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THE ROLE OF THE PROTEASOME IN HUNTINGTON’S DISEASE

Sabine Schipper-Krom
THE ROLE OF THE PROTEASOME IN HUNTINGTON’S DISEASE

Sabine Schipper-Krom

“A man who dares to waste one hour of time has not discovered the value of life”

Charles R. Darwin (1809-1882)
THE ROLE OF THE PROTEASOME IN HUNTINGTON’S DISEASE

ACADEMISCH PROEFSCHRIFT

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## TABLE OF CONTENTS

1. Short introduction and outline of the thesis

2. The ubiquitin-proteasome system in huntington’s disease

3. Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

4. The DNAJB6 and DNAJB8 chaperones prevent intracellular aggregation of polyglutamine peptides

5. Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

6. Enlightening proteasomes: methods to visualize their intracellular distribution, activity and interactions

7. Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies

8. Modulating proteasome activity in huntington’s disease

9. Summary and conclusion

& Addendum
SHORT INTRODUCTION AND OUTLINE
OF THE THESIS
INTRODUCTION

The cell can be considered as a big and crowded but well-functioning factory. Therefore, it is understandable that the proteome, which determines the status of the cell, needs to be monitored, controlled and adjusted very tightly. Proteostasis is thus an important aspect to retain homeostasis in the cell. The proteostasis network includes processes like protein folding and refolding by chaperones and protein degradation in order to prevent the accumulation and aggregation of hazardous misfolded proteins. Protein degradation is a continuous process, not only because of the limited half-life of many proteins and their natural turnover, but proteins can also become damaged by physiological conditions like stress and inadequate biogenesis [1, 2]. Moreover, aging, environmental and pathological conditions can induce increased damage or misfolding of proteins [3]. The latter can result in an overload of proteins targeted for degradation and consequently the misfolded proteins can sequester or interact with normal proteins causing loss of function.

Chaperones are one of the key players in the proteostasis network as they are involved in refolding proteins, prevention of misfolding and aggregation, and ultimately assist protein targeting towards the degradation machineries [4-6]. Upregulation of the amounts of chaperones can be a protective mechanism to prevent protein accumulation and aggregation during aging but also in various neurodegenerative diseases [7, 8]. Degradation of intracellular proteins occurs via two pathways, the ubiquitin-proteasome system (UPS) and autophagy. Autophagy is a lysosome-dependent degradation pathway which is generally held responsible for degradation of long-lived cytoplasmic proteins, large protein complexes and organelles [9]. The UPS mainly degrades short-lived and misfolded proteins. In this pathway, proteins are marked for degradation by a poly-ubiquitin chain that is recognized by the 19S cap of the proteasome [10]. This cap unfolds the protein, allowing access into the 20S proteolytic core where the protein is hydrolyzed into smaller peptides, ranging from 3 to 22 amino acids [11]. These peptides are subsequently processed into single amino acids by downstream peptidases or are presented by MHC class I molecules to the immune system.

While the UPS and autophagy are normally efficient in the turn-over of intracellular proteins, various neurodegenerative diseases, including Huntington’s disease (HD), are hallmark by the accumulation of misfolded and aggregating proteins. HD is one of the nine polyglutamine (polyQ) disorders that are caused by an expansion of the polyQ tract in the disease-related protein, which is huntingtin in the case of HD, and causes misfolding of the protein [12-14]. Patients suffering from this fatal disease have late-onset movement disorders due to a slowly progressive degeneration of the brain. While the exact mechanism behind the disease is still unknown, expansion of the polyQ tract of 37 glutamates or more results in aggregate formation in neuronal cells. The length of the expansion is inversely related with the severity of the disease, since a longer expansion induces an earlier onset of the disease and more severe symptoms. N-terminal fragments of the huntingtin protein, including the expanded polyQ tract, are considered to initiate aggregation, which mainly occurs in the nuclei of affected neurons [15]. As a consequence, the autophagic machinery is limited in targeting these nuclear aggregates, as autophagy is taking place in the cytoplasm. The alternative clearance pathway would be the UPS. However, the role of the UPS in HD remains elusive. The presence of proteasomes in polyQ aggregates in HD have led to the general idea that proteasomes are sequestered, leading to impairment of the UPS in HD [16]. In addition, proteasomes may be unable to degrade the polyQ tract, which would either lead to clogging of the proteasome by these polyQ fragments or the release of pure polyQ peptides [17, 18]. However, this data is conflicting with studies that monitored UPS activity in an HD mouse model, where aggregate formation did not necessarily led to the accumulation of short-lived proteins [19]. This indicates that the UPS is functional in these models. In this thesis, the role of proteasomes in HD is studied to explore whether the proteasome can serve as a target for therapeutic intervention.

