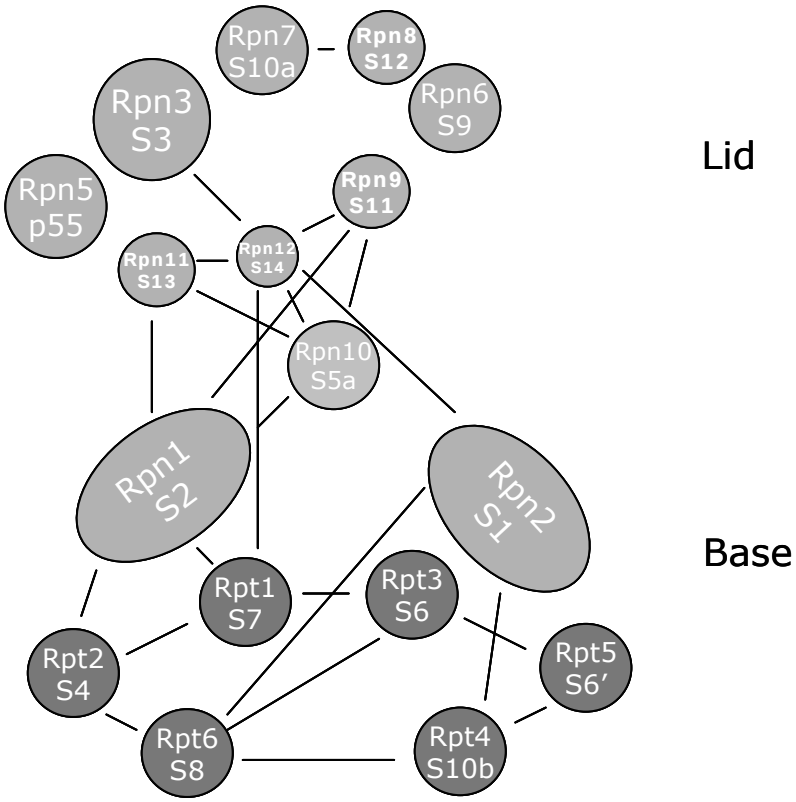

Figure 4. *Subunits of the 19S RP and their interactions with each other*. The lid and the base are indicated. The yeast/human nomenclature is used. Adjusted from biomol (www.proteasome.com).

Protein unfolding has to take place prior to translocation through the narrow opening (80), another function provided by the ATPases. The ATPase subunit Rpt5/S6' has polyUb binding capacity (154), providing an additional docking site for proteasome substrates. ATP hydrolysis is required for binding of the polyUb substrate to the Rpt5/S6' subunit. Remarkably, proteins that are degraded in a Ub-independent manner seem to be recognized by the same elements in the 19S RP that recognize Ub conjugates (306). ATP hydrolysis is not only required for binding of the 19S to the 20S but also for protein degradation (91). The conformational change that the ATPase subunits undergo with the ATPase cycle might function in unfolding and translocation. It remains unknown if binding of a polyubiquitinated substrate to the ATPase subunit Rpt5/S6' initiates unfolding and subsequent activation of translocation into the 20S CP. It has been suggested that upon ATP hydrolysis and substrate degradation the proteasome disassembles into the 20S CP and the 19S RP, the so called 'chew and spew' model (4). Even though the exact mechanism of how the assembly and disassembly takes place and what triggers it

remains unknown, it suggests that proteasomes are not stable particles but take part in a tightly controlled cycle of assembly and disassembly of the 20S, the 19S and the interacting proteins during protein degradation. With the disassembly of the proteasome peptides may be released.

The 19S lid is added on top of the 19S base and is composed of eight non-ATPase subunits, Rpn3/S3, Rpn5, Rpn6/S9, Rpn7/S10a, Rpn8/S12, Rpn9/S11, Rpn11/S13 and Rpn12/S14, most of which functions are not very well known (figure 4). The lid is believed to be anchored to the base by Rpn10/S5a (92). Rpn10/S5a is found associated to the base, where it binds the lid. In yeast Rpn10 is also found free in the cell and was suggested to bind and transport polyubiquitinated proteins to the proteasome (70) (see also chapter 4.5). In addition, Rpn10/S5a was the first proteasomal subunit that has been found to bind polyUb conjugates through its ubiquitin interacting motif (UIM) domains (See also chapter 4.5).

Another function of the 19S cap is deubiquitination of substrates bound to the 19S. In addition to tightly associated DUBs to the proteasome, the Rpn11/S13 subunit of the lid has been associated with metalloprotease activity, responsible for deubiquitination of polyUb chains (287, 297) (See also chapter 3.4).

The 19S RP can also function independent of the 20S CP or at least independent of the proteolytic activity of the 26S proteasome. The 19S RP is involved in elongation processes in transcription (75, 194) and plays a role in DNA repair (231, 282). How exactly the proteasome regulate these processes independent of its proteolytic activity remains to be determined. Possibly, the chaperone activity of the ATPases functions in remodelling of protein conformations or interactions. In line with this idea, the 19S ATPases have been shown to have chaperone activity and can fold a substrate without degrading it, even in the presence of the 20S CP (20).

4.4 Alternative 20S activators

Two alternative complexes have been found associated with the 20S CP in mammalian cells; 11S REG or PA28α/β and REGγ/PA28γ. The complex formation between the 20S and PA28α/β can be stimulated with the

immune-regulatory cytokine IFNγ (221). In contrast to the 19S regulator, the PA28α/β complex can stimulate the hydrolysis of small peptides but can not unfold or deubiquitinate proteins (66, 173). PA28α/β consists of two subunits, α and β which form a heteroheptamer, that can attach to either one or both sites of the 20S CP in an ATP-independent way. Even though these two subunits are expressed in many tissues, they are particularly abundant in immune tissues but virtually absent from the brain (222). Proteasomes generate the vast majority of 8-11 peptide residues presented on MHC class I molecules and PA28α/β contributes to MHC class I presentation (222). Mice lacking PA28α/β have impaired MHC class I-restricted antigen presentation (193, 219). It has been suggested that PA28α/β stimulates MHC class I presentation by opening a wide channel through the α ring of the proteasome, leading to an increased release of large peptides with a proper size for antigen presentation (288). Alternatively, PA28α/β might alter proteasomal cleavage sites within a polypeptide, thereby generating unique epitopes (193). Another option might be that the PA28α/β complex binds directly to the peptide loading complex, thereby making a channel from the peptide to empty MHC class I molecules (222).

In contrast to the predominant cytoplasmic localization of the PA28α/β complex, PA28γ has highest expression in the nucleus. Furthermore, PA28γ does not respond to induction of IFNγ and forms a homoheptameric ring that can also attach to one or either site of the 20S CP. Unlike PA28α/β, PA28γ is also present in organisms lacking an adaptive immune system, and is thought to be an evolutionary precursor of the PA28 α and β subunits (313). Mice lacking PA28γ show a subtle growth retardation, suggesting a role in cell proliferation and body growth, but have no obvious defects in their immune system consistent with a different function for PA28γ compared to PA28α/β (191).

4.5 Proteasome associated proteins

S5a/Rpn10 was the first Ub binding protein to be discovered (60). However, deleting the gene in yeast did not reduce viability and only affected degradation of a small group of substrates, indicating there must be additional pathways for substrate recognition. In yeast, most of the

Rpn10 is free and not part of the proteasome (276), suggesting that substrate recognition might involve transient interactions with the proteasome rather than intrinsic proteasome subunits.

Efficient delivery of proteins to the proteasome involves in many cases specific Ub binding proteins (187). These proteins include co-chaperones, multimeric ATPases and UBL-UBA shuttle proteins (187) (figure 5). None of these shuttling factors is part of the proteasome but facilitate a transient interaction between substrate and degradation machinery providing more efficient proteolysis. Additionally, shuttle factors might shield the polyubiquitinated substrate from DUBs on their way to the proteasome to ensure degradation (215). Considering the large amount of different substrate proteins that are degraded by the proteasome, multiple carriers might work in parallel, and selection of substrates might possible depend on the length of the Ub chain (226).

UBL-UBA proteins are a group of shuttle factors including Rad23, Ddi1 and Dsk2. The UBA domain facilitates binding to polyUb chains and the UBL domain can interact with the proteasome (70). Rad23 is protected from degradation through one of its UBA domains (107). One of the mammalian Rad23 homologues, hHR23B, can bind Rpn10/S5a through its UBL domain, but is also able to bind the proteasome independently of Rpn10/S5a (242), indicating that there might be at least one additional site for binding of Ub or UBL domains. There is no indication in the literature that monoUb can target proteins for proteasomal degradation. Therefore, binding of proteins such as Rad23, with a UBL domain, might be different from polyUb binding. The binding might also be prolonged since DUBs are not able to cleave the UBL domain, as would be possible for monoubiquitinated proteins, which would lead to rapid release of the substrate.

Interestingly, UBB^{+1} contains a UFD signal which resembles the uncleavable UBL domain. The UBL domain can bind to the proteasome in a Ub-independent manner and we show in **paper III** that the bulk of UBB^{+1} associated with the proteasome lacks polyUb chains. Possibly, binding of UBB^{+1} to the proteasome might resemble more the binding of shuttle proteins then polyubiquitinated substrates.

Recently, a five amino acids conserved domain has been discovered in several UBL domains of different proteins such as Parkin, Dsk2 and

Ubp6, which is important for proteasome binding (272). This so called proteasome-interacting motif (PIM) is however not found in all UBL domains and it remains to be determined if PIM domains are present in other proteins as a general conserved motif for proteasomal interaction. Interestingly, one of the missense mutations in parkin linked to PD is located within the codon encoding one of the conserved amino acids of the PIM motif suggesting that the PIM domain may have biological significance. In addition, PIM motifs have been found in some transcription factors. Since the 19S RP is involved in transcription, PIMs in transcription factors might help to recruit to 19S cap to the transcription machinery.

Chaperones and proteasomes represent the two main pathways to prevent the accumulation of misfolded proteins. Degradation and (re)folding have in general been studied separately, however, there seems to be a tight regulation between the pathways. One example is the Bag1 protein that contains an UBL domain at its N-terminus to provide interaction with the proteasome (171). With its C-terminus Bag1 can interact with the Hsp70 chaperone that is involved in the refolding of misfolded proteins (262). Another component of this complex is CHIP. CHIP can interact directly with both Hsp70 and Bag1 and is thought to act as a Ub ligase that ubiquitinates unfolded proteins, thereby targeting them to the proteasome (58, 192).

Similar to the ATPases of the 19S base, the AAA-ATPase valosin-containing protein (VCP) (also known as CDC48 in yeast, p97 in metazoans, or VAT in archea), has been shown to contain unfoldase activity (93, 312). Additionally, VCP can physically interact with the proteasome as well as polyubiquitinated substrates and polyUb chains (51). VCP has indeed been shown to function as a shuttle factor by binding to polyubiquitinated IκB and recruiting it to the proteasome (50). Moreover, yeast cells lacking Cdc48 accumulated large quantities of polyubiquitinated proteins, indicating that VCP may serve as a general escort protein (51, 289).

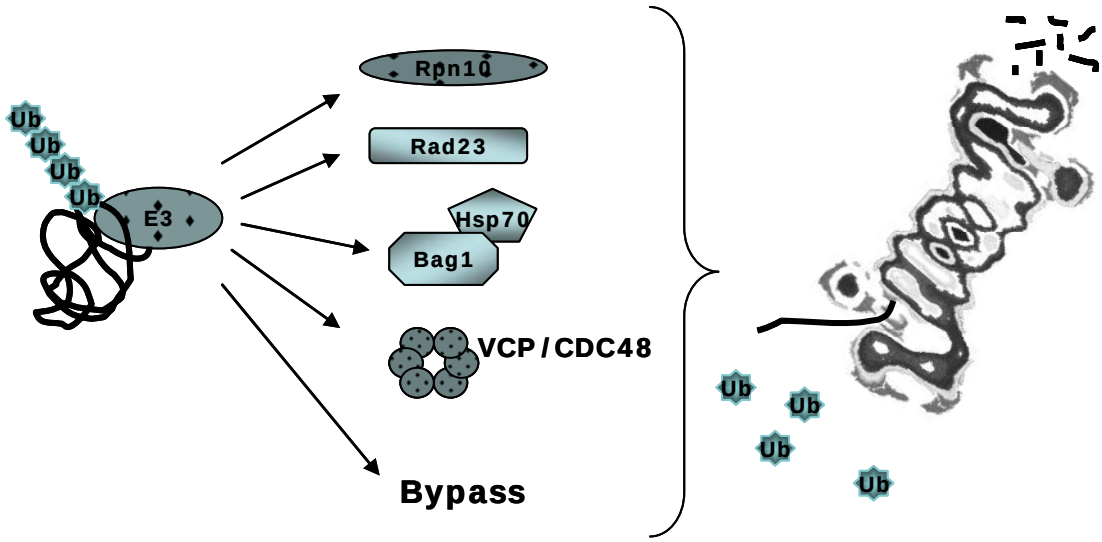

Figure 5. *Proteins involved in shuttling polyubiquitinated proteins to the proteasome*. Polyubiquitinated proteins can bind to the proteasome and be degraded without interference of additional enzymes. Alternatively, several proteins such as Rpn10, Rad23, Bag1/Hsp70 and VCP can bind both polyUb chains and the proteasome, thereby shuttling proteins that are destined for degradation to the proteasome. Once bound to the proteasome deubiquitination takes place and the substrate is unfolded and hydrolysed into small peptides independent from the shuttle factors. Adjusted from Hartmann-Petersen and coworkers (105).

4.6 Proteasome localization and regulation

The proteasome is localized in the cytosol and the nucleus (291). Besides cytosolic and nuclear proteins, the proteasome can also degrade proteins from the ER lumen and membrane (110) and cell surface proteins (17). Cellular localization studies demonstrated that proteasomes are approximately equally divided over the cytoplasm and nucleus (213, 225). Within these two compartments, proteasomes can diffuse rapidly. Proteasome subunits are assembled in the cytoplasm and only a few of the α subunits contain a nuclear localisation signal (278). When the nuclear envelope disintegrates during mitosis, proteasomes can rapidly diffuse in the dividing cell allowing the cytoplasmic and nuclear pool of proteasomes to mix. When cell division is complete, the restored nuclear envelope forms a barrier preventing transport of proteasomes from the nucleus into the cytoplasm. Additionally, slow transport of intact proteasomes from the cytoplasm into the nucleus is possible (225). The degradation of ER proteins takes place through retranslocation of ER membrane and lumen proteins into the cytosol, a pathway referred to as ER-associated degradation (ERAD). The ubiquitination and degradation

machinery is thus excluded from the ER lumen. The degradation machinery is furthermore thought to be excluded from the nucleoli, even though it has been suggested that proteasome localization and degradation might take place in nucleoli as well (177, 258). Within the nucleus there seem to be proteolytic centres for degradation such as promyelocytic leukemia (PML) bodies (291) or other focal subdomains (228).

Besides the interaction of the proteasomal subunits that regulates proteasome assembly, proteasome expression is also under regulatory control. In yeast, Rpn4 is a major player in regulating proteasome levels (293). Rpn4 was originally described as a subunit of the proteasome but functions as a transcriptional activator that binds to a proteasome associated control element found upstream of most proteasome genes in yeast. In addition, proteasome associated control elements have been found in a number of promoters of genes related to the UPS. Once Rpn4 induces proteasome formation, it is destroyed by mature proteasomes in an autoregulatory feedback mechanism (175). However, no homologue of Rpn4 or its DNA binding element has been found so far in mammalian cells. Regulation of proteasomes is important under cellular stress conditions such as heat shock or the accumulation of misfolded proteins. However the molecular mechanisms controlling constitutive and stress-induced regulation of protein gene expression are less well understood. Cells treated with proteasome inhibitor upregulate proteasomal subunits (183) or proteasome activity (157). Interestingly, antioxidants, negatively affecting the cell integrity, can indirectly activate transcription through NF-E2-related factor 2 (Nrf2) related signalling (198). Activation of Nrf2 by antioxidants, activates numerous genes, including subunits of the proteasome (150). Like Rpn4, Nrf2 levels are regulated by hydrolysis by the proteasome (198), so Nrf2 seems to function in manner that slightly resembles Rpn4.

5. GFP-BASED REPORTER SUBSTRATES TO MONITOR FUNCTIONALITY OF THE UPS

5.1 Fluorescent UPS substrates

The work described in this thesis is based on engineered GFP-based substrates for the UPS that enables monitoring the functionality of the UPS in living cells. Most studies on the UPS focus on one aspect such as substrate recognition, ubiquitination, degradation, substrate binding, deubiquitination, but do not give a complete picture of the UPS as an integral system. To be able to do so, several reporter substrates have been developed, based on GFP or the yellow fluorescent protein (YFP) (235). These stable fluorescent proteins were converted to proteasomal substrates by insertion of different constitutively active degradation signals which converted the fluorescent proteins into short-lived proteins due to rapid proteasomal degradation (see also chapter 3.5). Cells expressing these reporter proteins emit low fluorescence due to rapid turnover of the fluorescent protein. However, any obstruction of the UPS, for example by the administration of proteasome inhibitors, induces a dramatic increase in fluorescence (54). Most of the work in this thesis is based on the N-end rule substrate Ub-R-GFP or the UFD substrate Ub^{G76V}-GFP (see chapter 3.5). Two additional reporters, YFP-CL1 and CD3δ–YFP, were used in **paper IV** (184) (figure 6). The CL1 peptide has been found in a yeast screen for sequences that destabilize β-galactosidase in a Ubc6- and Ubc7-dependent manner (89). CL1 is a 16 amino acid degron that resembles a hydrophobic peptide. While normally located inside globular protein molecules or buried in membranes, exposure of a hydrophobic stretch might be recognized by the UPS. Fusion of CL1 to the C-terminus of YFP leads to rapid proteasomal turnover of this reporter substrate (184). CD3δ is part of the T-cell receptor and a well-established ERAD substrate (294). When expressed in the absence of other T-cell receptor subunits, CD3δ is retranslocated into the cytosol and degraded by the proteasome. These four different reporter substrates cover a wide spectrum of substrates: cytosolic, nuclear, ER and misfolded proteins.

Accumulation of these reporter substrates, measured by the fluorescence emitted by the reporter correlates with an induction of G2/M cell cycle arrest and apoptosis (54). Detectable levels of fluorescence preceded induction of apoptosis indicating the usefulness of these fluorescent reporters for an inhibited UPS. Several other fluorescent protein-based reporters with different degradation signals have been developed (235). Recently, a transgenic mouse model has been developed based on the UFD reporter protein (168). This model shows functionality of the reporter even in an *in vivo* model, and provides the opportunity to study the status of the UPS in neurodegeneration when crossed with mouse models for neurodegenerative disorders (19).