Outline of the thesis

With respect to the role of proteasomes in HD, many studies report contradictory results. In chapter 2 we discuss the background of HD and the UPS system, and review studies focused on proteasomes in HD and the hypotheses that were formulated in these studies. On the basis of the data presented, we hypothesize new strategies to improve mutant huntingtin (mHtt) degradation by modulating proteasomes. Because in vitro degradation studies of polyQ peptides suggests the inability of proteasomes to cleave within glutamines sequences, the toxic fragment hypothesis was formulated. This hypothesis states that the flanking sequences of mHtt are degraded by proteasomes but that pure polyQ peptides are released in the cell. In chapter 3, we designed polyQ constructs that mimic the polyQ peptides which may be released by the proteasome. We examined whether these peptides induce the same disease-related aggregation phenotype as polyQ-expanded proteins, indicating that polyQ peptides are initiators of aggregation, which may be a general mechanism behind all polyQ diseases. A comparative screen of multiple chaperones identified the chaperones DNAJB6 and DNAJB8 as suppressors of aggregation and toxicity induced by polyQ proteins. In chapter 4, we studied whether these chaperones prevent polyQ peptide aggregation. Additionally, we used chaperone mutants and FLIM microscopy to determine the mechanism of these chaperones in preventing polyQ aggregation. Since insufficient degradation of polyQ fragments was shown in in vitro experiments, using isolated proteasomes and short polyQ peptides, we examined in chapter 5 whether proteasomes can or cannot degrade polyQ-expanded mHtt fragments in living cells. We studied the role of proteasomes in mHtt degradation and generated short-lived mHtt variants that were directly targeted towards proteasomes in cells. In addition, we studied degradation of mHtt by proteasomes in vitro. In both cases we observed efficient degradation of the mHtt protein. To study the role of proteasomes in mHtt degradation in more detail, we explored and designed in chapter 6 various methods to study proteasomal functioning. We used fluorescent tags and activity probes to visualize proteasomal distribution, activity and interactions. These methods were subsequently used to study proteasomal behavior in mHtt-expressing cells. In chapter 7, various microscopical studies were used to explore proteasomal dynamics in polyQ-expressing cells. We analyzed whether proteasomes are indeed irreversibly sequestered into IBs, and whether this leads to proteasome inactivation. Because our studies indicate that proteasomes are still active in aggregates, that they are only reversibly recruited to aggregating mHtt and can still degrade mHtt, it raises the question whether proteasome activity can...
be modified to improve mHtt degradation. In chapter 8 we studied the direct effects of proteasome modulation on mHtt degradation, both in vitro and in cellular systems.

REFERENCES


THE UBIQUITIN-PROTEASOME SYSTEM IN HUNTINGTON’S DISEASE

ARE PROTEASOMES IMPAIRED, INITIATORS OF DISEASE OR COMING TO THE RESCUE?

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ABSTRACT

Huntington’s disease is a progressive neurodegenerative disease, caused by a polyglutamine expansion in the huntingtin protein. A prominent hallmark of the disease is the presence of intracellular aggregates initiated by N-terminal huntingtin-fragments containing the polyglutamine repeat, which recruit components of the Ubiquitin Proteasome System. While it is commonly thought that proteasomes are irreversibly sequestered into these aggregates leading to impairment of the UPS, the data on proteasomal impairment in Huntington’s disease is contradictory. In addition, it has been suggested that proteasomes are unable to actually cleave polyglutamine sequences in vitro, thereby releasing aggregation-prone polyQ peptides in cells. Here we discuss how the proteasome is involved in the various stages of polyglutamine aggregation in Huntington’s disease, and how alterations in activity may improve clearance of mutant huntingtin fragments.