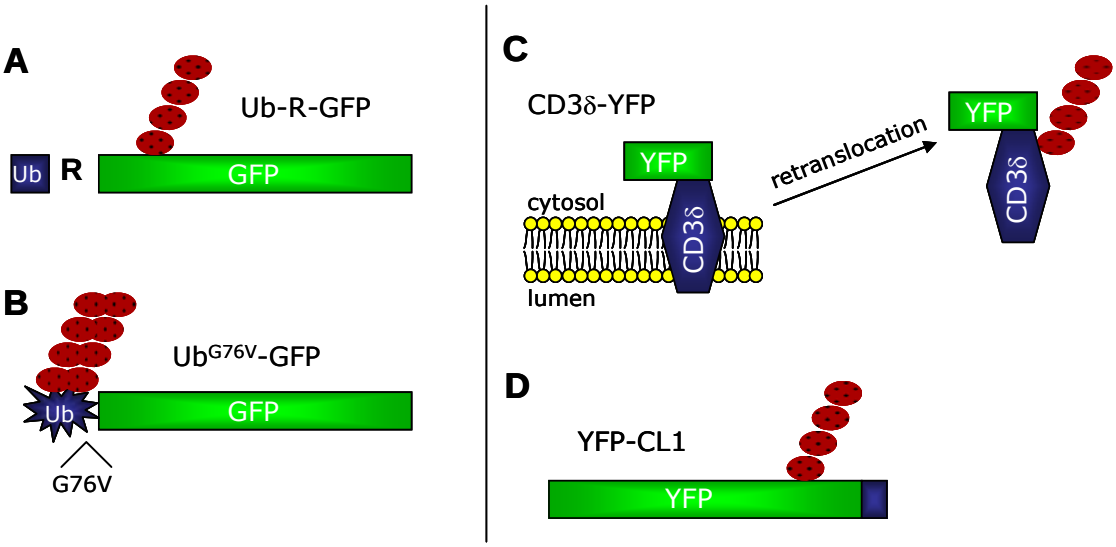

Figure 6. *Schematic representation of the GFP-based reporters.* A) N-end rule substrate. Ub is cleaved off by DUBs, and the exposed Arg (R) at the N-terminus of GFP leads to rapid ubiquitination of GFP. B) UFD substrate. Due to the G76V mutation Ub can not be cleaved by DUBs and remains fused to GFP. Ub itself is then acceptor for ubiquitination at two different Lys residues. C) ERAD substrate. In absence of its binding partners CD3δ is dislocated from the ER-membrane to the cytosol and ubiquitinated. D) CL1 is a hydrophobic peptide that resembles a misfolded domain.

5.2 Fluorescent reporters to monitor the UPS in neurodegeneration

There are several indications for a possible role of the UPS in neurodegenerative diseases (38), but convincing evidence is still lacking (see also chapter 6.4). This is largely due to the fact that functional

studies on the UPS have proven to be a major challenge owing to the complexity of the system. The usage of fluorescent UPS reporters might be a useful tool in this respect since it can evaluate the functionality of the UPS as an integrated system. Moreover, the availability of reporters that represent different types of UPS substrates; nuclear, cytosolic, ER associated and misfolded proteins provide the opportunity to evaluate different ubiquitination pathways.

Based on the accumulation of the UPS substrate GFP-CL1 in cell lines expressing the aggregation-prone mutant huntingtin (Htt) or mutant CFTRΔF508 it was suggested that the UPS might be disturbed (11). Accumulation of GFP-CL1 was also found in a number of similar investigations studying the mutant rhodopsin (120), a protein linked to the inherited form of retinitis pigmentosa, mutant α-synuclein, which is associated with PD (214), and mutant androgen receptor responsible for spinobulbar muscular atrophy (SBMA) (174). Accumulation of PEST-GFP, a Ub independent reporter also accumulated upon expression of mutant ataxin-1 (205), responsible for spinocerebellar ataxia (SCA) type 1, suggesting that it is not just one specific degradation pathway that is affected. In contrast, neither the UFD nor the N-end rule reporter substrates accumulated in cell lines expressing mutant ataxin-1 (**paper I** & unpublished results L.G.G.C. Verhoef, K. Lindsten and N.P. Dantuma).

In a recent study by Bennett and co-workers the GFP-CL1 reporter was specifically targeted to either the nucleus or the cytoplasm (13) and the effect of the aggregation-prone mutant Htt or ataxin-1 on the reporter in either the same or the opposite compartment was examined. The reporter accumulated in both the cytosol and the nucleus independent of the presence or the location of the aggregated proteins. No correlation was found between accumulation of the reporter and the presence of aggregated proteins suggesting that cellular inclusions are not the direct cause of an impaired UPS. Thus the molecular mechanism behind the disturbance of the UPS, as well as the nature of the inhibitory proteins remains to be resolved.

The aberrant Ub UBB^{+1} is encoded by an abnormal transcript of the Ub B gene that has a dinucleotide deletion due to a process known as molecular misreading (275). Although the transcript can be found in both normal and affected cells, the protein product exclusively accumulates in

affected cells in a number of neurodegenerative diseases, such as Alzheimer's disease (78). *In vitro* studies showed that UBB^{+1} can be polyubiquitinated and that the presence of ubiquitinated UBB^{+1} inhibits *in vitro* degradation of proteasome substrates (155). Also in reporter cell lines expressing Ub-R-GFP or Ub^{G76V}-GFP, UBB^{+1} causes a general UPS dysfunction that correlates with cell cycle arrest (**paper II**). Interestingly, UBB^{+1} itself is a UFD substrate of the UPS that is cleared in many cells by proteasomal degradation, while in some cells it accumulates and causes UPS dysfunction. The molecular mechanism of the inhibitory activity of UBB^{+1} is not well understood. However, resistance against proteasomal degradation seems to be a prerequisite to cause UPS dysfunction (**paper III**).

Recently, the first transgenic reporter mouse model for the UPS was generated based on the UFD reporter substrate (168). Crosses of these mice with a knock-in mouse model for mutant ataxin-7, which is responsible for SCA7, provided the first opportunity to study the functionality of the UPS in neurodegeneration *in vivo* (19). Analysis of these crosses did not reveal accumulation of the reporter substrate early in the disease, suggesting that a full block of the UPS is not directly responsible for the disease. Later in disease progression an accumulation of the reporter substrate was found, however, detailed analysis revealed that this increase was not due to increased protein levels but to increased transcription of the reporter transgene probably as a consequence of transcriptional dysregulation. Many of the neurodegenerative diseases, also SCA7, have been reported to display transcriptional dysregulation (151). Moreover, ataxin-7 is thought to be a subunit of the GCN5 histone acetyltransferase-containing coactivator complexes TFTC/STAGA (108). Mutant ataxin-7 caused histone H3 hyperacetylation as a result from an increased recruitment of the TFTC/STAGA complex to specific promoters. Additionally, this SCA7 mouse model showed severe chromatin decondensation.

The reporter substrates to measure functionality of the UPS will provide a more complete picture of the behaviour or different classes of UPS substrates, however, the exact role of the UPS in neurodegeneration needs further investigation.

6. THE UPS AND NEURODEGENERATIVE DISORDERS

6.1 Neurodegeneration and polyglutamine diseases

Human neurodegenerative disorders are a group of clinically and pathologically diverse disorders. These diseases include AD, PD, Huntington's disease (HD), ALS and prion encephalopathies. The diseases are characterized by selective loss of neurons, in specific but different regions of the brain. The result is disruption of motor, sensory or cognitive systems, resulting in severe disability of the patients. Many of these diseases have an age of onset around midlife, even in the case of the inherited diseases. The slow disease progression is probably not due to a single hit-and-run event, but rather a multiple-step process involving environmental, epigenetic and genetic events. Strikingly, many of these disorders have protein aggregation and IB formation in common (230). Aggregation reflects protein misfolding, which is a central theme of these diseases. Therefore the term 'conformational diseases' was introduced to describe all those diseases that arise 'when a protein undergoes a change in size or fluctuation in shape, with resultant self-association and tissue deposition' (25, 148) (figure 7).

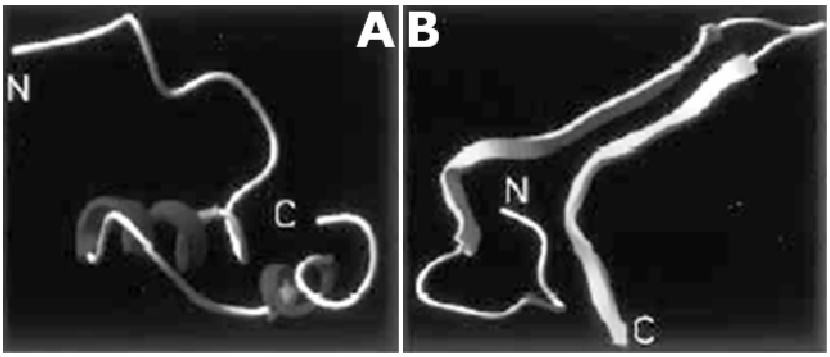

Figure 7. *Conformational change of a model protein*. A) represents a protein in its normal folded conformation. In B) the proteins underwent a conformational change which led to the formation of β-sheets instead of the α helices in A).

One specific group of neurodegenerative diseases are the so called polyGln diseases. These consist of a heterogeneous group of nine inheritable diseases caused by expansion of a the nucleotide CAG repeat (48). It includes HD, six different types of spinocerebellar ataxia (SCA 1,

2, 3, 6, 7 & 17), SBMA and dentatorubal pallydolusian atrophy (DRPLA). So far, the exact function of most of the proteins is unknown. The aberrant protein is in each case different as well as the specific region of the brain that is affected. Nevertheless, these diseases have several characteristics in common. First, they are all characterized by progressive neurodegeneration, striking in midlife and causing increasing neuronal dysfunction and eventually neuronal loss 10-20 years after the onset of symptoms (311). Second, expanded polyGln repeats confer a gain-of-function (83). For a few polyGln diseases homozygosity has been reported (139, 238) which correlates with advanced age of onset and increased diseases severity. Because expanded polyGln tracts have deleterious properties, higher levels of the mutant protein would intensify the toxic effects. The disease develops when the number of repeats exceeds approximately 35 glutamines. An exception is ataxin-6 with repeats of 21 glutamines or more causing SCA6 (309, 310). Third, despite a general expression pattern of the aberrant gene throughout the brain and other tissues, only a certain subset of neurons is affected. The reason of this neuronal selectivity is unknown (48). In a very late stage of the disease progression overlap occurs when massive and less specific neuronal loss occurs. In juvenile forms, caused by very long glutamine repeats, the disease is much more severe and shows an overlap in the phenotypes (49). Fourth, there is an inverse correlation between the length of the repeat and the age of onset: longer repeats give rise to an earlier age of onset and a more severe disease pathology (85). Fifth, as mentioned earlier for other neurodegenerative disorders, polyGln diseases are characterized by protein misfolding and aggregation. Despite the fact that the polyGln containing proteins have nothing in common except for the expanded polyGln tract, polyGln disorders are strikingly similar in many aspects of the pathophysiology, indicating a common mechanism underlying these diseases.

Interestingly, the polyGln tract on its own was thought to be the direct cause responsible for the diseases, which was supported by the fact that polyGln peptides can cause a similar pathological phenotype (295). However, more recent studies have shown that the protein context of the polyGln repeat is involved in the pathology of the disease as well (29, 71, 152).

6.2 Protein aggregation

Protein aggregation can occur i.e. due to a mutation in the sequence of a protein, or a genetic alteration that causes an elevation in the amounts of a normal protein. It can occur in the absence of genetic alterations possible triggered by environmental stress or aging. Alternatively, aggregation might occur as a biological function (160, 254, 255). The mutant proteins form fibrillar IBs called amyloid (230) although also amorphous IBs exist (147). The processes leading to protein misfolding are not fully understood. Since the clinical manifestations take decades to develop, neurons possibly die from cumulative damage. This implies that the probability that neurons die increases with age. However, Clarke and co-workers showed that the kinetics of neuronal death appear to be exponential. They proposed a 'one-hit model' for neurodegeneration that refers to the catastrophic event that leads to apoptosis (41). Based on this 'one-hit model' Perutz and Windle suggested that aggregate formation might require an early nucleation event. It is however becoming more clear that protein aggregation might be a complex process involving several kinds of intermediates, also referred to as protofibrils or microaggregates (104). A protofibril is smaller then a mature fibril and probably can polymerize into mature fibrils or IBs (143). The formation of large visible inclusions might involve the microtubule organization centre (MTOC) which transports the misfolded proteins to a perinuclear localization (114, 274) (see figure 8).

The term aggresome was given to deposits of misfolded proteins with a perinuclear localization (132). It was suggested that the formation of aggresomes is a general response of the cell when the amount of misfolded proteins exceed the capacity of the proteasome. This idea was strengthened by the fact that proteasome inhibitors provoke the formation of aggresomes (132). Inhibition of aggresome formation enhanced the toxicity of the mutant androgen receptor. It has been suggested that aggresomes might protect the cells through enhancing degradation of the mutant protein (268).

Lysosomes can degrade more complex structures then the UPS, including protein complexes and organelles. Lysosomal degradation can be mediated by autophagy, a process that involves the formation of double membrane structures called autophagic vacuoles which fuse with

lysosomes to form autolysosomes where their contents are then degraded by acidic lysosomal hydrolases (300). Several studies have reported structures that are positive for both lysosomal proteins and mutant misfolded proteins suggesting a role for autophagy in the clearance of misfolded proteins in neurodegeneration (56, 140, 196, 260). Indeed, recent evidence provided a role for autophagy in the degradation of aggregation-prone proteins (220). A study by Iwata and colleagues showed that autophagy is limited to cytosolic proteins (124) and provides a possible explanation why accumulation of mutant misfolded proteins in the nucleus might be more toxic for the cell (144, 240).

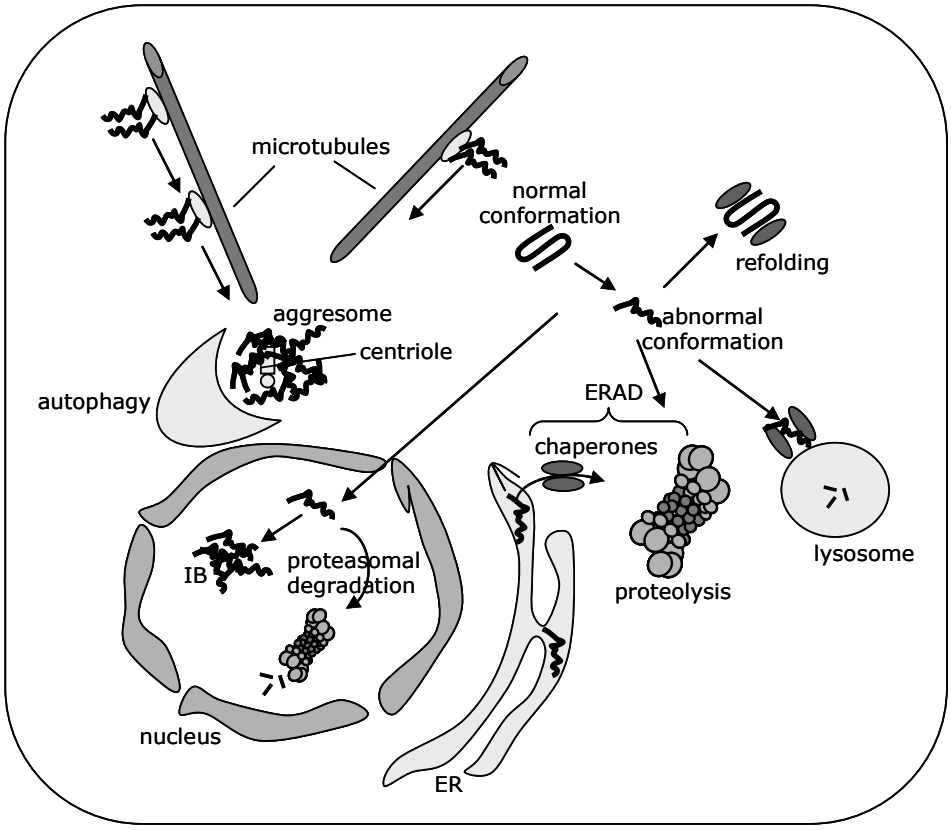

Figure 8. *Cellular defences against misfolded proteins in the cell*. Misfolded proteins in the cell can be refolded by chaperones. If the protein cannot be refolded it can be targeted to the lysosome or proteasome for degradation. Alternatively the (mis)folded protein can be translocated into the nucleus where it can form IBs or be degraded by the proteasome. Misfolded proteins inside the ER can be retranslocated to the cytosol where degradation takes place (ERAD). Misfolded proteins in the cytosol can be transported along microtubules to the MTOC to form an aggresome. Adjusted from Ross & Poirier (229).

Protein aggregation can also serve a biological function. Dendritic cells use the formation of aggresome-like structures for temporal storage of peptides for MHC class I presentation to regulate their immune functions (160), and proteins with prion-like properties involve their stable conformational state of the protein in long-term memory (254, 255).

Recruitment of chaperones in IBs and the upregulation of chaperones under conditions of an excess of aggregation-prone and misfolded proteins implicates an important role for chaperones in neurodegeneration. Chaperones provide a first line of defence against misfolded proteins and have been proven to be potent suppressors of neurodegeneration (190). *In vitro* and *in vivo* studies have shown that overexpression of the chaperones Hsp40 or Hsp70 increased solubility of polyGln proteins, suppressed the formation of IBs and reduced toxicity (47, 189, 279).

A key question that remains disputed is whether the soluble or aggregated mutant proteins in IBs are the toxic forms in these neurodegenerative disorders. Even though IBs are a characteristic of these neurodegenerative diseases, a correlation between IBs and clinical features is not always present (101, 149, 270). Crossing a SCA1 mouse into a mouse that lacks the Ub ligase E6-AP expression resulted in a reduced number of neurons with IBs but pathology got worse indicating that IBs are protective for the cell (46). Additionally, another mouse model for SCA1 was generated in which the self-association region of ataxin-1 was deleted. These mice showed a similar pattern of pathology as with the self-association domain but without the formation of IBs (144). Controversially, the formation of IBs has been shown to precede the neurological phenotype and to be the underlying effect of disease (56). In addition, IBs appear to be 'sticky' structures, interacting with many other proteins, thereby deplete proteins from their cellular environment which might contributes to disease progression (64).

6.3 Mechanisms of pathogenesis

Given that the pathogenesis of the polyGln diseases is due to a gain of function mechanism of the expanded polyGln repeat, what features do these repeats have that renders them toxic to neuronal cells?

It has been suggested that polyGln repeats are substrates of transglutaminases, which are calcium-dependent enzymes that catalyse protein cross-linking (97). The ability of transglutaminase to catalyse cross-linking increases with the length of the glutamine tract and stimulate formation of insoluble aggregates (134). It is however not known if transglutamination of polyGln occurs *in vivo*. Nevertheless, transglutaminase inhibitors in cell culture could partially rescue the apoptotic effect of atrophin-1 with expanded glutamine stretches (119) and elevated expression levels of transglutaminases are found in HD brain (128, 137). In addition, transglutaminases dramatically increased inhibition of the UPS caused by mutant androgen receptor (174).

Perutz and colleagues proposed an alternative model in which two antiparallel β-strands of polyGln repeats can be linked together by hydrogen bonds and undergo polymerization and subsequent aggregation by polar zipper formation (211). However, so far there is no proof for the existence of polar zippers in IBs.

Regardless if IBs are toxic or protective for the cell (discussed in chapter 6.2) changes in protein conformation could allow the mutant protein to recruit normal cellular proteins through aberrant interactions. Some interactions might be inappropriately enhanced, others lost.

Nuclear localization appears to be crucial for the toxicity of some of the polyGln diseases (144). In addition, transcriptional changes often precede pathogenesis (165, 299), suggesting that transcriptional dysregulation might be an important factor in the pathogenesis of polyGln disorders (303). This can be regulated by several mechanisms. Expanded polyGln proteins have been shown to interact with several transcriptions factors which might thereby end up in the IBs (163, 178, 252, 261). Mutant polyGln proteins might alternatively interfere with transcription through direct interaction of transcription factors, dissociating them from their target promoters (32). Additionally, ataxin-7 is part of the SAGA-complex and expansion of the polyGln repeat disturbs histone acetyl transferase (HAT) function of the complex (180, 200).

Mutant polyGln proteins can interfere with other mechanisms such as neuronal trafficking (158, 267) or synaptic transmission (264), thereby contributing to neurotoxicity. In addition to a gain-of-function, loss-of-function also plays a role in pathogenesis. The function of several polyGln

proteins is known; ataxin-3 functions as a DUB, ataxin-7 is part of the SAGA complex, ataxin-6 is part of a calcium channel (81), Htt is thought to play a role in brain development and neuronal survival (86, 224) and the affected protein in SBMA is the androgen receptor. The specific loss-of-function of each of these proteins contributes to pathogenesis.

Nuclear localization might be an important factor for pathogenesis in at least some of the polyGln diseases (144, 240). Htt has been shown to shuttle between the nucleus and cytoplasm. Nuclear export is provided through interaction with the nuclear pore protein translocated promoter region (Tpr). Presumably, the expanded polyGln tract of mutant Htt interferes with interaction with Tpr and Htt accumulates in the nucleus (44).

Nuclear localization of some of the polyGln proteins is dependent on cleavage of the full length protein. This suggest that processing of the full length protein to liberate a shorter peptide that can enter the nucleus is required for toxicity (49). At least for Htt, ataxin-3 and the androgen receptor, cleavage is thought to be carried out by caspases (284). The IBs possibly recruit procaspases which become activated and trigger apoptosis (236). PolyGln proteins are furthermore capable of activating apoptotic pathways indirectly through mitochondrial and ER stress (see also chapter 7) (166, 277).

6.4 Involvement of the UPS in neurodegeneration

Alterations in protein degradation by the UPS are believed to be involved in neurodegenerative diseases (245). The presence of components of the UPS and the refolding machinery in IBs suggest an attempt of the cell to refold or degrade the misfolded proteins. Another indication for a possible role of the UPS in neurodegeneration came from genetic studies showing a direct link between aberrations in proteins involved in the UPS resulting in neurodegeneration. Familiar PD for example can be caused by mutation in the Ub ligase parkin or in the DUB UCH-L1 (256). However, for the sporadic late onset diseases, which represent the majority of the cases, such a link is absent.

It has been reported that polyGln proteins interact with components of the UPS. Htt and a mutant form of Ub, UBB^{+1}, were found

ubiquitinated and interacted with E2-25K (135, 257). Similar, ataxin-7 was found associated with a subunit of the 19S RP, S4 (176). Expansion of the glutamine repeat of ataxin-7 resulted in a decreased interaction with S4, suggesting that stabilization of ataxin-7contributes to pathogenesis. Similar to ataxin-7, stabilization of expanded ataxin-1 has been reported (29). Alternatively, the interaction between ataxin-7 and S4 resulted in relocalization and altered expression levels of S4 and some other proteasome subunits which may affect assembly and functionality of the proteasome. Thus it is possible that interactions of the mutant polyGln proteins with the proteasome interfere with the function of the UPS.

Ataxin-3 is an interesting protein in the context of the UPS. First, it has been shown to function as a DUB through its Josephin domain (33). Second, ataxin-3 has a UIM domain (27) through which it interacts with polyubiquitinated proteins. It furthermore interacts with Rad23 and VCP and plays a possible role as a shuttle factor (65). Third, normal and expanded ataxin-3 are substrates of the proteasome (14). Fourth, ataxin-3 has been shown to be an efficient suppressor of polyGln induced neurotoxicity *in vivo* (280), which dependents on its Ub-associated activities and proteasome function.

The question remains why misfolded proteins in neurodegeneration accumulate and are not rapidly destroyed by the UPS. In case of polyGln diseases, it has been suggested that the glutamine repeat is indigestible for the proteasome (285). The proteasome will hydrolyse the protein except for the polyGln tract which will be released into the cytoplasm or nucleus. In this way the proteasome might even contribute to pathogenesis by releasing aggregation-prone (possible even more toxic) peptides. Nevertheless, we (**paper I**) and others have shown that soluble polyGln proteins (provided with a strong degradation signal) can be efficiently degraded by the proteasome in a Ub-dependent manner, independent of the length of the repeat suggesting that the proteasome is capable of digesting proteins with an expanded polyGln tract (14, 186, 286). It is however possible that also in these studies the polyGln protein is degraded but the polyGln tract itself is not, even though short glutamine peptides have not been detected (**paper I**, data not shown). Targeting polyGln proteins with a strong degradation signal resulted in

addition in a decrease of IBs and a reduced toxicity, possibly due to the accelerated turnover of the soluble polyGln proteins (**paper I**).

Expanded polyGln proteins might adopt energetically stable structures, which resist unfolding and therefore impede proteasomal degradation. In **paper I** we show that engineered or natural mutant polyGln proteins resist proteasomal degradation once aggregated. Additionally, proteins that co-aggregate with the expanded polyGln proteins are thereby also stabilized (**paper I**). If the amount of misfolded proteins exceeds the degradation capacity of the proteasome, IBs may develop which in turn causes redistribution of proteasomes (**paper I**, (126, 176)). Proteasome redistribution might contribute to disease progression by disturbing proteolysis and subsequent vital cellular functions.

Overexpression of a dominant negative Ub ligase in cell culture resulted in less IBs formed by mutant Htt but acceleration in pathogenesis (240). In line with this notion, mice expressing mutant ataxin-1 crossed with mice that lacked a Ub ligase developed fewer IBs but had an accelerated pathology (46) suggesting that proteasomal degradation might play a role in the turnover of these misfolded proteins.

Inhibition of the UPS has also been suggested to play a role in the disease progression of neurodegenerative disorders. Proteasome activity was decreased in an inducible cell line expressing mutant Htt, measured both in the soluble and insoluble fractions, suggesting that soluble and aggregates Htt can inhibit proteolytic activity of the proteasome (126). The decrease in proteasome activity was correlated to cell death. In contrast, Michalik and van Broeckhoven showed that proteasome activity was unaltered in cells expressing soluble polyGln proteins (186). However, the proteolytic capacity of the proteasome exceeds the level required for its household activities, since as much as 80% of the rate limiting chymotrypsin-like activity of the proteasome can be blocked without affecting cell viability (11, 54). Using a stable cell line expressing the reporter protein GFP-CL1, Bence and colleagues showed that IBs can cause a general impairment of the UPS (11). GFP-CL1 cell lines expressing the aggregation-prone proteins mutant Htt or mutant CFTRΔF508, accumulated the reporter in cells containing IBs. Accumulation of the reporter was also found in similar studies on the

mutant rhodopsin, a protein linked to the inherited form of retinitis pigmentosa (120), mutant α-synuclein, which is associated to PD (214) and mutant androgen receptor responsible for SBMA (174). Degradation of another reporter, PEST-GFP, was inhibited by mutant ataxin-1, suggesting that aggregation-prone proteins have a general inhibitory effect on the UPS (205). The mechanism behind this inhibition remains however unknown (see also chapter 6.3), but a recent report by Bennett and colleagues suggested that it is not caused by direct inhibition of the proteasome nor by sequestration into IBs (13). GFP-CL1 reporters were specifically targeted to either the nucleus or the cytosol and the effect of cytosolic or nuclear inclusions on the degradation of the reporter was examined. GFP fluorescence was found in both cellular compartments independent of the presence of IBs, indicating that the IBs are not a direct cause of UPS impairment. We showed in **paper I** however that IB formation of ataxin-1 did not cause accumulation of the transiently cotransfected reporters Ub-R-GFP and Ub^{G76V}-GFP. In addition, studies performed with human cervix carcinoma HeLa cells stably expressing the Ub^{G76V}-GFP substrate did not reveal any accumulation of the reporter in the presence of mutant ataxin-1 (unpublished observations L.G.G.C. Verhoef, K. Lindsten & N.P. Dantuma). While a cell specific effect can not be excluded, it is tempting to speculate that polyGln proteins might not cause an overall block of the UPS but instead impair degradation of a specific subset of proteasome substrates.

6.5 UBB^{+1}, a mutant form of ubiquitin and neurodegeneration

Not only mutations at DNA level but also transcriptional misreading of correct genetic information can lead to the formation of aberrant proteins. Molecular misreading leads to a dinucleotide deletion in mRNA transcripts (275). GAGAG motifs turned out to be hotspots for ΔGA deletions, especially when the transcriptional rate is very high. The exact mechanism causing this misreading is not known although an attractive explanation would be that the RNA polymerase slips over two nucleotides due to problems with the repetitive sequence. The dinucleotide deletion

results in a protein which is normal up to the site of the deletion followed by translation of the +1 reading frame. Often these +1 reading frames result in a premature stop codon giving rise to a truncated protein. Molecular misreading resulting in +1 proteins has been found in the β amyloid precursor protein (βAPP^{+1}) and the Ub B gene product (UBB^{+1}).

Both UBB^{+1} and βAPP^{+1} were found accumulated in protein deposits in brains of patients with AD while not observed in control brains (275). The accumulation of UBB^{+1} was later found to be a more widespread phenomena in conformational diseases; accumulating in brains of patients with different polyGln diseases as HD and SCA-3 (57), progressive supranuclear palsy (76), Pick's disease, frontotemporal dementia and argyrophilic grain disease (78), but not in α-synucleopathies such as PD and multiple system atrophy (78). UBB^{+1} accumulation is also found in livers of patients with α1-antitrypsin deficiency (292) and accumulated in IBs, i.e. Mallory bodies in the liver of patients with steatohepatitis and hepatocellular carcinoma (182).

The inability to detect UBB^{+1} or βAPP^{+1} in control brains is not due to the lack of transcription since the aberrant transcripts are present, but indicates clearance of the mutant proteins in healthy brains, although a decrease in translation cannot be excluded. However UBB^{+1} can be detected in control brains of elderly patients suggesting a role of aging in either molecular misreading or the efficiency of clearing misfolded proteins (78). When not efficiently cleared from the cells, UBB^{+1} acts as a potent inhibitor of the UPS, which possibly might play a role in accelerating neurodegeneration. In line with this idea, Song and co-workers appointed a critical role for UBB^{+1} in the neurotoxicity of AD (257). E2-25K/Hip-2, the E2 capable of polyubiquitinating UBB^{+1} is upregulated in neurons exposed to the Aβ peptide. Furthermore, E2-25K/Hip-2 is required for Aβ induced neurotoxicity, as well as for neurotoxicity mediated by UBB^{+1}, suggesting UBB^{+1} as a link between Aβ and dysfunction of the UPS.

Ub is translated as a precursor-protein, either as a fusion with ribosomal proteins or as multiple head-to-tail copies. In either case single Ub moieties are generated by cleavage by UCH (figure 9). In the case of UBB^{+1}, the GAGAG hot spot leading to a frame shift is at the end of the first Ub moiety. Also in UBB^{+1}, the GA deletion leads to a premature

stopcodon giving rise to an Ub molecule with an additional frame shifted 19 amino acids, here referred to as the +1 tail (figure 9). Moreover, the frame shift also gives rise to a change of the last Gly of Ub to a tyrosine (Tyr), making it unsusceptible for cleavage by UCH, and rendering it insensitive to activation by E1 so it cannot be part of a Ub chain. However, due to the fact that all Lys residues are present, UBB^{+1} itself can be targeted for ubiquitination.

.

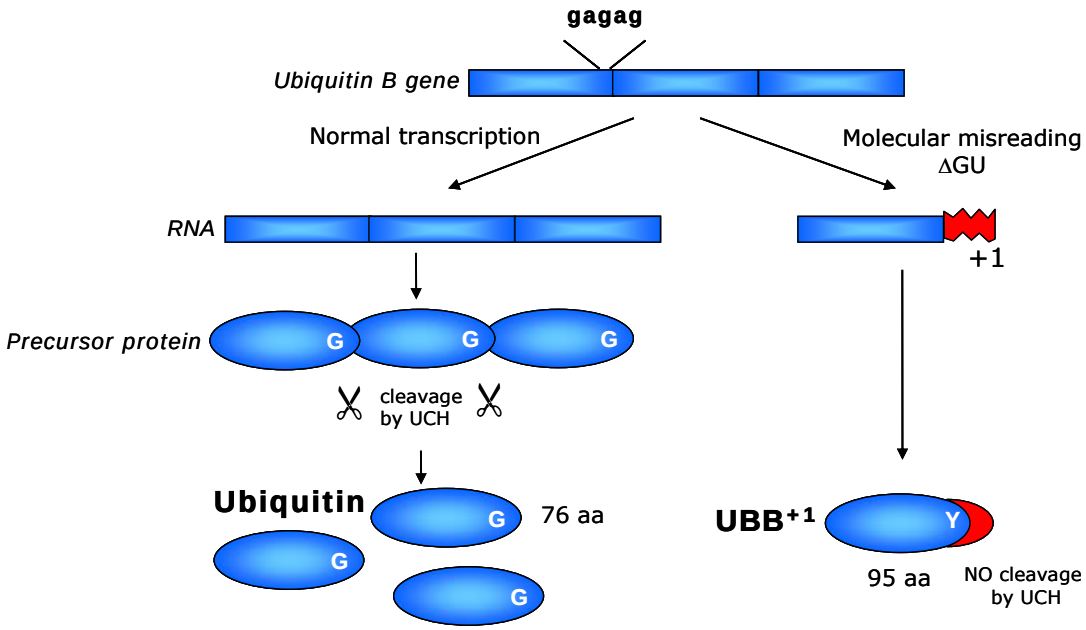

Figure 9. *Generation of Ub and UBB^{+1} from the Ub B gene*. The Ub B gene encode three head-to-tail copies of Ub with a GAGAG nucleotide repeat near the end of the first Ub. Transcription and translation lead to a precursor protein that can be cleaved by UCH into three single Ub molecules, each with a Gly at position 76. Molecular misreading can take place in or adjacent to the GAGAG repeat leading to a frame shift deletion in the RNA. Translation of this product results in one Ub molecule with an additional 19aa. Additionally, the Gly at position 76 is replaced by a Tyr at position 76 which is insensitive for cleavage by UCH.

UBB^{+1} was shown *in vitro* to be resistant against deubiquitination (155). The formation of polyubiquitinated UBB^{+1} inhibited proteasomal degradation *in vitro*, similar to proteasomal inhibition of unanchored Ub chains when not deubiquitinated (155). This inhibition was suggested as a mechanism for UBB^{+1} mediated toxicity in AD. The presence of UBB^{+1} in brains of patients with neurodegenerative disorders combined with the fact that *in vitro* UBB^{+1} could inhibit proteasomal degradation led us to investigate if expression of UBB^{+1} in cells had an effect on the UPS

(**paper II**). Expression of UBB^{+1} in HeLa or neuroblastoma cell lines expressing either the Ub^{G76V}-GFP or the N-end rule Ub-R-GFP reporter revealed also inhibition of the UPS *in vivo*. Accumulation of polyubiquitinated proteins suggested that UBB^{+1} inhibits the UPS at another step then ubiquitination. To investigate if ubiquitination of UBB^{+1} was required for its inhibitory effect, Lys^{48} was mutated to an arginine ($UBB^{+1\ K48R}$). Surprisingly, $UBB^{+1\ K48R}$ was still found ubiquitinated, and therefore Lys^{29} was mutated as well ($UBB^{+1\ K29,48R}$). Either Lys^{29} or Lys^{48} was sufficient for ubiquitination, whereas mutation of both basically prevented ubiquitination. The inhibitory effect on the UPS was lost when both Lys residues were mutated.

Ubiquitination at both Lys^{29} and Lys^{48} has been shown before for UFD substrates (131, 146). The fact the UBB^{+1} can be ubiquitinated at these two Lys residues plus the fact that $UBB^{+1\ K29,48R}$ has higher steady state levels then UBB^{+1} led us to investigate if UBB^{+1} is a UFD substrate and can be degraded by the proteasome. Indeed polyubiquitinated UBB^{+1} is degraded by the proteasome and resembles a UFD substrate.

Besides the aggregation-prone polyGln proteins, UBB^{+1} is another protein that can resist proteasomal degradation. We set out to investigate the underlying mechanism behind the resistance against degradation and the inhibitory effect of UBB^{+1} on the UPS (**paper III**).

The difference between Ub and UBB^{+1} are the additional 19 amino acids encoded by the +1 open reading frame. Database searches revealed no homology of this 19 amino acid expansion with any other polypeptide. Ub is a tightly folded structure. It is unlikely that the 19 amino acids are part of this structure but more likely will be an unstructured peptide. Since unfolded and unstructured polypeptides are prone to aggregation which can have stabilizing effects (**paper I**) we set out to determine if insertion of the extension of UBB^{+1} in proteasome substrates could affect the turnover of these proteasomal substrates similar to other aggregation-prone domains. Degradation of Ub^{G76V}-GFP or Ub-R-GFP was however not affected by the extension with the 19 amino acids of UBB^{+1} suggesting that the +1 tail is not a transferable element with a stabilizing capacity on other substrates. Moreover, UBB^{+1} did not form visible IBs also arguing against aggregation as a possible stabilizing mechanism.

The major difference between the UFD substrates Ub^{G76V}-GFP and UBB^{+1} is the limited length of the C-terminal extension of UBB^{+1}. We wondered if this limited size could be the reason for poor degradation of UBB^{+1}. We therefore generated an engineered UFD substrate by truncating Ub^{G76V}-non fluorescent GFP (nfGFP) to the same size as UBB^{+1}. This resulted in two proteins of the same size with an N-terminal Ub but unrelated C-terminal extensions. ^{myc}Ub-19aa, like UBB^{+1} resisted proteasomal degradation suggesting that the stability of these UFD substrates is due to the length of the C-terminus and independent of its sequence. We hypothesized that these proteins are too short to be efficiently degraded. To test this possibility we stepwise truncated the C-terminus of $^{myc}Ub^{G76V}$-nfGFP to maintain engineered UFD substrates with different lengths of the C-terminal extension. Surprisingly, a minor difference in length of the C-terminal extension had dramatic consequences for proteolysis. While ^{myc}Ub-20aa is a stable protein, ^{myc}Ub25aa was rapidly degraded. Possibly, ^{myc}Ub-20aa is too short to reach from the binding site at the proteasome to the centre of the ATPases that provide translocation of the protein into the catalytic core leading to degradation of the protein. Additionally strong binding of the uncleavable Ub moiety of the UFD protein might counteract the inwards movement of the protein into the 20S CP.

Similar to $^{myc}UBB^{+1}$, the stable ^{myc}Ub-20aa also inhibits the UPS. Degradation of ^{myc}Ub-25aa however, was accompanied by the loss of inhibitory effect on the UPS. Thus, the inability to degrade UFD proteins is required for inhibition of the UPS.

The question remains exactly how these natural or designed UFD proteins inhibit proteasomal degradation. Both ^{myc}Ub-25aa and its stable counterpart ^{myc}Ub-20aa interact with the proteasome. However, there seems to be more of the stable ^{myc}Ub-20aa bound to the proteasome compared to ^{myc}Ub-25aa. It is tempting to speculate that lack of degradation and resistance against deubiquitination traps ^{myc}Ub-20aa bound to the proteasome. Consequently, by occupying the proteasome, these substrates may affect degradation of other proteasomal substrates.