INTRODUCTION

Huntington’s disease

Huntington’s disease (HD) is one of nine polyglutamine (polyQ) disorders known to date, which are caused by an expansion in the CAG repeat sequence of the encoding DNA that is subsequently translated into a polyQ expansion within the disease-related protein [1, 2]. The presence of a glutamine repeat within proteins is a common feature mainly in transcription factors, and may mediate in protein-protein interactions [3, 4]. However, when the polyQ repeat exceeds a length of around 37 glutamines the expansion becomes disease causing [5, 6]. The severity of the disease is correlated with the length of the polyQ expansion, as an increasing repeat length correlates with earlier onset of disease and more severe symptoms [1]. There is strong evidence that the polyQ expansion induces a gain of function since insertion of an expanded CAG sequence in the hypoxanthine phosphoribosyltransferase (HPRT) gene, an HD unrelated gene which is not involved in any polyQ disorders, induced late-onset neurodegeneration and premature death in a mouse model similar to transgenic HD mouse models [7]. In addition, over-expression of polyQ peptides in transgenic mice caused a neurodegenerative phenotype demonstrating that the polyQ stretch by itself induces toxicity [8]. Still, a loss of wildtype huntingtin (Htt) function may also contribute to the disease when considering the broad spectrum of functions which are ascribed to wildtype Htt [9]. The Htt protein, affected in HD, is an ubiquitously expressed protein which is proposed to be important in embryonal development, transcriptional regulation, axonal and vesicle transport and has an anti-apoptotic function [10]. Although Htt is ubiquitously expressed, the earliest neuropathological changes in HD are found in the striatum and cerebral cortex, which are involved in motor control, cognition, and sensory pathways [11]. This leads to a cognitive decline in a progressive manner and manifests in motor dysfunction and severe dementia [12]. Furthermore, HD is characterized by psychiatric and emotional disturbances [13]. The fact that particular brain regions are more affected than others suggest that specific neurons are more vulnerable to Htt-induced toxicity probably due to cell-specific gene expression, protein-protein interaction or posttranslational protein modification [14, 15].

While the exact disease mechanisms behind HD remain elusive, many cellular pathways including transcriptional dysregulation, activation of apoptotic pathways, altered neurotransmitter release, mitochondrial dysfunction and oxidative stress were found to be affected and therefore subjected to research for therapeutic intervention [16]. An important pathological hallmark of all polyQ disorders is the presence of intracellular protein aggregates, similar as observed in other neurodegenerative disorders like Parkinson’s Disease and Alzheimer’s Disease. In the case of HD, aggregates found in human HD postmortem brain are composed of mutant Htt (mHtt) N-terminal fragments containing the polyQ stretch [17, 18]. The N-terminal mHtt fragments are highly prone to aggregate in the cell, and accumulating evidence suggests that especially small aggregates of oligomeric mHtt cause cellular toxicity [19-21]. Improving the clearance of these intermediate aggregates or monomeric mHtt fragments should therefore be a therapeutic target to prevent or delay the onset of HD.

The Ubiquitin Proteasome System

The two main pathways involved in the degradation of intracellular proteins are the Ubiquitin Proteasome System (UPS) and autophagy. Degradation via the UPS is essential for the clearance of short-lived and misfolded proteins, while autophagy mostly targets long-lived proteins and large structures like protein aggregates or organelles [22, 23]. Both cellular pathways are involved in polyQ protein clearance but at different levels. Degradation of mHtt via macro-autophagy requires targeting of proteins towards lysosomes, which is initiated by engulfment of proteins into autophagosomes. These subsequently fuse with lysosomes to form autolysosomes, resulting in breakdown of their contents by hydrolytic enzymes [24, 25]. However, aggregates of N-terminal mHtt fragments are mainly present in the cell nucleus in human HD postmortem brains [17, 18], while macro-autophagy is a cytoplasmic degradation pathway and therefore not sufficiently effective in clearing nuclear mHtt aggregates. To target nuclear mHtt fragments, the UPS gets into the picture, as proteasomes are present in both the cytoplasm and nucleus. Indeed, various studies indicate that the UPS is involved in processing both wildtype and mHtt [26, 27]. The UPS is mainly involved in maintaining cellular homeostasis via degradation of short-lived regulatory proteins like transcription factors and cell cycle regulatory proteins but also has a protective function since it is responsible for the degradation of damaged and misfolded proteins [28]. Most proteins designated for destruction by the UPS are first tagged by a poly-Ubiquitin chain, which is an ATP-dependent process that occurs via a three steps process. First, Ub is activated by an E1 ubiquitin-activating enzyme, followed by binding to an E2 conjugating enzyme, and finally the binding of the Ubiquitin (Ub) moiety to a lysine residue within the targeted protein via an E3-ligase. Subsequent ubiquitination of the conjugated Ub leads to a poly-Ub chain which designates the protein for targeting towards the 26S proteasome, were the substrates are recognized, unfolded and degraded [29]. The 26S proteasome includes two major complexes, the 20S core proteasome...
and the 19S regulatory particle. The 19S regulatory particle recognizes and de-ubiquitates the poly-ubiquitinated substrate, unfolds the protein and guides it through the 20S core [30-32]. The 20S core is a cylindrical complex consisting of four rings stacked on top of each other, while each ring contains seven subunits [33-35] (Fig. 1A). The two outer rings consist of α-subunits that close the interior of the barrel shaped complex, whereas the inner two rings are composed of seven β-subunits including three subunits with catalytic activity. These three active subunits, referred to as β1, β2, and β5, have caspase-like activity which cleaves behind acidic residues, trypsin-likes activity which cleaves after basic residues and chymotrypsin-like activity which cleaves behind hydrophobic residues, respectively. When unfolded substrates enter the hollow cavity of the 20S complex their amino acid chains are then attacked by the N-terminal threonine residue of the catalytic subunits [33, 34, 36, 37] (Fig. 1B). After cleavage, peptides are released into the cellular environment, where they are further processed by peptidases for antigen presentation or recycled into amino acids.

![Fig. 1. Representation of the 26S proteasome.](image)

(A) The 26S proteasome consists of a 20S core capped by one or two 19S activators. The catalytic subunits β1, β2 and β5 are represented in blue in the b-ring. (B) A schematic model of the 20S core, indicating the presence of cleavage sites inside the barrel-shaped structure.

THE ROLE OF THE PROTEASOME IN HUNTINGTON’S DISEASE

Proteasomes in HD: The good, the bad or the ugly?

Various studies indicate that the UPS is involved in processing mHtt since aggregates induced by mHtt are positively stained for Ub and proteasome subunits in human HD postmortem brains, in HD transgenic R6/2 mice that express polyQ-expanded mHtt-exon1(Q145) and in cell culture [17, 38-40]. Also soluble mHtt is poly-ubiquitinated in cells transfected with mHtt and in HD patient material, suggesting that mHtt can be targeted by the UPS [41-43]. Though, recently it was shown in cell culture that mHtt inclusions are initially devoid of ubiquitin and that soluble mHtt is not extensively ubiquitinated [44]. Furthermore, in vitro data suggested that proteasomes may actually be unable to degrade the polyQ repeat present in proteins, as purified mammalian 26S proteasomes were only able to cleave within the flanking sequences or after the first glutamine of a polyQ-containing peptide, while the remaining polyQ repeat was released by the proteasome [45].