There is no evidence in the literature suggesting that monoubiquitination can target proteins for proteasomal degradation. Moreover, a chain of minimal four Ub molecules is required for

proteasomal binding (269). Surprisingly, binding of the UFD substrates is predominantly found in its unmodified form, suggesting that monoUb is sufficient at least to maintain proteasomal binding. Polyubiquitinated species of UBB^{+1} are seen *in vivo* as well as *in vitro* (155, 167). Possibly, polyubiquitination of UFD substrates is required for initial binding to the proteasome but rapid deubiquitination leaves the unmodified form bound to the proteasome. Deubiquitination is thought to play a regulatory role in proteasomal degradation, and can rescue proteins from destruction (102). Long polyUb chains might be required to prevent release by deubiquitination from the proteasome prior to degradation. Remarkably, polyUb chains of UBB^{+1} are often not more then a few Ub molecules. UBB^{+1} is insensitive to deubiquitination due to its uncleavable Ub molecule. Our results suggest that to maintain proteasomal binding this uncleavable Ub is sufficient. Long Ub chains might therefore not be required. Additional experiments will have to be done to provide more answers.

7. PROTEIN QUALITY CONTROL, ER STRESS AND NEURODEGENERATION

The ER is the major site for synthesis of membrane and secretory proteins. It receives newly synthesized proteins from the cytosol via a narrow channel formed by the Sec61 complex, the translocon (67). In the ER the proteins encounter an elaborate system of chaperones and can undergo post-translational modifications that together catalyse the folding and assembly of the polypeptides allowing them to fold correctly in their native conformation. Correctly folded proteins are packaged into transport vesicles that deliver them to their final destination in or outside the cell. Immature molecules are retained in the ER until maturation is complete. The sequence of events in the ER is strictly regulated as the release of unfolded proteins would have disastrous consequence for the cell. The ER quality control monitors protein folding and assembly and prevents the transport of immature molecules. The ER is also challenged with proteins that become irreversible misfolded. Errors in transcription or translation or environmental stress such as elevated temperature and oxidative damage are examples leading to the formation of aberrant proteins. The importance of quality control mechanisms is underscored by the presence of several human diseases caused by aberrant folded proteins.

The misfolded proteins in the cell have three fates: refolding by chaperones, destruction by proteases or aggregation. Chaperones are a diverse group of proteins that can bind and stabilize non-native conformations of other proteins. Under conditions of stress, when proteins become unfolded and require protection and stabilization, the concentration of chaperones is greatly increased. The ER has its own set of chaperones many of which are also involved in protein quality control, such as BiP, calnexin and calreticulin and GRP94 (69). The main quality control in the ER consists of a retention-based system that recognizes and retains incompletely folded or unassembled oligomers inside the ER.

When proteins, despite the effort of ER chaperones, are not properly folded, they have to be eliminated. It turned out that not proteases inside the ER are responsible for the degradation of misfolded ER client proteins but the UPS. The UPS is however not present in the

lumen of the ER and aberrant or misfolded proteins have to be retranslocated into the cytosol where they undergo ubiquitination and subsequent degradation by the proteasome, a process called ERAD (185). Retranslocation is thought to process through the Sec61 channel (271). When polyubiquitination is prevented, some substrates can not be retranslocated into to cytosol (127, 248, 271) indicating that ubiquitination is not only required for proteasomal degradation but also participates in the transfer of proteins across the ER membrane. Several E2 and E3 enzymes involved the ERAD pathway have been found associated to the ER (28, 84). In addition, the cytosolic chaperone VCP, together with its cofactors Ufd1 and Npl4, is thought to be involved in 'pulling' the polyubiquitinated protein across the ER membrane (9, 127) which is dependent on VCP's ATPase activity (298, 307).

When misfolded proteins are not refolded or destroyed the third option is aggregate formation. The accumulation of misfolded proteins inside the ER elicits a stress-response, the unfolded protein response (UPR), that will induce apoptosis unless the proper ER environment is restored on time (304). The UPR is characterised by a shutdown of global protein synthesis and activation of expression of genes coding for ER residence proteins that are involved in the folding and processing reactions (244). The ER chaperone GRP78 plays a major role in the ER stress response together with three ER membrane proteins, ATF6, PERK and IRE1 (207) (figure 10). Activation of the UPR is required for the cell to withstand the stressful conditions associated with ER dysfunction. When the stress sustains and is not restored, it will result in ER stress induced apoptosis through CHOP, caspase 12 or mitochondrial cytochrome c release (207).

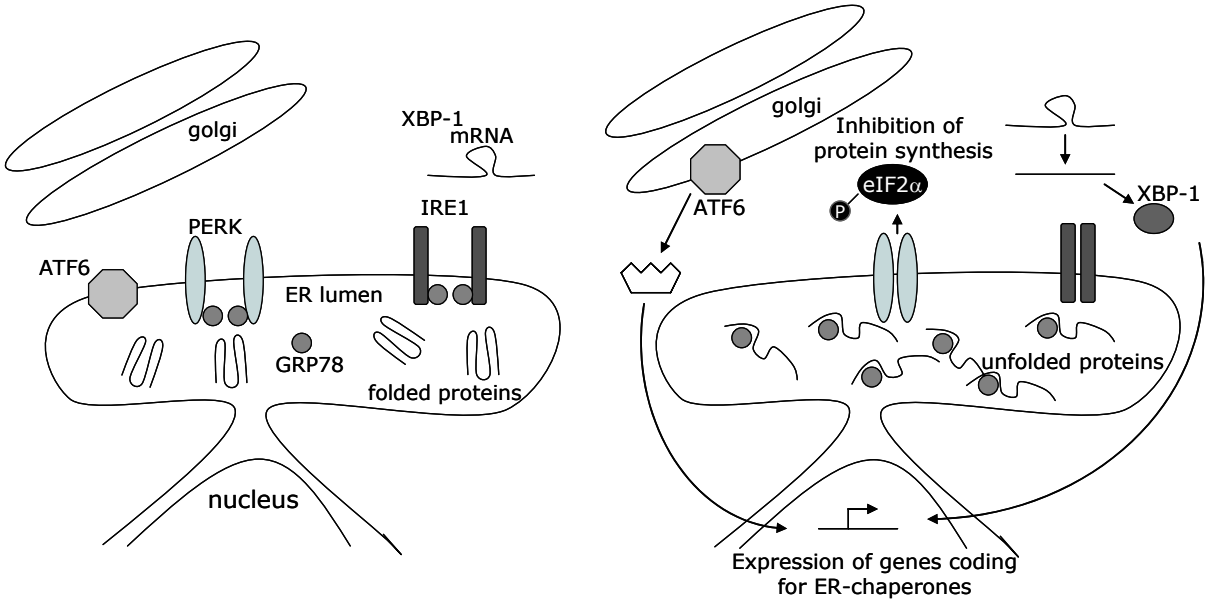

Figure 10. *The UPR*. Under normal conditions, GRP78 is bound to ATF6, PERK and IRE1 keeping them in an inactive state. When the amount of misfolded proteins exceeds the levels of free GRP78, it dissociates form the three membrane proteins leading to their activation. Dimerized PERK will phosphorylate the α-subunit of eIF2 resulting in a shutdown in global protein synthesis. Dimerization of IRE1 results in endonuclease activity, cleaving a sequence out of the XBP-1 mRNA. XBP-1 is then translocated to the nucleus where it functions as a transcription factor for genes involve din folding and processing of misfolded proteins. ATF6 will translocate into the golgi where it is cleaved by proteases to form an active transcription factor that induced expression of ER stress genes including GRP78. Adjusted from Paschen & Mengesdorf (207).

Several studies support the idea that ER stress is involved in neurodegenerative diseases. High ER calcium levels are required for protein folding inside the ER and mutations in presenilins, involved in AD, have been shown to disturb the calcium levels (159, 302). Mutation variants of presenilin 1 and 2 have also been shown to impair functioning of the ER stress inducers PERK, IRE1 and ATF6 (138, 239). Parkin, a Ub ligase, might protect neurons through the degradation of unfolded proteins (122). Loss of parkin function, involved in PD, might result in aggregation of unfolded proteins, thereby possibly impairing proteasome function leading to dysfunction of ERAD and subsequent ER stress (232). Several other proteins have been shown to inhibit the UPS, such as polyGln proteins or UBB^{+1} and might thereby contribute to dysfunction of ERAD and induction of ER stress. However, inhibition of the UPS is followed by rapid cell death and is therefore unlikely to be responsible for the long-term gradual accumulation of misfolded proteins as seen in

neurodegenerative disorders. In **paper IV** we studied therefore the effect of ER stress on the functionality of the UPS.

To monitor the functionality of the UPS, human melanoma MelJuSo cell lines stably transfected with YFP-based reporter proteins were generated. Four different reporter substrates were used. Three cytosolic/nuclear substrates which have been described before: the N-end rule substrate Ub-R-YFP, the UFD substrate Ub^{G76V}-YFP and YFP-CL1 (see also chapter 3.5) (11, 54). In addition, the ERAD substrate CD3δ was fused to YFP, generating CD3δ-YFP. All four cell lines expressing the different reporter substrates have low levels of YFP due to rapid turnover. Inhibition of the proteasome by chemical inhibitors resulted in a dramatic increase in YFP fluorescence, demonstrating that these reporter substrates can be used to monitor functionality of the UPS. Moreover, the four reporter substrates represent a versatile tool to measure three different pools of UPS substrates; cytosolic, nuclear and misfolded proteins.

ER stress can be induced by several chemicals, such as thapsigargin, which depletes the ER calcium storage by specific inhibition of ER calcium ATPases, tunicamycin which inhibits N-linked glycosylation, or the reducing agent dithiothreitol (DTT). Induction of ER stress resulted in increased levels of CD3δ-YFP similar to chemical inhibition of the proteasome, suggesting that ER stress severely impairs the degradation of ERAD substrates. Interestingly, induction of ER stress caused a small but highly significant accumulation of both the soluble and misfolded cytosolic/nuclear substrates. The increase of reporter levels was not due to an increase in protein synthesis but due to a delay in proteasomal degradation. Expression levels of two of the 20S core subunits of the proteasome were however unaltered under conditions of ER stress. Measuring proteolytic activity of the proteasome by cleavage of fluorogenic substrates revealed no decrease in proteolytic activity either. Neither expression levels of the proteasome nor its activity was altered, suggesting that the 20S CP of the proteasome is unaffected under conditions of ER stress and thereby likely not responsible for the accumulation of the reporter substrates.

Degradation of the disease related protein UBB^{+1}, a problematic proteasomal substrate, was more dramatically affected. The steady state

levels of UBB^{+1} increased due to ER stress and consequently caused an increase in inhibition of the UPS. ER stress can cause accumulation of the toxic proteasome substrate UBB^{+1} which results in a general blockage of the UPS. It has been suggested that polyGln proteins can cause ER stress. We therefore tested if polyGln proteins can cause a similar accumulation of UBB^{+1} as seen by chemical induction of ER stress. Cotransfections of the mutant polyGln proteins Htt or ataxin-1 with UBB^{+1} revealed an increase in detectable UBB^{+1} levels in cells with aggregated mutant polyGln proteins (unpublished observations L.G.G.C. Verhoef & N.P. Dantuma). Similar to our results, de Pril and co-workers showed an increase of UBB^{+1} in cells expressing mutant polyGln proteins (57). Even though induction of ER stress has not been measured under these conditions it is tempting to speculate that the accumulation of the aggregation-prone mutant polyGln proteins induced ER stress which compromises the UPS resulting in accumulation of UBB^{+1}.

In conclusion, ER stress causes impairment of degradation of ERAD substrates and a delay in degradation of nuclear/cytosolic substrates. In addition, aggresome formation of YFP-CL1 has been detected in a small percentage of cells when the UPS is compromised, suggesting that a mild inhibition of the UPS is sufficient to induce the formation of aggresomes. Despite the fact that aggresome formation might be a protective mechanism of the cell against an excess of aberrant proteins, it is found as an initial stage in neurodegenerative diseases. Although it remains to be resolved what the limiting factor is causing the delay in proteasomal degradation, ER stress has a subtle negative effect on the functionality of the UPS. While a full block of the UPS has been suggested in the pathology of neurodegenerative diseases, it is hard to reconcile with the long-term slow progression of these diseases since a full block of the UPS results in cell-cycle arrest and apoptosis. A mild inhibited UPS as discovered in ER stressed cells might be an explanation for the slow progression of neurodegenerative diseases.

8. CONCLUSIONS AND FUTURE PROSPECTS

In **paper I** we investigated the degradation of soluble and aggregated polyGln proteins by the proteasome. Mutant polyGln proteins carrying a strong degradation signal were degraded by the proteasome in a Ub-dependent way. However, IB formation rendered the mutant polyGln proteins resistant to proteasomal degradation. The formation of IBs led to redistribution of proteasomes to the IBs. Additionally, proteins with a short non-pathological polyGln repeat interacted and co-aggregated with expanded polyGln proteins which resulted in stabilization of the co-aggregated protein. It has been suggested that mutant polyGln proteins can cause a general blockage of the UPS. In contrast, both soluble and aggregated polyGln proteins did not cause accumulation of the Ub^{G76V}-GFP reporter substrate. This suggests that mutant polyGln proteins do not cause a general inhibition of the UPS. It is tempting to speculate that misfolded or aggregation-prone proteins block the turnover of certain but not all substrates. Further research comparing the turnover of different reporter substrates should provide more answers. Additional crossing of the GFP-reporter mice with mouse models for neurodegeneration shed light on a possible role of the UPS in neurodegenerative diseases.

Introduction of a strong degradation signal in the polyGln proteins resulted in a reduction of IBs and toxicity. Thus, targeting these proteins for degradation might be an interesting therapeutic approach. Strategies have been developed to redirect the cellular ubiquitination machinery to a target proteins-of-interest. One method was based on the expression of a chimeric ligase adaptor harbouring a binding motif for the target protein (308). The second approach utilizes small peptides that form a trimeric complex with the target protein and the Ub ligase (234). It would be interesting to investigate the possibility to target polyGln proteins for degradation through the above mentioned techniques. The interaction between a short glutamine stretch and an expanded repeat might be a possible binding target.

UBB^{+1} (**paper II**) is an aberrant form of Ub that accumulated in the brains of patients suffering from various neurodegenerative disorders.

UBB^{+1} is a UFD substrate that can be degraded in a Ub-dependent manner by the proteasome. In addition, it is a potent inhibitor of the UPS in cells and this inhibitory activity is dependent on ubiquitination of UBB^{+1}. The underlying mechanism of the accumulation of UBB^{+1} remained unknown. This was the focus of investigation in the study presented in **paper III**. UBB^{+1} is too short for efficient proteasomal degradation, and consequently accumulates in the cell. The lack of degradation is required for the inhibitory effect of UBB^{+1} on the UPS. Additionally, a minimal length of UFD substrates is required for efficient proteasomal degradation. Together these data provide a mechanistic insight in the requirements for turnover of UFD substrates and shed light on a possible mechanism of UPS inhibition of UBB^{+1} in neurodegeneration. Several questions remain:

First, how does UBB^{+1} interact with the proteasome? Is polyubiquitination a transient state to provide proteasome interaction or is UBB^{+1} capable of binding to the proteasome in an unmodified form? Additionally, with which subunit does UBB^{+1} interact and is this different from non-UFD substrates?

Second, does binding of UBB^{+1} compete with the binding of other proteasome targets? UBB^{+1} might compete with the proteasomal binding of other polyubiquitinated substrates. In addition, the uncleavable Ub moiety resembles an uncleavable UBL domain, suggesting that binding of these proteins to the proteasome might be similar. Competition experiments with shuttle factors such as Rad23 or VCP might provide some answers.

Third, how exactly can UBB^{+1} inhibit the UPS? Analysis of interaction between the proteasome and UBB^{+1} suggest that UBB^{+1} may be 'stuck' at the proteasome, thereby preventing recruitment of other proteasomal substrates and delaying their degradation. Showing that the degradation of UBB^{+1} is indeed delayed once it interacts with the proteasome would give a final answer to that question. Instead of being stuck at the proteasome, another possibility is that UBB^{+1} is released from the proteasome and inhibits the UPS at another level then the proteasome.

Fourth, what role does UBB^{+1} play in neurodegeneration?

An important unresolved question in many neurodegenerative diseases is why the UPS fails to destroy the misfolded proteins. It has been suggested that aggregation-prone misfolded proteins can cause a general blockage of the UPS and thereby allow the accumulation of the toxic mutant proteins (11, 120, 214). A general feature in almost all of the neurodegenerative disorders is the slow progression of the disease over many years or even decades. A full block of the UPS is accompanied by apoptosis and is therefore hard to reconcile with the slow progression of these diseases. The UPS is also involved in degradation of misfolded proteins from the ER through ERAD. Accumulation of misfolded proteins in the ER causes ER stress and a subsequent UPR to restore the integrity of the ER. ER stress has been implicated in the pathogenesis of neurodegenerative diseases but it is not completely clear what the exact role of ER stress is during the slow progression of the diseases. In **paper IV** we show that ER stress has a subtle effect on the functionality of the UPS, which might explain the long-term slow progression of the disease. The degradation of ERAD substrates is blocked during ER stress and the degradation of cytosolic and nuclear substrates is delayed. Since the expression levels and the catalytic activity of the 20S CP seem unaltered during ER stress, the inhibition has to take place at another level. The four reporter substrates represent a large fraction of different proteasomal substrates. The degradation of each substrate is affected by the induction of ER stress making it unlikely that one specific factor is affected, like an E2/E3 enzyme or a shuttle factor, but rather a more general key player. Further research has to be done to provide additional answers.

9. ACKNOWLEDGEMENTS

Looking back over the past five years puts a smile on my face; so many new experiences, so many new friends. Thanks to all of you I met in the past years and made my stay into a nice one.

My journey started at the 2nd of January 2001. When I arrived at city terminalen I found you there **Nico**, waiting for me while reading Scientific American. They just published an article on the proteasome which you told me enthusiastically about in the subway on the way to my new room. This turned not out to be an exception, you have a great enthusiasm for science…and you managed to transfer quite a bit of that to me☺ Thank you for your supervision, your inspiration, your amazing knowledge but also for the nice dinners outside work. You always managed to get a great team around you and I really enjoyed being part of that! **Maria**, you provided the opportunity to work with Nico in your lab. Thank you for accepting me as an undergraduate and providing the opportunity to stay 4 additional years. **Victoria**, we started together and we finish together; you were there every day. After 'living together' for five years, the next lab will be without you☹ How to survive??? You are so jumpy, full of energy and craziness; thanks for bringing this to the lab! I will never forget the crazy times in the lab, the coffees and beers outside, shopping, that midsummer…I will miss you…and YES…weeee madeeee it…oh yes we did, we did makeeee it *Adjusted text from The Presidents…sing along loud* **Florian**, my early morning coffee-mate. A pity I did not meet you earlier when we had the chance in Cologne…It was a lot of fun that you managed to bring Sinterklaas to Sweden, I guess it was the singing, right?? Thanks for dragging me to the gym together with Victoria. Many thanks for helping me out in the last months with 'confocallen' proof-reading or just cheering me up with a little chat. Whether it is just a cup of coffee, a chat, a beer after work, or many in the MTC pubs, it is always nice to have you around! When do we go shopping again? **Deborah**, you were always there for coffee and the necessary hours of blabbing. I am very grateful for the self-evident way you provided help. Cloning, proof-reading, taking over my journal club, no favour was too big for you to accept...I really appreciate you helped me out so many times. Thanks for your down-to-earth perspective on life! Even though you like terrible music, and also share it with us in the lab, it was great having you around! **Christian**…bitte danke…thanks for enlarging my German vocabulary, if I ever go to Germany this is definitely going to help me☺ You are simply great fun and a pleasure to have around in 'da lab'! **Christa**, thanks for sharing the taste of good house and trance music. I don't understand the others don't like it;-) And most importantly for introducing the concept

'healthy cake'. **Sasha**, thank you for providing two extra hands and helping me out in the last moments of my stay here when I needed it so much! **Kristina**, you helped me a great deal; starting up when I came to the lab, getting around, sharing science, and even providing an apartment. But more importantly, thanks for being such a good friend, always there to listen and ready for a chat. **Stijn**, thanks for your never-ending help in cloning, and especially for being like a supervisor when Nico was in Holland. It is very nice you are still around even though you left our group! **Marianne Z.**, it was great fun having you around! **Sai**, goede morgen en veel succes in Groningen! But stop washing vegetables in the washing machine, that's freaky;-) **Aude**, have fun in Stockholm!