One possible consequence of the ineffective degradation of polyQ sequences could be the clogging of proteasomes by long polyQ repeats. Proteasomes generate peptides with an average length of 3-9 amino acids, and these peptides do not exceed a length of 22 amino acids [46]. When confronted with a polyQ-expanded protein, the undigested polyQ peptide is much longer, which may then be unable to diffuse out of the narrow α-pore thereby clogging the proteasome, resulting in proteasomal impairment. This hypothesis was supported by FRET experiments showing a stable interaction between the proteasomal catalytic immuno-subunit LMP2 and mHtt, although it should be noted that the proteasomal fluorophore was on the outside of the proteasome core, and may thus reflect proteasome binding to Htt aggregates [47]. An alternative consequence would be the release of the polyQ peptides generated by the proteasome into the cellular environment, which subsequently would initiate aggregation. Indeed, when mimicking this polyQ peptide release in cells, polyQ peptides exceeding the disease-related threshold of around 40 glutamines showed resistance to degradation, leading to their accumulation and subsequent aggregation [48]. To prevent their accumulation, proteases and peptidases downstream the proteasome should target these polyQ peptides. One of the peptidases shown to be able to target polyQ sequences is Puromycin Sensitive Aminopeptidase (PSA), which is however, only able to degrade peptides up to 33 amino acids in length [49]. Surprisingly, PSA could still reduce aggregation and toxicity induced by polyQ-expanded peptides or mHtt, but this appears to occur via activation of the autophagy pathway and not via direct degradation by the peptidase [50]. Together this data indicates that proteasomes can not process polyQ fragments, which consequently could result in proteasome impairment.

Indeed, various studies in cell models and in patient material, have reported that the UPS is impaired in HD, which could be the underlying cause of the neurotoxicity. To examine the UPS pathway at different stages of mHtt degradation, a combination of different assays was used to detect alterations in the UPS in striatal cells derived from httQ111 knock-in mice which express full length mHtt at endogenous levels [51]. By using small fluorogenic proteasome substrates, as well as various short-lived luciferase reporters which act at different levels of the UPS, it was shown that the UPS is affected at two different levels. A change in activity of the 20S proteasome was detected, as the caspase-like and chymotrypsin-like activities were down-regulated, whereas the trypsin-like activity was highly upregulated. Importantly, no effect on the degradation of short-lived proteins that did not require ubiquitination was detected, whereas an increase in the half-life of a poly-ubiquitinated reporter was observed, indicating that there was a defect in recognition, de-ubiquitination or unfolding by the 19S cap. Since an increase in trypsin-like activity was also observed after a stress response upon ATP depletion, it was suggested that expression of mHtt may have caused this change in proteasome activity by a similar, indirect mechanism. In agreement with these results, a decrease in proteasomal caspase-like and chymotrypsin-like activity was also
detected both in postmortem brain material and in skin fibroblasts of HD patients, by using small fluorescent peptides [52]. Together, these studies suggest an overall proteasome impairment both in cells and patient material.

Furthermore, co-expression of mHtt with proteasomal subunits in cells also revealed that recruitment of proteasomes into HD aggregates seems to be irreversible, as fluorescence recovery after photo-bleaching (FRAP) experiments in living cells using fluorescently-tagged proteasomes showed no recovery of bleached proteasomes that resided in aggregates [47]. These findings led to the conclusion that proteasomes are trapped into polyQ aggregates, which would lead to impairment of the UPS due to Ub and proteasome depletion and even direct blockage or clogging of proteasomes. When using small fluorogenic substrates to quantify proteasome activity, a decrease in proteasomal activity was detected in the soluble fraction of neuronal cells stably expressing an N-terminal fragment of mHttQ150, whereas an increased proteasome activity was detected in the insoluble cell fraction containing aggregates [53]. Since there was also a decrease in the degradation of the proteasomal substrate p53, it was concluded that the sequestration of proteasomes into aggregates caused impaired proteasome functionality and neurotoxicity in the cell. This impairment due to sequestration in aggregates was further confirmed by groups using a short-lived GFP reporter, which has a CL-1 degron signal fused to the C-terminus of GFP, thereby targeting GFP for proteasomal degradation [54]. When this UPS reporters was co-transfected with polyQ proteins in HEK293 cells, intracellular GFP fluorescence increased 2-3-fold compared to control cells, indicating that proteasome impairment occurred in polyQ protein expressing cells [55]. The increase in fluorescence and thus proteasome impairment was even higher in polyQ-aggregate-containing cells, although it cannot be excluded that this could be due to higher expression levels of the introduced cDNAs in these cells. A global proteasome impairment was reported when mHtt aggregates were present in either the nucleus or the cytoplasm, using GFP expression levels of the introduced cDNAs in these cells. A global proteasome impairment was even higher in polyQ-aggregate-containing cells, although it cannot be excluded that this could be due to higher expression levels of the introduced cDNAs in these cells. A global proteasome impairment was also more recently underscored in R6/2 mice crossed with transgenic mouse/human HttQ94 minus in the forebrain, the earlier reported UPS impairment could not be detected [63]. But an increase in both the trypsin- and chymotrypsin-like activity was observed, similar to the increase in activities observed in cells expressing so-called immuno-proteasomes (induced upon treatment with IFNγ, as discussed below). Indeed, labeling for immuno-proteasome subunits confirmed the presence of immuno-proteasomes in brains of HD mice. The absence of proteasome impairment was also more recently underscored in R6/2 mice crossed with transgenic mice expressing different short-lived GFP reporters. Both GFP and Ub-G76V-GFP, where the GFP protein is fused to a non-cleavable Ub acting as a Ub-fusion degradation (UFD) signal, have been used as a proteasomal activity marker [61, 64, 65]. In both mouse models no inhibitory effect by mHtt on proteasomes was detected, contradicting the evidence for proteasome impairment in HD.