Ulrika…where to start. Countless hours of chatting, drinking wine, good food, dancing, parties, more wine, weird SMSes late at night, long and short walks, great holidays, and hopefully much more to come! Thanks for learning me not to waste water, no my father does not make it;-) I really enjoyed all the parties you took me along to and introducing me to so many of your great friends…most importantly…for introducing me to **Lisa**. Cause there is no better way to start a day, to get through a day or to end a day then by a little chat on the messenger with you. You always managed to make me laugh or cheer me up☺ Thank you for not being a scientist and for sharing soo much bästisar-love! MUAAH! Ullies o Lisa, ni två är verkligen bäst…hoppas ni kommer så oftast som möjligt till Milano och jag lovar att aldrig lämna Sverige igen;-) Tack att ni lärde mig konceptet "snygg och skön" och att alkohol gör dig smart. Och naturligtvis allt svenskt ni lärt mig...hur konstigt som hellst...allt var användbart☺ Tack för att ni finns gummiesar♥

My lovely sambo's, **Andre** & **Jose**. Even though more rare then anticipated, I honestly loved the times we did share the apartment together. **Andre**, you make me feel beautiful, put a smile on my face. Gossiping with you is the best…hi hi. Such a pity our roads did split up too early, I hope we manage to organise a yearly get-together, wherever we are. The idea that you nevertheless will be at my defence makes my party complete!! **Ze**, you are so sweet, gorgeous and a hard-working example! It was fun moving to CMB and to pass by your 'window' so often;-) Thanks for being my mail-man, and for always making time for a kiss and good morning in the corridor! Don't forget the veggies now I will be gone☺ A big kiss to both of you! **Micke**, your stories were always a bit weirder, more disgusting or just 'bajs'…I laughed so much with you! It was great having you on the other end of the computer going through the same the last weeks... Du är verkligen en bra kompis! **Elena**, thanks for being such a sweet friend, always ready for a hug and nice talk! You are absolutely a great cook; I hope we can cook together again some time soon (read: you cook, I

bring the wine and cut some veggies:-;-)) **Anna C.**, I really miss you around. Thanks for those many hours in THE pub with beer, club-sandwich and much to talk about, puss puss. **Marianne J.**, min 'svenska mamma'☺ Tack för att du alltid lyssnade till mina många timmer av inte-viktigt information, tittade på bilder av mina brors barn, och hjäpte med allt möjligt. Jag njöt väldigt mycket av din kladdkaka varje år! Tack för att du var där..hoppas du fortsätta njuta av din pension, och att vi inte tappar kontakten! **Yvonne** du såg till att allt funkade…alltid! Ett stort tack för all hjälp!

To the other people that passed the MGM lab; **Anna B.**, thanks for the fun and relaxed atmosphere you brought with you...a pity you stayed so short! Thanks for sharing you apartment, Barcelona is great! Even more fun when you were also there (despite the heat☹) Good luck finishing up!! **Rebecca**, you are sooo sweet, it was great meeting you! **Moniek**, it was really fun with you in Stockholm. The present and previous MGM lab members: **Cheng-Hong**, **Daorong**, **Ainers**, **Anatoly**, **Piri**, **LiQi**, **Victor**, **Anders**, **Sara**, **Abela**, **Vanessa** and **Elio**, thanks for creating a great atmosphere! The MTC pub crew, **Elio**, **Danika**, **Alberto** and **Rutger**, you made those Friday nights a lot of fun!! **Ying**, it's great to see you always smiling! **Birgitta,** thanks for always making the FACS work and making me feel welcome back at MTC☺

Lina and **Per-Henrik**, thanks for sharing space with us which made the big quiet room into a tiny loud space. Thank for borrowing reagents and equipment, the chats, help with teaching and of course keeping me updated on the latest sailing boats. The rest of the cell biology crew: **Ales**, **Raju**, **Dima** (thanks for teaching and sharing gradient stuff!!), **Giorgio**, **Ben**, **Sasha**, **Oksana**, **Karin**, **Tove**, **Björn**, **Birgitta**, **Örjan**, **Sergej** & **Sergey**, **Lars-Göran**, **Ulf**, **Sara**, **Lars**, **Bertil**, **Andreas**, **Arne**, **Christina**, **Eva** and **Inger**, thanks for all those Friday meetings and the celebrations together.

Zdravko, thanks for fixing everything with a smile. **Tony** & **Lia**, er gaat niks boven een praatje en een grapje in het Nederlands☺. **Helder** & **Jorge**, thanks for sharing idea's, reagents, feelings and parties; you guys are great, beijos beijos! **Tiago**, you were one of my first friends in Stockholm, thanks for all those nights at Berns, sharing our birthday together, nice dinners and parties…I really had a lot of fun!! I sincerely hope we don't loose contact!

Johan, **Johanna** & **Kristoffer** thanks for great Greek memories. When do we start the next ginotomics study?

A big thanks to **Martin Scheffner** and the rest of his lab. You even came in on a Saturday to teach me how to isolate those enzymes, thanks for having me in your lab! **Patrick Young** and **Josefin**, thanks for teaching me of what turned out not to be so easy essays☺

Mirjam & **Wendelien**, Ik mis al onze theekransjes samen, 'moederkoeken bakken', toetjes dinner, etentjes in alternative restaurants, kortom, ik mis mijn biofriends®. Hopelijk houden we contact; Biofiends® forever! **Martijn** thanks for sharing great music. You are a big source of inspiration, I enjoy spending time with you! En ik verwacht natuurlijk wel een vraag tijdens mijn promotie;-). **Brenda**, pippi hier… Het blijft lachen met jou. You manage to shock me every time with your stories;-) Great you finally have broadband as well. It was really fun with you, **Bianca** and **kikker** in Stockholm. I promise to finally come over to see your new house before you move out again☺. **Eric**, despite the fact I always manage to loose contact, one telephone call when I am in Holland is enough to get in touch again. Thanks for keeping the line open, for many long walks, fast rides in Norway and nice dinners. You are a great friend! **Hans**, I am really not the best in keeping contact, maar zoals jij het een keer zo mooi zei: 'uit het oog maar niet uit het hart'. Ik kom je nu echt snel een keer opzoeken!

Jarno, thanks for being my big brother. A big thanks to you and **Susan**, for picking me up from the airport, for always letting me sleep over, the cheese fondue with 'cheesefondue music', all apple updates, even when unwanted;-), the pictures, although very few for a photographer; the little beautiful photo album is still waiting to be filled☺ **Thomas**, **Ralph** & **Britt**, leuk dat ik altijd met jullie mocht komen spelen en dat jullie zo vaak via de webcam wilde kletsen…dikke kus! To my parents, **Ben** & **Rika**, woorden schieten te kort. The endless love and support, no matter what road I decided to take means the world to me! Thanks for the endless phone calls, especially when I needed someone to talk to, which was often☺ The webcam, the best present ever, to make Roermond feel next door. It made the chatting sessions not only more frequent but also affordable. Thanks for all the ' bijklets' emails, to keep me updated on all that happened in Holland☺ And of course for those great rides on the motorbike, I hope we'll plan one soon again. I have a good feeling about the 'italian-Rika&Lisette-team' to win this year☺. And last, but certainly not least, **Anders**, you make my life wonderful! Att vakna med dig på morgonen, somna med dig på kvällen och allt där emellan är fantastiskt! Tack för att du delat denna resa med mig. Tack för så mycket kärlek, stöd och förståelse, särskilt under de senaste månaderna. Även om våra vägar nu kommer delas under ett tag, finns du alltid i mitt hjärta och jag är säker på att vi kommer hitta ett sätt att få det att fungera. Pussje!!

Ce vediamo a Milano!!

10. REFERENCES

1. **Amerik, A. Y., and M. Hochstrasser.** 2004. Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta. **1695:**189-207.
2. **Ardley, H. C., and P. A. Robinson.** 2005. E3 ubiquitin ligases. Essays Biochem. **41:**15-30.
3. **Arrigo, A. P., K. Tanaka, A. L. Goldberg, and W. J. Welch.** 1988. Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalian cells (the proteasome). Nature. **331:**192-4.
4. **Babbitt, S. E., A. Kiss, A. E. Deffenbaugh, Y. H. Chang, E. Bailly, H. Erdjument-Bromage, P. Tempst, T. Buranda, L. A. Sklar, J. Baumler, E. Gogol, and D. Skowyra.** 2005. ATP hydrolysis-dependent disassembly of the 26S proteasome is part of the catalytic cycle. Cell. **121:**553-65.
5. **Baboshina, O. V., and A. L. Haas.** 1996. Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5. J Biol Chem. **271:**2823-31.
6. **Bachmair, A., D. Finley, and A. Varshavsky.** 1986. In vivo half-life of a protein is a function of its amino-terminal residue. Science. **234:**179-86.
7. **Baek, K. H.** 2003. Conjugation and deconjugation of ubiquitin regulating the destiny of proteins. Exp Mol Med. **35:**1-7.
8. **Baumeister, W., B. Dahlmann, R. Hegerl, F. Kopp, L. Kuehn, and G. Pfeifer.** 1988. Electron microscopy and image analysis of the multicatalytic proteinase. FEBS Lett. **241:**239-45.
9. **Bays, N. W., S. K. Wilhovsky, A. Goradia, K. Hodgkiss-Harlow, and R. Y. Hampton.** 2001. HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. Mol Biol Cell. **12:**4114-28.
10. **Benaroudj, N., E. Tarcsa, P. Cascio, and A. L. Goldberg.** 2001. The unfolding of substrates and ubiquitin-independent protein degradation by proteasomes. Biochimie. **83:**311-8.
11. **Bence, N. F., R. M. Sampat, and R. R. Kopito.** 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. Science **292:**1552-5.
12. **Beninga, J., K. L. Rock, and A. L. Goldberg.** 1998. Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. J Biol Chem. **273:**18734-42.
13. **Bennett, E. J., N. F. Bence, R. Jayakumar, and R. R. Kopito.** 2005. Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol Cell **17:**351-65.
14. **Berke, S. J., Y. Chai, G. L. Marrs, H. Wen, and H. L. Paulson.** 2005. Defining the role of ubiquitin-interacting motifs in the polyglutamine disease protein, ataxin-3. J Biol Chem. **280:**32026-34 Epub 2005 Jul 21.
15. **Berndt, C., D. Bech-Otschir, W. Dubiel, and M. Seeger.** 2002. Ubiquitin system: JAMMing in the name of the lid. Curr Biol. **12:**R815-7.
16. **Boname, J. M., and P. G. Stevenson.** 2001. MHC class I ubiquitination by a viral PHD/LAP finger protein. Immunity. **15:**627-36.
17. **Bonifacino, J. S., and A. M. Weissman.** 1998. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu Rev Cell Dev Biol. **14:**19-57.
18. **Borodovsky, A., B. M. Kessler, R. Casagrande, H. S. Overkleeft, K. D. Wilkinson, and H. L. Ploegh.** 2001. A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. Embo J. **20:**5187-96.
19. **Bowman, A. B., S. Y. Yoo, N. P. Dantuma, and H. Y. Zoghbi.** 2005. Neuronal dysfunction in a polyglutamine disease model occurs in the absence of ubiquitin-proteasome system impairment and inversely correlates with the degree of nuclear inclusion formation. Hum Mol Genet **14:**679-91 Epub 2005 Jan 20.
20. **Braun, B. C., M. Glickman, R. Kraft, B. Dahlmann, P. M. Kloetzel, D. Finley, and M. Schmidt.** 1999. The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat Cell Biol. **1:**221-6.
21. **Byrd, C., G. C. Turner, and A. Varshavsky.** 1998. The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. Embo J. **17:**269-77.

22. **Cadwell, K., and L. Coscoy.** 2005. Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. Science **309:**127-30.
23. **Cao, P. R., H. J. Kim, and S. H. Lecker.** 2005. Ubiquitin-protein ligases in muscle wasting. Int J Biochem Cell Biol. **37:**2088-97 Epub 2004 Dec 14.
24. **Capili, A. D., D. C. Schultz, I. F. Rauscher, and K. L. Borden.** 2001. Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains. Embo J. **20:**165-77.
25. **Carrell, R. W., and D. A. Lomas.** 1997. Conformational disease. Lancet **350:**134-8.
26. **Carter, S., S. Urbe, and M. J. Clague.** 2004. The met receptor degradation pathway: requirement for Lys48-linked polyubiquitin independent of proteasome activity. J Biol Chem **279:**52835-9 Epub 2004 Oct 05.
27. **Chai, Y., S. S. Berke, R. E. Cohen, and H. L. Paulson.** 2004. Poly-ubiquitin binding by the polyglutamine disease protein ataxin-3 links its normal function to protein surveillance pathways. J Biol Chem. **279:**3605-11 Epub 2003 Nov 5.
28. **Chen, B., J. Mariano, Y. C. Tsai, A. H. Chan, M. Cohen, and A. M. Weissman.** 2006. The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. Proc Natl Acad Sci U S A. **103:**341-6 Epub 2006 Jan 3.
29. **Chen, H. K., P. Fernandez-Funez, S. F. Acevedo, Y. C. Lam, M. D. Kaytor, M. H. Fernandez, A. Aitken, E. M. Skoulakis, H. T. Orr, J. Botas, and H. Y. Zoghbi.** 2003. Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. Cell. **113:**457-68.
30. **Chen, L., and K. Madura.** 2002. Rad23 promotes the targeting of proteolytic substrates to the proteasome. Mol Cell Biol. **22:**4902-13.
31. **Chen, P., P. Johnson, T. Sommer, S. Jentsch, and M. Hochstrasser.** 1993. Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MAT alpha 2 repressor. Cell. **74:**357-69.
32. **Chen-Plotkin, A. S., G. Sadri-Vakili, G. J. Yohrling, M. W. Braveman, C. L. Benn, K. E. Glajch, D. P. Dirocco, L. A. Farrell, D. Krainc, S. Gines, M. E. Macdonald, and J. H. Cha.** 2006. Decreased association of the transcription factor Sp1 with genes downregulated in Huntington's disease. Neurobiol Dis **23:**23.
33. **Chow, M. K., J. P. Mackay, J. C. Whisstock, M. J. Scanlon, and S. P. Bottomley.** 2004. Structural and functional analysis of the Josephin domain of the polyglutamine protein ataxin-3. Biochem Biophys Res Commun. **322:**387-94.
34. **Chung, K. K., Y. Zhang, K. L. Lim, Y. Tanaka, H. Huang, J. Gao, C. A. Ross, V. L. Dawson, and T. M. Dawson.** 2001. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med. **7:**1144-50.
35. **Ciechanover, A.** 2005. Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. Cell Death Differ. **12:**1178-90.
36. **Ciechanover, A.** 2006. The ubiquitin proteolytic system: from an idea to the patient bed. Proc Am Thorac Soc. **3:**21-31.
37. **Ciechanover, A., and R. Ben-Saadon.** 2004. N-terminal ubiquitination: more protein substrates join in. Trends Cell Biol. **14:**103-6.
38. **Ciechanover, A., and P. Brundin.** 2003. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron **40:**427-46.
39. **Ciechanover, A., S. Elias, H. Heller, S. Ferber, and A. Hershko.** 1980. Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. J Biol Chem. **255:**7525-8.
40. **Ciehanover, A., Y. Hod, and A. Hershko.** 1978. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. Biochem Biophys Res Commun. **81:**1100-5.
41. **Clarke, G., R. A. Collins, B. R. Leavitt, D. F. Andrews, M. R. Hayden, C. J. Lumsden, and R. R. McInnes.** 2000. A one-hit model of cell death in inherited neuronal degenerations. Nature **406:**195-9.
42. **Coffino, P.** 2001. Antizyme, a mediator of ubiquitin-independent proteasomal degradation. Biochimie. **83:**319-23.
43. **Cook, J. C., and P. B. Chock.** 1992. Isoforms of mammalian ubiquitin-activating enzyme. J Biol Chem. **267:**24315-21.

44. **Cornett, J., F. Cao, C. E. Wang, C. A. Ross, G. P. Bates, S. H. Li, and X. J. Li.** 2005. Polyglutamine expansion of huntingtin impairs its nuclear export. Nat Genet **37:**198-204 Epub 2005 Jan 16.
45. **Coscoy, L., and D. Ganem.** 2003. PHD domains and E3 ubiquitin ligases: viruses make the connection. Trends Cell Biol. **13:**7-12.
46. **Cummings, C. J., E. Reinstein, Y. Sun, B. Antalffy, Y. Jiang, A. Ciechanover, H. T. Orr, A. L. Beaudet, and H. Y. Zoghbi.** 1999. Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. Neuron **24:**879-92.
47. **Cummings, C. J., Y. Sun, P. Opal, B. Antalffy, R. Mestril, H. T. Orr, W. H. Dillmann, and H. Y. Zoghbi.** 2001. Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. Hum Mol Genet. **10:**1511-8.
48. **Cummings, C. J., and H. Y. Zoghbi.** 2000. Fourteen and counting: unraveling trinucleotide repeat diseases. Hum Mol Genet **9:**909-16.
49. **Cummings, C. J., and H. Y. Zoghbi.** 2000. Trinucleotide repeats: mechanisms and pathophysiology. Annu Rev Genomics Hum Genet. **1:**281-328.
50. **Dai, R. M., E. Chen, D. L. Longo, C. M. Gorbea, and C. C. Li.** 1998. Involvement of valosin-containing protein, an ATPase Co-purified with IkappaBalpha and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IkappaBalpha. J Biol Chem. **273:**3562-73.
51. **Dai, R. M., and C. C. Li.** 2001. Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. Nat Cell Biol. **3:**740-4.
52. **Dantuma, N. P., T. A. M. Groothuis, F. A. Salomons, and J. Neefjes.** 2006. A dynamic ubiquitin equilibrium couples proteasomal degradation to chromatin remodeling. J. Cell Biol. **in Press**.
53. **Dantuma, N. P., S. Heessen, K. Lindsten, M. Jellne, and M. G. Masucci.** 2000. Inhibition of proteasomal degradation by the gly-Ala repeat of Epstein-Barr virus is influenced by the length of the repeat and the strength of the degradation signal. Proc Natl Acad Sci U S A **97:**8381-5.
54. **Dantuma, N. P., K. Lindsten, R. Glas, M. Jellne, and M. G. Masucci.** 2000. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat Biotechnol **18:**538-43.
55. **Dantuma, N. P., and M. G. Masucci.** 2002. Stabilization signals: a novel regulatory mechanism in the ubiquitin/proteasome system. FEBS Lett **529:**22-6.
56. **Davies, S. W., M. Turmaine, B. A. Cozens, M. DiFiglia, A. H. Sharp, C. A. Ross, E. Scherzinger, E. E. Wanker, L. Mangiarini, and G. P. Bates.** 1997. Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell. **90:**537-48.
57. **de Pril, R., D. F. Fischer, M. L. Maat-Schieman, B. Hobo, R. A. de Vos, E. R. Brunt, E. M. Hol, R. A. Roos, and F. W. van Leeuwen.** 2004. Accumulation of aberrant ubiquitin induces aggregate formation and cell death in polyglutamine diseases. Hum Mol Genet. **13:**1803-13 Epub 2004 Jun 15.
58. **Demand, J., S. Alberti, C. Patterson, and J. Hohfeld.** 2001. Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr Biol. **11:**1569-77.
59. **Desterro, J. M., M. S. Rodriguez, and R. T. Hay.** 1998. SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. Mol Cell. **2:**233-9.
60. **Deveraux, Q., V. Ustrell, C. Pickart, and M. Rechsteiner.** 1994. A 26 S protease subunit that binds ubiquitin conjugates. J Biol Chem. **269:**7059-61.
61. **Dhananjayan, S. C., A. Ismail, and Z. Nawaz.** 2005. Ubiquitin and control of transcription. Essays Biochem. **41:**69-80.
62. **Dick, T. P., A. K. Nussbaum, M. Deeg, W. Heinemeyer, M. Groll, M. Schirle, W. Keilholz, S. Stevanovic, D. H. Wolf, R. Huber, H. G. Rammensee, and H. Schild.** 1998. Contribution of proteasomal beta-subunits to the cleavage of peptide substrates analyzed with yeast mutants. J Biol Chem. **273:**25637-46.
63. **Ditzel, M., R. Wilson, T. Tenev, A. Zachariou, A. Paul, E. Deas, and P. Meier.** 2003. Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. Nat Cell Biol. **5:**467-73.
64. **Donaldson, K. M., W. Li, K. A. Ching, S. Batalov, C. C. Tsai, and C. A. Joazeiro.** 2003. Ubiquitin-mediated sequestration of normal cellular proteins into polyglutamine aggregates. Proc Natl Acad Sci U S A **100:**8892-7. Epub 2003 Jul 11.

65. **Doss-Pepe, E. W., E. S. Stenroos, W. G. Johnson, and K. Madura.** 2003. Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis. Mol Cell Biol. **23:**6469-83.
66. **Dubiel, W., G. Pratt, K. Ferrell, and M. Rechsteiner.** 1992. Purification of an 11 S regulator of the multicatalytic protease. J Biol Chem. **267:**22369-77.
67. **Eichler, J., and F. Duong.** 2004. Break on through to the other side--the Sec translocon. Trends Biochem Sci. **29:**221-3.
68. **Eletr, Z. M., D. T. Huang, D. M. Duda, B. A. Schulman, and B. Kuhlman.** 2005. E2 conjugating enzymes must disengage from their E1 enzymes before E3-dependent ubiquitin and ubiquitin-like transfer. Nat Struct Mol Biol. **12:**933-4 Epub 2005 Sep 4.
69. **Ellgaard, L., and A. Helenius.** 2003. Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol. **4:**181-91.
70. **Elsasser, S., and D. Finley.** 2005. Delivery of ubiquitinated substrates to protein-unfolding machines. Nat Cell Biol **7:**742-9.
71. **Emamian, E. S., M. D. Kaytor, L. A. Duvick, T. Zu, S. K. Tousey, H. Y. Zoghbi, H. B. Clark, and H. T. Orr.** 2003. Serine 776 of ataxin-1 is critical for polyglutamine-induced disease in SCA1 transgenic mice. Neuron. **38:**375-87.
72. **Etlinger, J. D., and A. L. Goldberg.** 1977. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. Proc Natl Acad Sci U S A. **74:**54-8.
73. **Fan, C. M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-kappa B by processing of p105 through an ATP-dependent pathway. Nature. **354:**395-8.
74. **Feany, M. B., and W. W. Bender.** 2000. A Drosophila model of Parkinson's disease. Nature. **404:**394-8.
75. **Ferdous, A., F. Gonzalez, L. Sun, T. Kodadek, and S. A. Johnston.** 2001. The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. Mol Cell. **7:**981-91.
76. **Fergusson, J., M. Landon, J. Lowe, L. Ward, F. W. van Leeuwen, and R. J. Mayer.** 2000. Neurofibrillary tangles in progressive supranuclear palsy brains exhibit immunoreactivity to frameshift mutant ubiquitin-B protein. Neurosci Lett. **279:**69-72.
77. **Finley, D., S. Sadis, B. P. Monia, P. Boucher, D. J. Ecker, S. T. Crooke, and V. Chau.** 1994. Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. Mol Cell Biol. **14:**5501-9.
78. **Fischer, D. F., R. A. De Vos, R. Van Dijk, F. M. De Vrij, E. A. Proper, M. A. Sonnemans, M. C. Verhage, J. A. Sluijs, B. Hobo, M. Zouambia, E. N. Steur, W. Kamphorst, E. M. Hol, and F. W. Van Leeuwen.** 2003. Disease-specific accumulation of mutant ubiquitin as a marker for proteasomal dysfunction in the brain. Faseb J. **17:**2014-24.
79. **Flick, K., I. Ouni, J. A. Wohlschlegel, C. Capati, W. H. McDonald, J. R. Yates, and P. Kaiser.** 2004. Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain. Nat Cell Biol. **6:**634-41 Epub 2004 Jun 20.
80. **Forster, A., and C. P. Hill.** 2003. Proteasome degradation: enter the substrate. Trends Cell Biol. **13:**550-3.
81. **Frontali, M.** 2001. Spinocerebellar ataxia type 6: channelopathy or glutamine repeat disorder? Brain Res Bull. **56:**227-31.
82. **Gaczynska, M., A. L. Goldberg, K. Tanaka, K. B. Hendil, and K. L. Rock.** 1996. Proteasome subunits X and Y alter peptidase activities in opposite ways to the interferon-gamma-induced subunits LMP2 and LMP7. J Biol Chem. **271:**17275-80.
83. **Galvao, R., L. Mendes-Soares, J. Camara, I. Jaco, and M. Carmo-Fonseca.** 2001. Triplet repeats, RNA secondary structure and toxic gain-of-function models for pathogenesis. Brain Res Bull. **56:**191-201.
84. **Gardner, R. G., A. G. Shearer, and R. Y. Hampton.** 2001. In vivo action of the HRD ubiquitin ligase complex: mechanisms of endoplasmic reticulum quality control and sterol regulation. Mol Cell Biol. **21:**4276-91.
85. **Gatchel, J. R., and H. Y. Zoghbi.** 2005. Diseases of unstable repeat expansion: mechanisms and common principles. Nat Rev Genet. **6:**743-55.
86. **Gauthier, L. R., B. C. Charrin, M. Borrell-Pages, J. P. Dompierre, H. Rangone, F. P. Cordelieres, J. De Mey, M. E. MacDonald, V. Lessmann, S. Humbert, and F. Saudou.** 2004. Huntingtin controls neurotrophic support and

survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell. **118:**127-38.
87. **Gavioli, R., T. Frisan, S. Vertuani, G. W. Bornkamm, and M. G. Masucci.** 2001. c-myc overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells. Nat Cell Biol. **3:**283-8.
88. **Geier, E., G. Pfeifer, M. Wilm, M. Lucchiari-Hartz, W. Baumeister, K. Eichmann, and G. Niedermann.** 1999. A giant protease with potential to substitute for some functions of the proteasome. Science. **283:**978-81.
89. **Gilon, T., O. Chomsky, and R. G. Kulka.** 1998. Degradation signals for ubiquitin system proteolysis in Saccharomyces cerevisiae. Embo J **17:**2759-66.
90. **Glas, R., M. Bogyo, J. S. McMaster, M. Gaczynska, and H. L. Ploegh.** 1998. A proteolytic system that compensates for loss of proteasome function. Nature. **392:**618-22.
91. **Glickman, M. H., and A. Ciechanover.** 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev **82:**373-428.
92. **Glickman, M. H., D. M. Rubin, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried, and D. Finley.** 1998. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell. **94:**615-23.
93. **Golbik, R., A. N. Lupas, K. K. Koretke, W. Baumeister, and J. Peters.** 1999. The Janus face of the archaeal Cdc48/p97 homologue VAT: protein folding versus unfolding. Biol Chem. **380:**1049-62.
94. **Goldberg, M. S., S. M. Fleming, J. J. Palacino, C. Cepeda, H. A. Lam, A. Bhatnagar, E. G. Meloni, N. Wu, L. C. Ackerson, G. J. Klapstein, M. Gajendiran, B. L. Roth, M. F. Chesselet, N. T. Maidment, M. S. Levine, and J. Shen.** 2003. Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. J Biol Chem. **278:**43628-35 Epub 2003 Aug 20.
95. **Goldstein, G., M. Scheid, U. Hammerling, D. H. Schlesinger, H. D. Niall, and E. A. Boyse.** 1975. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc Natl Acad Sci U S A. **72:**11-5.
96. **Graham, R. W., D. Jones, and E. P. Candido.** 1989. UbiA, the major polyubiquitin locus in Caenorhabditis elegans, has unusual structural features and is constitutively expressed. Mol Cell Biol. **9:**268-77.
97. **Green, H.** 1993. Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism. Cell. **74:**955-6.
98. **Greene, J. C., A. J. Whitworth, I. Kuo, L. A. Andrews, M. B. Feany, and L. J. Pallanck.** 2003. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proc Natl Acad Sci U S A. **100:**4078-83 Epub 2003 Mar 17.
99. **Groll, M., M. Bajorek, A. Kohler, L. Moroder, D. M. Rubin, R. Huber, M. H. Glickman, and D. Finley.** 2000. A gated channel into the proteasome core particle. Nat Struct Biol. **7:**1062-7.
100. **Groll, M., L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H. D. Bartunik, and R. Huber.** 1997. Structure of 20S proteasome from yeast at 2.4 A resolution. Nature. **386:**463-71.
101. **Gutekunst, C. A., S. H. Li, H. Yi, J. S. Mulroy, S. Kuemmerle, R. Jones, D. Rye, R. J. Ferrante, S. M. Hersch, and X. J. Li.** 1999. Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J Neurosci. **19:**2522-34.
102. **Guterman, A., and M. H. Glickman.** 2004. Deubiquitinating enzymes are IN/(trinsic to proteasome function). Curr Protein Pept Sci. **5:**201-11.
103. **Hanson, P. I., and S. W. Whiteheart.** 2005. AAA+ proteins: have engine, will work. Nat Rev Mol Cell Biol. **6:**519-29.
104. **Harper, J. D., C. M. Lieber, and P. T. Lansbury, Jr.** 1997. Atomic force microscopic imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid-beta protein. Chem Biol. **4:**951-9.
105. **Hartmann-Petersen, R., M. Seeger, and C. Gordon.** 2003. Transferring substrates to the 26S proteasome. Trends Biochem Sci. **28:**26-31.
106. **Hatakeyama, S., M. Yada, M. Matsumoto, N. Ishida, and K. I. Nakayama.** 2001. U box proteins as a new family of ubiquitin-protein ligases. J Biol Chem. **276:**33111-20 Epub 2001 Jul 2.

107. **Heessen, S., M. G. Masucci, and N. P. Dantuma.** 2005. The UBA2 domain functions as an intrinsic stabilization signal that protects Rad23 from proteasomal degradation. Mol Cell. **18:**225-35.
108. **Helmlinger, D., S. Hardy, G. Abou-Sleymane, A. Eberlin, A. B. Bowman, A. Gansmuller, S. Picaud, H. Y. Zoghbi, Y. Trottier, L. Tora, and D. Devys.** 2006. Glutamine-expanded ataxin-7 alters TFTC/STAGA recruitment and chromatin structure leading to photoreceptor dysfunction. PLoS Biol. **4:**e67 Epub 2006 Feb 28.
109. **Hershko, A., and A. Ciechanover.** 1998. The ubiquitin system. Annu Rev Biochem **67:**425-79.
110. **Hicke, L.** 1999. Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol. **9:**107-12.
111. **Hicke, L.** 2001. Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol. **2:**195-201.
112. **Hipp, M. S., B. Kalveram, S. Raasi, M. Groettrup, and G. Schmidtke.** 2005. FAT10, a Ubiquitin-Independent Signal for Proteasomal Degradation. Mol Cell Biol **25:**3483-91.
113. **Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis, and S. Jentsch.** 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature. **419:**135-41.
114. **Hoffner, G., P. Kahlem, and P. Djian.** 2002. Perinuclear localization of huntingtin as a consequence of its binding to microtubules through an interaction with beta-tubulin: relevance to Huntington's disease. J Cell Sci. **115:**941-8.
115. **Hofmann, R. M., and C. M. Pickart.** 1999. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell. **96:**645-53.
116. **Holzl, H., B. Kapelari, J. Kellermann, E. Seemuller, M. Sumegi, A. Udvardy, O. Medalia, J. Sperling, S. A. Muller, A. Engel, and W. Baumeister.** 2000. The regulatory complex of Drosophila melanogaster 26S proteasomes. Subunit composition and localization of a deubiquitylating enzyme. J Cell Biol. **150:**119-30.
117. **Hoppe, T., K. Matuschewski, M. Rape, S. Schlenker, H. D. Ulrich, and S. Jentsch.** 2000. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. Cell. **102:**577-86.
118. **Hough, R., G. Pratt, and M. Rechsteiner.** 1986. Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. J Biol Chem. **261:**2400-8.
119. **Igarashi, S., R. Koide, T. Shimohata, M. Yamada, Y. Hayashi, H. Takano, H. Date, M. Oyake, T. Sato, A. Sato, S. Egawa, T. Ikeuchi, H. Tanaka, R. Nakano, K. Tanaka, I. Hozumi, T. Inuzuka, H. Takahashi, and S. Tsuji.** 1998. Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. Nat Genet. **18:**111-7.
120. **Illing, M. E., R. S. Rajan, N. F. Bence, and R. R. Kopito.** 2002. A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system. J Biol Chem **277:**34150-60 Epub 2002 Jun 28.
121. **Imai, Y., M. Soda, H. Inoue, N. Hattori, Y. Mizuno, and R. Takahashi.** 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell. **105:**891-902.
122. **Imai, Y., M. Soda, and R. Takahashi.** 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J Biol Chem. **275:**35661-4.
123. **Itier, J. M., P. Ibanez, M. A. Mena, N. Abbas, C. Cohen-Salmon, G. A. Bohme, M. Laville, J. Pratt, O. Corti, L. Pradier, G. Ret, C. Joubert, M. Periquet, F. Araujo, J. Negroni, M. J. Casarejos, S. Canals, R. Solano, A. Serrano, E. Gallego, M. Sanchez, P. Denefle, J. Benavides, G. Tremp, T. A. Rooney, A. Brice, and J. Garcia de Yebenes.** 2003. Parkin gene inactivation alters behaviour and dopamine neurotransmission in the mouse. Hum Mol Genet. **12:**2277-91 Epub 2003 Jul 22.
124. **Iwata, A., J. C. Christianson, M. Bucci, L. M. Ellerby, N. Nukina, L. S. Forno, and R. R. Kopito.** 2005. Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation. Proc Natl Acad Sci U S A. **102:**13135-40 Epub 2005 Sep 2.

125. **Jackson, P. K., A. G. Eldridge, E. Freed, L. Furstenthal, J. Y. Hsu, B. K. Kaiser, and J. D. Reimann.** 2000. The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. **10:**429-39.
126. **Jana, N. R., E. A. Zemskov, G. Wang, and N. Nukina.** 2001. Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum Mol Genet **10:**1049-59.
127. **Jarosch, E., C. Taxis, C. Volkwein, J. Bordallo, D. Finley, D. H. Wolf, and T. Sommer.** 2002. Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat Cell Biol **4:**134-9.
128. **Jeitner, T. M., M. B. Bogdanov, W. R. Matson, Y. Daikhin, M. Yudkoff, J. E. Folk, L. Steinman, S. E. Browne, M. F. Beal, J. P. Blass, and A. J. Cooper.** 2001. N(epsilon)-(gamma-L-glutamyl)-L-lysine (GGEL) is increased in cerebrospinal fluid of patients with Huntington's disease. J Neurochem. **79:**1109-12.
129. **Jentsch, S., W. Seufert, and H. P. Hauser.** 1991. Genetic analysis of the ubiquitin system. Biochim Biophys Acta. **1089:**127-39.
130. **Johnson, E. S., B. Bartel, W. Seufert, and A. Varshavsky.** 1992. Ubiquitin as a degradation signal. Embo J. **11:**497-505.
131. **Johnson, E. S., P. C. Ma, I. M. Ota, and A. Varshavsky.** 1995. A proteolytic pathway that recognizes ubiquitin as a degradation signal. J Biol Chem. **270:**17442-56.
132. **Johnston, J. A., C. L. Ward, and R. R. Kopito.** 1998. Aggresomes: a cellular response to misfolded proteins. J Cell Biol **143:**1883-98.
133. **Kahle, P. J., and C. Haass.** 2004. How does parkin ligate ubiquitin to Parkinson's disease? EMBO Rep. **5:**681-5.
134. **Kahlem, P., C. Terre, H. Green, and P. Djian.** 1996. Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. Proc Natl Acad Sci U S A. **93:**14580-5.
135. **Kalchman, M. A., R. K. Graham, G. Xia, H. B. Koide, J. G. Hodgson, K. C. Graham, Y. P. Goldberg, R. D. Gietz, C. M. Pickart, and M. R. Hayden.** 1996. Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. J Biol Chem. **271:**19385-94.
136. **Kanayama, A., R. B. Seth, L. Sun, C. K. Ea, M. Hong, A. Shaito, Y. H. Chiu, L. Deng, and Z. J. Chen.** 2004. TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell. **15:**535-48.
137. **Karpuj, M. V., H. Garren, H. Slunt, D. L. Price, J. Gusella, M. W. Becher, and L. Steinman.** 1999. Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei. Proc Natl Acad Sci U S A. **96:**7388-93.
138. **Katayama, T., K. Imaizumi, N. Sato, K. Miyoshi, T. Kudo, J. Hitomi, T. Morihara, T. Yoneda, F. Gomi, Y. Mori, Y. Nakano, J. Takeda, T. Tsuda, Y. Itoyama, O. Murayama, A. Takashima, P. St George-Hyslop, M. Takeda, and M. Tohyama.** 1999. Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. Nat Cell Biol. **1:**479-85.
139. **Kawakami, H., H. Maruyama, S. Nakamura, Y. Kawaguchi, A. Kakizuka, M. Doyu, and G. Sobue.** 1995. Unique features of the CAG repeats in Machado-Joseph disease. Nat Genet. **9:**344-5.
140. **Kegel, K. B., M. Kim, E. Sapp, C. McIntyre, J. G. Castano, N. Aronin, and M. DiFiglia.** 2000. Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. J Neurosci. **20:**7268-78.
141. **Kisselev, A. F., T. N. Akopian, and A. L. Goldberg.** 1998. Range of sizes of peptide products generated during degradation of different proteins by archaeal proteasomes. J Biol Chem. **273:**1982-9.
142. **Kisselev, A. F., T. N. Akopian, K. M. Woo, and A. L. Goldberg.** 1999. The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. J Biol Chem. **274:**3363-71.
143. **Klein, W. L., G. A. Krafft, and C. E. Finch.** 2001. Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci. **24:**219-24.
144. **Klement, I. A., P. J. Skinner, M. D. Kaytor, H. Yi, S. M. Hersch, H. B. Clark, H. Y. Zoghbi, and H. T. Orr.** 1998. Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. Cell. **95:**41-53.