How to explain these apparently opposite findings in proteasome activity?

**Aggregate formation rescues proteasome function**

As mentioned above, *in vitro* experiments do not show any impairment when proteasomes were incubated with isolated mHtt aggregates and although proteasomes are associated with aggregates, cells still contain a large fraction of proteasomes that are not associated [56, 66]. Together with the observation that proteasomal impairment can already occur before aggregate formation, this argues against a sequestration model. Moreover, a potential protective role was suggested for aggregates when cultured striatal neurons expressing GFP-tagged mHtt-exon1 were visualized by means of an automated fluorescence microscope and followed in time [21]. Surprisingly, neurons that formed large aggregates (called inclusion bodies or IBs) showed a reduction in diffuse mHtt and a
The Ubiquitin-Proteasome System in Huntington’s Disease

The observed UPS impairment may therefore reflect the inability of cells to maintain protein homeostasis. This model would be in line with the observed transient accumulation of proteasome activity [66]. This suggests that diffuse, oligomeric mHtt can cause proteasomal impairment, whereas IBs do not interfere with the UPS. However, Hipp et al. showed that proteasomes do not become clogged in vitro by mHtt. This study also excludes in vitro competition between ubiquitinated mHtt and other ubiquitinated proteins for 26S proteasome-dependent degradation [44]. Together, this suggests that mHtt does not directly affect proteasomal activity, but rather maintaining mHtt’s solubility will place a burden on the total protein homeostasis machinery. The chaperone network would then become overloaded by aggregation prone mHtt, leading to an overload of proteins that depend on folding and a collapse of the proteolysis network. The observed UPS impairment may therefore reflect the inability of cells to maintain protein homeostasis. This model would be in line with the observed transient accumulation of proteasome reporters in inducible HD94 mice that were crossed with transgenic Ub-G76V-GFP mice [68]. Upon expression of the HD94 gene in two month old mature mice, a modest increase of the GFP reporter was measured in the first four weeks, indicating a decrease in UPS activity, followed by a decrease in GFP levels when aggregates appeared. When these mice received riluzole (an aggregation preventing compound) an increase in proteasomal reporter levels was again detected. These studies suggest that IB formation is not the bottle neck in progressive neurodegeneration, but that high levels of aggregation-prone mHtt can frustrate the UPS indirectly.

Still, one would expect that the observed sequestration of proteasomes into IBs would affect UPS function. Recent data from our lab suggests that proteasomes are not irreversibly sequestered in mHtt aggregates, but can still move outwards. When fluorescently-tagged proteasomes were co-expressed with fluorescently-tagged polyQ peptides in HeLa cells (green and red, respectively, Fig. 2), proteasomes were recruited into the polyQ aggregate. While the proteasome is present in the core of the aggregate in newly formed polyQ aggregates (Fig. 2, upper panel), the proteasomes only occupy the outskirts of larger aggregates in contrast to the polyQ peptides (Fig. 2, lower two panels). This suggests that polyQ fragments but not proteasomes are irreversibly sequestered. This shift to the outside of the large aggregate probably occurs slowly in time and would explain why no rapid exchange of proteasomes could be observed by photobleaching experiments [47]. Since proteasomes are apparently dynamically recruited to mHtt aggregates, is it then possible to stimulate proteasome activity to improve its capacity to reduce the burden of mHtt?