145. **Kloetzel, P. M.** 2004. The proteasome and MHC class I antigen processing. Biochim Biophys Acta. **1695:**225-33.
146. **Koegl, M., T. Hoppe, S. Schlenker, H. D. Ulrich, T. U. Mayer, and S. Jentsch.** 1999. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell. **96:**635-44.
147. **Kopito, R. R.** 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. **10:**524-30.
148. **Kopito, R. R., and D. Ron.** 2000. Conformational disease. Nat Cell Biol. **2:**E207-9.
149. **Kuemmerle, S., C. A. Gutekunst, A. M. Klein, X. J. Li, S. H. Li, M. F. Beal, S. M. Hersch, and R. J. Ferrante.** 1999. Huntington aggregates may not predict neuronal death in Huntington's disease. Ann Neurol. **46:**842-9.
150. **Kwak, M. K., N. Wakabayashi, J. L. Greenlaw, M. Yamamoto, and T. W. Kensler.** 2003. Antioxidants enhance mammalian proteasome expression through the Keap1-Nrf2 signaling pathway. Mol Cell Biol. **23:**8786-94.
151. **La Spada, A. R., Y. H. Fu, B. L. Sopher, R. T. Libby, X. Wang, L. Y. Li, D. D. Einum, J. Huang, D. E. Possin, A. C. Smith, R. A. Martinez, K. L. Koszdin, P. M. Treuting, C. B. Ware, J. B. Hurley, L. J. Ptacek, and S. Chen.** 2001. Polyglutamine-expanded ataxin-7 antagonizes CRX function and induces cone-rod dystrophy in a mouse model of SCA7. Neuron. **31:**913-27.
152. **La Spada, A. R., and J. P. Taylor.** 2003. Polyglutamines placed into context. Neuron. **38:**681-4.
153. **Lam, Y. A., G. N. DeMartino, C. M. Pickart, and R. E. Cohen.** 1997. Specificity of the ubiquitin isopeptidase in the PA700 regulatory complex of 26 S proteasomes. J Biol Chem. **272:**28438-46.
154. **Lam, Y. A., T. G. Lawson, M. Velayutham, J. L. Zweier, and C. M. Pickart.** 2002. A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. Nature. **416:**763-7.
155. **Lam, Y. A., C. M. Pickart, A. Alban, M. Landon, C. Jamieson, R. Ramage, R. J. Mayer, and R. Layfield.** 2000. Inhibition of the ubiquitin-proteasome system in Alzheimer's disease. Proc Natl Acad Sci U S A **97:**9902-6.
156. **Lam, Y. A., W. Xu, G. N. DeMartino, and R. E. Cohen.** 1997. Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature. **385:**737-40.
157. **Lee, C. S., L. Y. Tee, T. Warmke, A. Vinjamoori, A. Cai, A. M. Fagan, and B. J. Snider.** 2004. A proteasomal stress response: pre-treatment with proteasome inhibitors increases proteasome activity and reduces neuronal vulnerability to oxidative injury. J Neurochem. **91:**996-1006.
158. **Lee, W. C., M. Yoshihara, and J. T. Littleton.** 2004. Cytoplasmic aggregates trap polyglutamine-containing proteins and block axonal transport in a Drosophila model of Huntington's disease. Proc Natl Acad Sci U S A. **101:**3224-9 Epub 2004 Feb 20.
159. **Leissring, M. A., Y. Akbari, C. M. Fanger, M. D. Cahalan, M. P. Mattson, and F. M. LaFerla.** 2000. Capacitative calcium entry deficits and elevated luminal calcium content in mutant presenilin-1 knockin mice. J Cell Biol. **149:**793-8.
160. **Lelouard, H., V. Ferrand, D. Marguet, J. Bania, V. Camosseto, A. David, E. Gatti, and P. Pierre.** 2004. Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. J Cell Biol. **164:**667-75 Epub 2004 Feb 23.
161. **Levitskaya, J., m. Coram, V. Levitsky, S. Imreh, P. M. Steigerwald-Mullen, G. Klein, M. G. Kurilla, and M. G. Masucci.** 1995. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr Virus nuclear antigen-1. Nature (London) **375:**685-688.
162. **Levitskaya, J., A. Sharipo, A. Leonchiks, A. Ciechanover, and M. G. Masucci.** 1997. Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. Proceedings of the National Academy of Sciences of the United States of America **94:**12616-21.
163. **Li, S. H., A. L. Cheng, H. Zhou, S. Lam, M. Rao, H. Li, and X. J. Li.** 2002. Interaction of Huntington disease protein with transcriptional activator Sp1. Mol Cell Biol. **22:**1277-87.
164. **Lin, L., and S. Ghosh.** 1996. A glycine-rich region in NF-kappaB p105 functions as a processing signal for the generation of the p50 subunit. Mol Cell Biol. **16:**2248-54.

165. **Lin, X., B. Antalffy, D. Kang, H. T. Orr, and H. Y. Zoghbi.** 2000. Polyglutamine expansion down-regulates specific neuronal genes before pathologic changes in SCA1. Nat Neurosci. **3:**157-63.
166. **Lindholm, D., H. Wootz, and L. Korhonen.** 2006. ER stress and neurodegenerative diseases. Cell Death Differ. **13:**385-92.
167. **Lindsten, K., F. M. de Vrij, L. G. Verhoef, D. F. Fischer, F. W. van Leeuwen, E. M. Hol, M. G. Masucci, and N. P. Dantuma.** 2002. Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation. J Cell Biol **157:**417-27. Epub 2002 Apr 29.
168. **Lindsten, K., V. Menendez-Benito, M. G. Masucci, and N. P. Dantuma.** 2003. A transgenic mouse model of the ubiquitin/proteasome system. Nat Biotechnol **21:**897-902 Epub 2003 Jul 20.
169. **Liu, C. W., M. J. Corboy, G. N. DeMartino, and P. J. Thomas.** 2003. Endoproteolytic activity of the proteasome. Science. **299:**408-11 Epub 2002 Dec 12.
170. **Lu, Z., S. Xu, C. Joazeiro, M. H. Cobb, and T. Hunter.** 2002. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol Cell. **9:**945-56.
171. **Luders, J., J. Demand, and J. Hohfeld.** 2000. The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J Biol Chem. **275:**4613-7.
172. **Lupas, A., W. Baumeister, and K. Hofmann.** 1997. A repetitive sequence in subunits of the 26S proteasome and 20S cyclosome (anaphase-promoting complex). Trends Biochem Sci. **22:**195-6.
173. **Ma, C. P., C. A. Slaughter, and G. N. DeMartino.** 1992. Identification, purification, and characterization of a protein activator (PA28) of the 20 S proteasome (macropain). J Biol Chem. **267:**10515-23.
174. **Mandrusiak, L. M., L. K. Beitel, X. Wang, T. C. Scanlon, E. Chevalier-Larsen, D. E. Merry, and M. A. Trifiro.** 2003. Transglutaminase potentiates ligand-dependent proteasome dysfunction induced by polyglutamine-expanded androgen receptor. Hum Mol Genet. **12:**1497-506.
175. **Mannhaupt, G., R. Schnall, V. Karpov, I. Vetter, and H. Feldmann.** 1999. Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett. **450:**27-34.
176. **Matilla, A., C. Gorbea, D. D. Einum, J. Townsend, A. Michalik, C. van Broeckhoven, C. C. Jensen, K. J. Murphy, L. J. Ptacek, and Y. H. Fu.** 2001. Association of ataxin-7 with the proteasome subunit S4 of the 19S regulatory complex. Hum Mol Genet. **10:**2821-31.
177. **Mattsson, K., K. Pokrovskaja, C. Kiss, G. Klein, and L. Szekely.** 2001. Proteins associated with the promyelocytic leukemia gene product (PML)-containing nuclear body move to the nucleolus upon inhibition of proteasome-dependent protein degradation. Proc Natl Acad Sci U S A **98:**1012-7.
178. **McCampbell, A., J. P. Taylor, A. A. Taye, J. Robitschek, M. Li, J. Walcott, D. Merry, Y. Chai, H. Paulson, G. Sobue, and K. H. Fischbeck.** 2000. CREB-binding protein sequestration by expanded polyglutamine. Hum Mol Genet. **9:**2197-202.
179. **McDonough, H., and C. Patterson.** 2003. CHIP: a link between the chaperone and proteasome systems. Cell Stress Chaperones. **8:**303-8.
180. **McMahon, S. J., M. G. Pray-Grant, D. Schieltz, J. R. Yates, 3rd, and P. A. Grant.** 2005. Polyglutamine-expanded spinocerebellar ataxia-7 protein disrupts normal SAGA and SLIK histone acetyltransferase activity. Proc Natl Acad Sci U S A. **102:**8478-82 Epub 2005 Jun 2.
181. **McNaught, K. S., D. P. Perl, A. L. Brownell, and C. W. Olanow.** 2004. Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. Ann Neurol **56:**149-62.
182. **McPhaul, L. W., J. Wang, E. M. Hol, M. A. Sonnemans, N. Riley, V. Nguyen, Q. X. Yuan, Y. H. Lue, F. W. Van Leeuwen, and S. W. French.** 2002. Molecular misreading of the ubiquitin B gene and hepatic mallory body formation. Gastroenterology. **122:**1878-85.
183. **Meiners, S., D. Heyken, A. Weller, A. Ludwig, K. Stangl, P. M. Kloetzel, and E. Kruger.** 2003. Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of Mammalian proteasomes. J Biol Chem **278:**21517-25 Epub 2003 Apr 3.

184. **Menendez-Benito, V., L. G. Verhoef, M. G. Masucci, and N. P. Dantuma.** 2005. Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. Hum Mol Genet. **14:**2787-99 Epub 2005 Aug 15.
185. **Meusser, B., C. Hirsch, E. Jarosch, and T. Sommer.** 2005. ERAD: the long road to destruction. Nat Cell Biol. **7:**766-72.
186. **Michalik, A., and C. Van Broeckhoven.** 2004. Proteasome degrades soluble expanded polyglutamine completely and efficiently. Neurobiol Dis **16:**202-11.
187. **Miller, J., and C. Gordon.** 2005. The regulation of proteasome degradation by multi-ubiquitin chain binding proteins. FEBS Lett. **579:**3224-30 Epub 2005 Mar 31.
188. **Mitsiades, C. S., N. Mitsiades, T. Hideshima, P. G. Richardson, and K. C. Anderson.** 2005. Proteasome inhibitors as therapeutics. Essays Biochem. **41:**205-18.
189. **Muchowski, P. J., G. Schaffar, A. Sittler, E. E. Wanker, M. K. Hayer-Hartl, and F. U. Hartl.** 2000. Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. Proc Natl Acad Sci U S A. **97:**7841-6.
190. **Muchowski, P. J., and J. L. Wacker.** 2005. Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci. **6:**11-22.
191. **Murata, S., H. Kawahara, S. Tohma, K. Yamamoto, M. Kasahara, Y. Nabeshima, K. Tanaka, and T. Chiba.** 1999. Growth retardation in mice lacking the proteasome activator PA28gamma. J Biol Chem. **274:**38211-5.
192. **Murata, S., Y. Minami, M. Minami, T. Chiba, and K. Tanaka.** 2001. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep. **2:**1133-8 Epub 2001 Nov 21.
193. **Murata, S., H. Udono, N. Tanahashi, N. Hamada, K. Watanabe, K. Adachi, T. Yamano, K. Yui, N. Kobayashi, M. Kasahara, K. Tanaka, and T. Chiba.** 2001. Immunoproteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta. Embo J. **20:**5898-907.
194. **Muratani, M., and W. P. Tansey.** 2003. How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol. **4:**192-201.
195. **Myung, J., K. B. Kim, and C. M. Crews.** 2001. The ubiquitin-proteasome pathway and proteasome inhibitors. Med Res Rev. **21:**245-73.
196. **Nagata, E., A. Sawa, C. A. Ross, and S. H. Snyder.** 2004. Autophagosome-like vacuole formation in Huntington's disease lymphoblasts. Neuroreport. **15:**1325-8.
197. **Nasmyth, K.** 2002. Segregating sister genomes: the molecular biology of chromosome separation. Science. **297:**559-65.
198. **Nguyen, T., P. J. Sherratt, H. C. Huang, C. S. Yang, and C. B. Pickett.** 2003. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. J Biol Chem. **278:**4536-41 Epub 2002 Nov 22.
199. **Nishikawa, H., S. Ooka, K. Sato, K. Arima, J. Okamoto, R. E. Klevit, M. Fukuda, and T. Ohta.** 2004. Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. J Biol Chem. **279:**3916-24 Epub 2003 Nov 24.
200. **Okazawa, H.** 2003. Polyglutamine diseases: a transcription disorder? Cell Mol Life Sci. **60:**1427-39.
201. **Pagano, M.** 1997. Cell cycle regulation by the ubiquitin pathway. Faseb J. **11:**1067-75.
202. **Palacino, J. J., D. Sagi, M. S. Goldberg, S. Krauss, C. Motz, M. Wacker, J. Klose, and J. Shen.** 2004. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem. **279:**18614-22 Epub 2004 Feb 24.
203. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell. **78:**773-85.
204. **Parag, H. A., B. Raboy, and R. G. Kulka.** 1987. Effect of heat shock on protein degradation in mammalian cells: involvement of the ubiquitin system. Embo J. **6:**55-61.
205. **Park, Y., S. Hong, S. J. Kim, and S. Kang.** 2005. Proteasome Function Is Inhibited by Polyglutamine-expanded Ataxin-1, the SCA1 Gene Product. Mol Cells **19:**23-30.
206. **Park, Y., Y. P. Hwang, J. S. Lee, S. H. Seo, S. K. Yoon, and J. B. Yoon.** 2005. Proteasomal ATPase-Associated Factor 1 Negatively Regulates Proteasome Activity by Interacting with Proteasomal ATPases. Mol Cell Biol **25:**3842-53.
207. **Paschen, W., and T. Mengesdorf.** 2005. Endoplasmic reticulum stress response and neurodegeneration. Cell Calcium. **38:**409-15.

208. **Patel, S., and M. Latterich.** 1998. The AAA team: related ATPases with diverse functions. Trends Cell Biol. **8:**65-71.
209. **Peng, J., D. Schwartz, J. E. Elias, C. C. Thoreen, D. Cheng, G. Marsischky, J. Roelofs, D. Finley, and S. P. Gygi.** 2003. A proteomics approach to understanding protein ubiquitination. Nat Biotechnol. **21:**921-6 Epub 2003 Jul 20.
210. **Perez, F. A., and R. D. Palmiter.** 2005. Parkin-deficient mice are not a robust model of parkinsonism. Proc Natl Acad Sci U S A. **102:**2174-9 Epub 2005 Jan 31.
211. **Perutz, M. F., T. Johnson, M. Suzuki, and J. T. Finch.** 1994. Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc Natl Acad Sci U S A **91:**5355-8.
212. **Pesah, Y., T. Pham, H. Burgess, B. Middlebrooks, P. Verstreken, Y. Zhou, M. Harding, H. Bellen, and G. Mardon.** 2004. Drosophila parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. Development. **131:**2183-94 Epub 2004 Apr 8.
213. **Peters, J. M., W. W. Franke, and J. A. Kleinschmidt.** 1994. Distinct 19 S and 20 S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. J Biol Chem. **269:**7709-18.
214. **Petrucelli, L., C. O'Farrell, P. J. Lockhart, M. Baptista, K. Kehoe, L. Vink, P. Choi, B. Wolozin, M. Farrer, J. Hardy, and M. R. Cookson.** 2002. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. Neuron **36:**1007-19.
215. **Pickart, C. M.** 2001. Mechanisms underlying ubiquitination. Annu Rev Biochem. **70:**503-33.
216. **Pickart, C. M., and M. J. Eddins.** 2004. Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta. **1695:**55-72.
217. **Pickart, C. M., and D. Fushman.** 2004. Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol. **8:**610-6.
218. **Piotrowski, J., R. Beal, L. Hoffman, K. D. Wilkinson, R. E. Cohen, and C. M. Pickart.** 1997. Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths. J Biol Chem. **272:**23712-21.
219. **Preckel, T., W. P. Fung-Leung, Z. Cai, A. Vitiello, L. Salter-Cid, O. Winqvist, T. G. Wolfe, M. Von Herrath, A. Angulo, P. Ghazal, J. D. Lee, A. M. Fourie, Y. Wu, J. Pang, K. Ngo, P. A. Peterson, K. Fruh, and Y. Yang.** 1999. Impaired immunoproteasome assembly and immune responses in PA28-/- mice. Science. **286:**2162-5.
220. **Ravikumar, B., R. Duden, and D. C. Rubinsztein.** 2002. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum Mol Genet **11:**1107-17.
221. **Realini, C., W. Dubiel, G. Pratt, K. Ferrell, and M. Rechsteiner.** 1994. Molecular cloning and expression of a gamma-interferon-inducible activator of the multicatalytic protease. J Biol Chem. **269:**20727-32.
222. **Rechsteiner, M., and C. P. Hill.** 2005. Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. Trends Cell Biol. **15:**27-33.
223. **Rechsteiner, M., and S. W. Rogers.** 1996. PEST sequences and regulation by proteolysis. Trends Biochem Sci. **21:**267-71.
224. **Reiner, A., I. Dragatsis, S. Zeitlin, and D. Goldowitz.** 2003. Wild-type huntingtin plays a role in brain development and neuronal survival. Mol Neurobiol. **28:**259-76.
225. **Reits, E. A., A. M. Benham, B. Plougastel, J. Neefjes, and J. Trowsdale.** 1997. Dynamics of proteasome distribution in living cells. Embo J. **16:**6087-94.
226. **Richly, H., M. Rape, S. Braun, S. Rumpf, C. Hoege, and S. Jentsch.** 2005. A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell. **120:**73-84.
227. **Rivett, A. J., and A. R. Hearn.** 2004. Proteasome function in antigen presentation: immunoproteasome complexes, Peptide production, and interactions with viral proteins. Curr Protein Pept Sci. **5:**153-61.
228. **Rockel, T. D., D. Stuhlmann, and A. von Mikecz.** 2005. Proteasomes degrade proteins in focal subdomains of the human cell nucleus. J Cell Sci. **118:**5231-42 Epub 2005 Oct 25.
229. **Ross, C. A., and M. A. Poirier.** 2005. Opinion: What is the role of protein aggregation in neurodegeneration? Nat Rev Mol Cell Biol. **6:**891-8.
230. **Ross, C. A., and M. A. Poirier.** 2004. Protein aggregation and neurodegenerative disease. Nat Med **10:**S10-7.

231. **Russell, S. J., S. H. Reed, W. Huang, E. C. Friedberg, and S. A. Johnston.** 1999. The 19S regulatory complex of the proteasome functions independently of proteolysis in nucleotide excision repair. Mol Cell. **3:**687-95.
232. **Ryu, E. J., H. P. Harding, J. M. Angelastro, O. V. Vitolo, D. Ron, and L. A. Greene.** 2002. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci. **22:**10690-8.
233. **Saeki, Y., Y. Tayama, A. Toh-e, and H. Yokosawa.** 2004. Definitive evidence for Ufd2-catalyzed elongation of the ubiquitin chain through Lys48 linkage. Biochem Biophys Res Commun. **320:**840-5.
234. **Sakamoto, K. M., K. B. Kim, A. Kumagai, F. Mercurio, C. M. Crews, and R. J. Deshaies.** 2001. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. Proc Natl Acad Sci U S A **98:**8554-9.
235. **Salomons, F. A., L. G. Verhoef, and N. P. Dantuma.** 2005. Fluorescent reporters for the ubiquitin-proteasome system. Essays Biochem. **41:**113-28.
236. **Sanchez, I., C. J. Xu, P. Juo, A. Kakizaka, J. Blenis, and J. Yuan.** 1999. Caspase-8 is required for cell death induced by expanded polyglutamine repeats. Neuron. **22:**623-33.
237. **Saric, T., S. C. Chang, A. Hattori, I. A. York, S. Markant, K. L. Rock, M. Tsujimoto, and A. L. Goldberg.** 2002. An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. Nat Immunol. **3:**1169-76 Epub 2002 Nov 18.
238. **Sato, K., K. Kashihara, S. Okada, T. Ikeuchi, S. Tsuji, T. Shomori, K. Morimoto, and T. Hayabara.** 1995. Does homozygosity advance the onset of dentatorubral-pallidoluysian atrophy? Neurology. **45:**1934-6.
239. **Sato, N., K. Imaizumi, T. Manabe, M. Taniguchi, J. Hitomi, T. Katayama, T. Yoneda, T. Morihara, Y. Yasuda, T. Takagi, T. Kudo, T. Tsuda, Y. Itoyama, T. Makifuchi, P. E. Fraser, P. St George-Hyslop, and M. Tohyama.** 2001. Increased production of beta-amyloid and vulnerability to endoplasmic reticulum stress by an aberrant spliced form of presenilin 2. J Biol Chem. **276:**2108-14 Epub 2000 Oct 12.
240. **Saudou, F., S. Finkbeiner, D. Devys, and M. E. Greenberg.** 1998. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell **95:**55-66.
241. **Saveanu, L., D. Fruci, and P. van Endert.** 2002. Beyond the proteasome: trimming, degradation and generation of MHC class I ligands by auxiliary proteases. Mol Immunol. **39:**203-15.
242. **Schauber, C., L. Chen, P. Tongaonkar, I. Vega, D. Lambertson, W. Potts, and K. Madura.** 1998. Rad23 links DNA repair to the ubiquitin/proteasome pathway. Nature. **391:**715-8.
243. **Schindler, U., H. Beckmann, and A. R. Cashmore.** 1993. HAT3.1, a novel Arabidopsis homeodomain protein containing a conserved cysteine-rich region. Plant J. **4:**137-50.
244. **Schroder, M., and R. J. Kaufman.** 2005. The mammalian unfolded protein response. Annu Rev Biochem. **74:**739-89.
245. **Schwartz, A. L., and A. Ciechanover.** 1999. The ubiquitin-proteasome pathway and pathogenesis of human diseases. Annu Rev Med. **50:**57-74.
246. **Schwartz, D. C., and M. Hochstrasser.** 2003. A superfamily of protein tags: ubiquitin, SUMO and related modifiers. Trends Biochem Sci. **28:**321-8.
247. **Serwold, T., F. Gonzalez, J. Kim, R. Jacob, and N. Shastri.** 2002. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. Nature. **419:**480-3.
248. **Shamu, C. E., D. Flierman, H. L. Ploegh, T. A. Rapoport, and V. Chau.** 2001. Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. Mol Biol Cell. **12:**2546-55.
249. **Shang, F., G. Deng, Q. Liu, W. Guo, A. L. Haas, B. Crosas, D. Finley, and A. Taylor.** 2005. Lys6-modified ubiquitin inhibits ubiquitin-dependent protein degradation. J Biol Chem. **280:**20365-74 Epub 2005 Mar 24.
250. **Sharipo, A., M. Imreh, A. Leonchiks, C. Branden, and M. G. Masucci.** 2001. cis-Inhibition of proteasomal degradation by viral repeats: impact of length and amino acid composition. FEBS Lett. **499:**137-42.
251. **Sharon, M., S. Witt, K. Felderer, B. Rockel, W. Baumeister, and C. V. Robinson.** 2006. 20S proteasomes have the potential to keep substrates in store for continual degradation. J Biol Chem **30:**30.

252. **Shimohata, T., T. Nakajima, M. Yamada, C. Uchida, O. Onodera, S. Naruse, T. Kimura, R. Koide, K. Nozaki, Y. Sano, H. Ishiguro, K. Sakoe, T. Ooshima, A. Sato, T. Ikeuchi, M. Oyake, T. Sato, Y. Aoyagi, I. Hozumi, T. Nagatsu, Y. Takiyama, M. Nishizawa, J. Goto, I. Kanazawa, I. Davidson, N. Tanese, H. Takahashi, and S. Tsuji.** 2000. Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. Nat Genet. **26:**29-36.
253. **Shimura, H., M. G. Schlossmacher, N. Hattori, M. P. Frosch, A. Trockenbacher, R. Schneider, Y. Mizuno, K. S. Kosik, and D. J. Selkoe.** 2001. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science. **293:**263-9 Epub 2001 Jun 28.
254. **Si, K., M. Giustetto, A. Etkin, R. Hsu, A. M. Janisiewicz, M. C. Miniaci, J. H. Kim, H. Zhu, and E. R. Kandel.** 2003. A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. Cell. **115:**893-904.
255. **Si, K., S. Lindquist, and E. R. Kandel.** 2003. A neuronal isoform of the aplysia CPEB has prion-like properties. Cell. **115:**879-91.
256. **Snyder, H., and B. Wolozin.** 2004. Pathological proteins in Parkinson's disease: focus on the proteasome. J Mol Neurosci. **24:**425-42.
257. **Song, S., S. Y. Kim, Y. M. Hong, D. G. Jo, J. Y. Lee, S. M. Shim, C. W. Chung, S. J. Seo, Y. J. Yoo, J. Y. Koh, M. C. Lee, A. J. Yates, H. Ichijo, and Y. K. Jung.** 2003. Essential role of E2-25K/Hip-2 in mediating amyloid-beta neurotoxicity. Mol Cell **12:**553-63.
258. **Song, Z., and M. Wu.** 2005. Identification of a novel nucleolar localization signal and a degradation signal in Survivin-deltaEx3: a potential link between nucleolus and protein degradation. Oncogene **28:**28.
259. **Spence, J., S. Sadis, A. L. Haas, and D. Finley.** 1995. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Mol Cell Biol. **15:**1265-73.
260. **Stefanis, L., K. E. Larsen, H. J. Rideout, D. Sulzer, and L. A. Greene.** 2001. Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. J Neurosci. **21:**9549-60.
261. **Steffan, J. S., A. Kazantsev, O. Spasic-Boskovic, M. Greenwald, Y. Z. Zhu, H. Gohler, E. E. Wanker, G. P. Bates, D. E. Housman, and L. M. Thompson.** 2000. The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. Proc Natl Acad Sci U S A. **97:**6763-8.
262. **Stuart, J. K., D. G. Myszka, L. Joss, R. S. Mitchell, S. M. McDonald, Z. Xie, S. Takayama, J. C. Reed, and K. R. Ely.** 1998. Characterization of interactions between the anti-apoptotic protein BAG-1 and Hsc70 molecular chaperones. J Biol Chem. **273:**22506-14.
263. **Sun, L., and Z. J. Chen.** 2004. The novel functions of ubiquitination in signaling. Curr Opin Cell Biol. **16:**119-26.
264. **Sun, Y., A. Savanenin, P. H. Reddy, and Y. F. Liu.** 2001. Polyglutamine-expanded huntingtin promotes sensitization of N-methyl-D-aspartate receptors via post-synaptic density 95. J Biol Chem. **276:**24713-8 Epub 2001 Apr 23.
265. **Suzuki, T., and A. Varshavsky.** 1999. Degradation signals in the lysine-asparagine sequence space. Embo J. **18:**6017-26.
266. **Swaminathan, S., A. Y. Amerik, and M. Hochstrasser.** 1999. The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. Mol Biol Cell. **10:**2583-94.
267. **Szebenyi, G., G. A. Morfini, A. Babcock, M. Gould, K. Selkoe, D. L. Stenoien, M. Young, P. W. Faber, M. E. MacDonald, M. J. McPhaul, and S. T. Brady.** 2003. Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport. Neuron. **40:**41-52.
268. **Taylor, J. P., F. Tanaka, J. Robitschek, C. M. Sandoval, A. Taye, S. Markovic-Plese, and K. H. Fischbeck.** 2003. Aggresomes protect cells by enhancing the degradation of toxic polyglutamine-containing protein. Hum Mol Genet. **12:**749-57.
269. **Thrower, J. S., L. Hoffman, M. Rechsteiner, and C. M. Pickart.** 2000. Recognition of the polyubiquitin proteolytic signal. Embo J. **19:**94-102.
270. **Tompkins, M. M., and W. D. Hill.** 1997. Contribution of somal Lewy bodies to neuronal death. Brain Res. **775:**24-9.
271. **Tsai, B., Y. Ye, and T. A. Rapoport.** 2002. Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. Nat Rev Mol Cell Biol. **3:**246-55.

272. **Upadhya, S. C., and A. N. Hegde.** 2003. A potential proteasome-interacting motif within the ubiquitin-like domain of parkin and other proteins. Trends Biochem Sci. **28:**280-3.
273. **Vabulas, R. M., and F. U. Hartl.** 2005. Protein synthesis upon acute nutrient restriction relies on proteasome function. Science. **310:**1960-3.
274. **Waelter, S., A. Boeddrich, R. Lurz, E. Scherzinger, G. Lueder, H. Lehrach, and E. E. Wanker.** 2001. Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. Mol Biol Cell **12:**1393-407.
275. **van Leeuwen, F. W., D. P. de Kleijn, H. H. van den Hurk, A. Neubauer, M. A. Sonnemans, J. A. Sluijs, S. Koycu, R. D. Ramdjielal, A. Salehi, G. J. Martens, F. G. Grosveld, J. Peter, H. Burbach, and E. M. Hol.** 1998. Frameshift mutants of beta amyloid precursor protein and ubiquitin-B in Alzheimer's and Down patients. Science **279:**242-7.
276. **van Nocker, S., S. Sadis, D. M. Rubin, M. Glickman, H. Fu, O. Coux, I. Wefes, D. Finley, and R. D. Vierstra.** 1996. The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in Saccharomyces cerevisiae and plays a nonessential, substrate-specific role in protein turnover. Mol Cell Biol. **16:**6020-8.
277. **Wang, H. L., T. H. Yeh, A. H. Chou, Y. L. Kuo, L. J. Luo, C. Y. He, P. C. Huang, and A. H. Li.** 2006. Polyglutamine-expanded ataxin-7 activates mitochondrial apoptotic pathway of cerebellar neurons by upregulating Bax and downregulating Bcl-x(L). Cell Signal. **18:**541-52 Epub 2005 Jun 16.
278. **Wang, H. R., M. Kania, W. Baumeister, and P. M. Nederlof.** 1997. Import of human and Thermoplasma 20S proteasomes into nuclei of HeLa cells requires functional NLS sequences. Eur J Cell Biol. **73:**105-13.
279. **Warrick, J. M., H. Y. Chan, G. L. Gray-Board, Y. Chai, H. L. Paulson, and N. M. Bonini.** 1999. Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. Nat Genet. **23:**425-8.
280. **Warrick, J. M., L. M. Morabito, J. Bilen, B. Gordesky-Gold, L. Z. Faust, H. L. Paulson, and N. M. Bonini.** 2005. Ataxin-3 suppresses polyglutamine neurodegeneration in Drosophila by a ubiquitin-associated mechanism. Mol Cell. **18:**37-48.
281. **Varshavsky, A.** 2006. The early history of the ubiquitin field. Protein Sci. **15:**647-54.
282. **Weeda, G., M. Rossignol, R. A. Fraser, G. S. Winkler, W. Vermeulen, L. J. van 't Veer, L. Ma, J. H. Hoeijmakers, and J. M. Egly.** 1997. The XPB subunit of repair/transcription factor TFIIH directly interacts with SUG1, a subunit of the 26S proteasome and putative transcription factor. Nucleic Acids Res. **25:**2274-83.
283. **Welchman, R. L., C. Gordon, and R. J. Mayer.** 2005. Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nat Rev Mol Cell Biol. **6:**599-609.
284. **Wellington, C. L., L. M. Ellerby, A. S. Hackam, R. L. Margolis, M. A. Trifiro, R. Singaraja, K. McCutcheon, G. S. Salvesen, S. S. Propp, M. Bromm, K. J. Rowland, T. Zhang, D. Rasper, S. Roy, N. Thornberry, L. Pinsky, A. Kakizuka, C. A. Ross, D. W. Nicholson, D. E. Bredesen, and M. R. Hayden.** 1998. Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. J Biol Chem. **273:**9158-67.
285. **Venkatraman, P., R. Wetzel, M. Tanaka, N. Nukina, and A. L. Goldberg.** 2004. Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing proteins. Mol Cell **14:**95-104.
286. **Verhoef, L. G., K. Lindsten, M. G. Masucci, and N. P. Dantuma.** 2002. Aggregate formation inhibits proteasomal degradation of polyglutamine proteins. Hum Mol Genet **11:**2689-700.
287. **Verma, R., L. Aravind, R. Oania, W. H. McDonald, J. R. Yates, 3rd, E. V. Koonin, and R. J. Deshaies.** 2002. Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science. **298:**611-5 Epub 2002 Aug 15.
288. **Whitby, F. G., E. I. Masters, L. Kramer, J. R. Knowlton, Y. Yao, C. C. Wang, and C. P. Hill.** 2000. Structural basis for the activation of 20S proteasomes by 11S regulators. Nature. **408:**115-20.
289. **Wilkinson, C. R., M. Seeger, R. Hartmann-Petersen, M. Stone, M. Wallace, C. Semple, and C. Gordon.** 2001. Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. Nat Cell Biol. **3:**939-43.

290. **Wing, S. S.** 2003. Deubiquitinating enzymes--the importance of driving in reverse along the ubiquitin-proteasome pathway. Int J Biochem Cell Biol. **35:**590-605.
291. **Wojcik, C., and G. N. DeMartino.** 2003. Intracellular localization of proteasomes. Int J Biochem Cell Biol. **35:**579-89.
292. **Wu, S. S., J. P. de Chadarevian, L. McPhaul, N. E. Riley, F. W. van Leeuwen, and S. W. French.** 2002. Coexpression and accumulation of ubiquitin +1 and ZZ proteins in livers of children with alpha(1)-antitrypsin deficiency. Pediatr Dev Pathol. **5:**293-8.
293. **Xie, Y., and A. Varshavsky.** 2001. RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc Natl Acad Sci U S A. **98:**3056-61.
294. **Yang, M., S. Omura, J. S. Bonifacino, and A. M. Weissman.** 1998. Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. J Exp Med. **187:**835-46.
295. **Yang, W., J. R. Dunlap, R. B. Andrews, and R. Wetzel.** 2002. Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells. Hum Mol Genet. **11:**2905-17.
296. **Yang, Y., I. Nishimura, Y. Imai, R. Takahashi, and B. Lu.** 2003. Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. Neuron. **37:**911-24.
297. **Yao, T., and R. E. Cohen.** 2002. A cryptic protease couples deubiquitination and degradation by the proteasome. Nature. **419:**403-7 Epub 2002 Sep 1.
298. **Ye, Y., H. H. Meyer, and T. A. Rapoport.** 2001. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature. **414:**652-6.
299. **Yoo, S. Y., M. E. Pennesi, E. J. Weeber, B. Xu, R. Atkinson, S. Chen, D. L. Armstrong, S. M. Wu, J. D. Sweatt, and H. Y. Zoghbi.** 2003. SCA7 knockin mice model human SCA7 and reveal gradual accumulation of mutant ataxin-7 in neurons and abnormalities in short-term plasticity. Neuron. **37:**383-401.
300. **Yorimitsu, T., and D. J. Klionsky.** 2005. Autophagy: molecular machinery for self-eating. Cell Death Differ. **12:**1542-52.
301. **Yoshida, Y., T. Chiba, F. Tokunaga, H. Kawasaki, K. Iwai, T. Suzuki, Y. Ito, K. Matsuoka, M. Yoshida, K. Tanaka, and T. Tai.** 2002. E3 ubiquitin ligase that recognizes sugar chains. Nature. **418:**438-42.
302. **Zatti, G., R. Ghidoni, L. Barbiero, G. Binetti, T. Pozzan, C. Fasolato, and P. Pizzo.** 2004. The presenilin 2 M239I mutation associated with familial Alzheimer's disease reduces Ca2+ release from intracellular stores. Neurobiol Dis. **15:**269-78.
303. **Zhai, W., H. Jeong, L. Cui, D. Krainc, and R. Tjian.** 2005. In vitro analysis of huntingtin-mediated transcriptional repression reveals multiple transcription factor targets. Cell. **123:**1241-53.
304. **Zhang, K., and R. J. Kaufman.** 2006. The unfolded protein response: a stress signaling pathway critical for health and disease. Neurology. **66:**S102-9.
305. **Zhang, M., and P. Coffino.** 2004. Repeat sequence of Epstein-Barr virus-encoded nuclear antigen 1 protein interrupts proteasome substrate processing. J Biol Chem. **279:**8635-41 Epub 2003 Dec 19.
306. **Zhang, M., C. M. Pickart, and P. Coffino.** 2003. Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate. Embo J. **22:**1488-96.
307. **Zhong, X., Y. Shen, P. Ballar, A. Apostolou, R. Agami, and S. Fang.** 2004. AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. J Biol Chem. **279:**45676-84 Epub 2004 Aug 24.
308. **Zhou, P., R. Bogacki, L. McReynolds, and P. M. Howley.** 2000. Harnessing the ubiquitination machinery to target the degradation of specific cellular proteins. Mol Cell **6:**751-6.
309. **Zhuchenko, O., J. Bailey, P. Bonnen, T. Ashizawa, D. W. Stockton, C. Amos, W. B. Dobyns, S. H. Subramony, H. Y. Zoghbi, and C. C. Lee.** 1997. Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. Nat Genet. **15:**62-9.
310. **Zoghbi, H. Y.** 1997. CAG repeats in SCA6. Anticipating new clues. Neurology. **49:**1196-9.
311. **Zoghbi, H. Y., and H. T. Orr.** 2000. Glutamine repeats and neurodegeneration. Annu Rev Neurosci **23:**217-47.

312. **Zwickl, P., W. Baumeister, and A. Steven.** 2000. Dis-assembly lines: the proteasome and related ATPase-assisted proteases. Curr Opin Struct Biol. **10:**242-50.
313. **Zwickl, P., E. Seemuller, B. Kapelari, and W. Baumeister.** 2001. The proteasome: a supramolecular assembly designed for controlled proteolysis. Adv Protein Chem. **59:**187-222.