**IMPROVING ACTIVITY OF PROTEASOMES TOWARDS POLYQ PROTEINS**

**Changing proteasomal activity**

While purified proteasomes are unable to degrade polyQ repeats, it appears that the UPS in living cells is somehow capable to degrade these polyQ proteins once they are targeted towards the proteasome. This could be due to various modulations in the UPS that occur after specific triggers from the cellular environment, such as alterations in proteasome composition or the recruitment of proteasome activators. If possible, it would be interesting to modify the proteasomal activity to increase cleavage of polyQ sequences? While the constitutive 26S proteasome is comprised of a 20S catalytic core and a 19S activator as described above, the 19S cap can be replaced by the proteasome activator (PA)28γ or the PA28αβ activating cap. Furthermore, the 20S catalytic subunits β1, β2 and β5 can be replaced by the immuno-subunits LMP2 (PSMB9), LMP7 (PSMB8) and Mecl-1 (PSMB10).

The 20S core has two mechanisms to prevent random cleavage of substrates. First, there is a narrow channel, the α-annulus, which closes the catalytic proteasome core to folded proteins (Fig. 1B) [69]. Secondly, the N-termini of the α-subunits form a closed gate which cannot even be entered by small substrates. Thus, for substrates to enter the 20S proteasome, opening of the α-gate is necessary. This can be achieved by the 19S cap, which recognizes ubiquitinated proteins, but also by other proteasome activators, jet via a different mechanism. PA28α, β, and γ are homologous and thus activate the proteasome in a similar fashion. PA28αβ forms a homohexameric ring while PA28βγ forms a homohexameric ring [70-72]. These activator rings can dock on the α-subunits of the 20S via binding of the PA28 C-termini into the pockets between the α-subunits, followed by opening of the proteasome [73, 74]. Unlike the 19S cap, the PA28 caps are ATP-independent and are unable to recognize ubiquitinated and folded proteins, but can stimulate the peptidase activity of the proteasome up to 200-fold dependent on the substrate [74-76]. PA28βγ...
expression is induced upon IFNγ stimulation or viral infections, like multiple other genes involved in the immune response, and is therefore proposed to have an important role in antigen processing and presentation [77, 78]. When PA28αβ binds the proteasome all three catalytic activities of the proteasome are increased [79], which is not due to a direct effect on the catalytic subunits, but rather by structural change of the 20S core increasing the accessibility of the catalytic subunits [78, 80]. Furthermore, binding of the PA28αβ ring will open the α-gate, increasing the uptake but also the release of peptides, which may explain the increase in generated peptides that are more suitable for MHC class I binding [81]. The function of PA28γ in the cell is more diverse, as multiple interaction partners and degradation targets have been identified confirming a role in various cellular processes including cell cycle regulation and apoptosis, both in a 20S-dependent or independent manner [82, 83]. Proteasome activation by PA28αβ mainly increases trypsin-like activity, suggesting a conformational change in the 20S core that covers the chymotrypsin-like and caspase-like site and exposes the trypsin-like site [79, 84, 85]. Despite the peptidease activity 20S-PA28γ proteasomes are able to cleave intact proteins via unstructured or linker regions [84, 86].

The proteasome activator PA28αβ

Expression of the proteasome activator PA28αβ in patient material increased UPS function in control cells but not in HD fibroblasts, suggesting that introduction of PA28αβ would not improve polyQ degradation in mHtt expressing cells [52]. However, these experiments were performed in cells already expressing mHtt, and it would be interesting to induce PA28αβ at an earlier stage prior to disease onset in order to study the direct effect of PA28αβ on polyQ degradation. Interestingly, PA28αβ activation of purified 20S proteasomes increased degradation of short Q-peptides consisting of 10 glutamines, and degradation of short peptides with a glutamine at position P1 was increased with PA28αβ present [45, 87]. The expression of PA28αβ could improve polyQ degradation via two potential pathways, either by so-called hybrid proteasomes or via a two-step, sequential cleavage pathway (Fig. 3). Hybrid proteasomes are composed of a 20S particle capped on one side by the 19S complex and on the other side by PA28αβ [88]. Here, the 19S cap would recognize and unfold mHtt fragments, whereas PA28αβ would operate as an exit channel for generated polyQ peptides, thereby preventing internal clogging of proteasomes (Fig. 3, route 1). Although polyQ degradation is not improved, by flushing the polyQ peptides the proteasome would at least remain active. The second possibility would be a sequential pathway involving two different composed proteasomes (Fig. 3, route 2). When indeed the 26S proteasome would be unable to cleave the polyQ sequences present in mHtt, it would release the resulting pure polyQ fragments into the cellular environment [45]. However, PA28αβ could bind to both sites of downstream 20S particles thereby opening both gates and enhance the proteasome activities towards polyQ peptide degradation [89]. The frequency of these PA28αβ capped proteasomes seems to be low since it was shown that only 4% of the total proteasome pool in rabbit spleen had this composition [90], although this number differs dependent on the cell type used (e.g. 15% in HeLa cells) and can be increased by IFNγ [91]. To our knowledge it is unknown whether these double-capped PA28αβ proteasomes are present in brain tissues and whether they are increased during HD.

The proteasome activator PA28γ

While PA28αβ is mainly present in immune-related cells and generally absent from the brain, PA28γ could be a better candidate for proteasome activation due to its high expression in neurons [92]. Furthermore, the nuclear localization of PA28γ, in contrast to PA28αβ which is mainly present in the cytoplasm, makes it an interesting proteasomal activator to target intranuclear mHtt. However, since expression of PA28γ inhibits the chymotrypsin-like activity, which seems to be the catalytic site important for cleaving polyQ peptides, it was speculated that down-regulation of PA28γ would reduce the disease phenotype [65, 85, 93]. When R6/2 mice were crossed with PA28γ KO mice, no difference was seen in the behavioral phenotype nor in aggregate formation. In contrast, expression of PA28γ showed a protective role in an HD cellular model, but the observed increase in viability of cells that were exposed to stress conditions could also be due to the role of PA28γ as an anti-apoptotic factor [94, 95]. Additionally, an intriguing mutation at lysine 188 in PA28γ altered the activation of the 20S proteasome due to destabilization of the PA28γ ring structure [85]. It is thought that due to this unstable conformation the 20S core is differently structured thereby exposing all active sites with an increase of the proteasome activities similar to the activation changes induced by PA28αβ. In vitro, it has been demonstrated that the mutated activator PA28γ(K188E) increased activation towards polyQ fragments, since Q10-peptides were degraded into fragments ranging between 1-9 glutamines [93]. This is in contrast to the earlier studies by Venkatraman and colleagues, were it was
shown that proteasomes could not cleave polyQ sequence [45]. Varying experimental conditions could explain these differences. As proposed for the PA28αβ, also PA28γ(K188E) could improve polyQ degradation in two different pathways, either as hybrid proteasomes or by improving cleavage of polyQ peptides released by upstream 26S proteasomes (Fig 3).

**Proteasome immuno-subunits**

Besides inducing PA28αβ, IFNγ also induces expression of the proteasome immuno-subunits LMP2 (β1i), LMP7 (β5i) and MEC1-1 (β2i) which replace the constitutive catalytic subunits β1, β5 and β2, respectively. Incorporation of these newly synthesized subunits happens in de novo formed proteasomes within a time span that is four times faster than assembly of constitutive proteasomes [96, 97]. More important is the induced change in proteasome activity, as replacement of the constitutive subunits by immuno-subunits leads to down-regulation of the caspase-like activity and up-regulation of the trypsin-like and chymotrypsin-like activity [98-100], although some discrepancies have been published on the induced alterations in proteasome activity and studies on activity changes induced by individual immuno-subunits also do not give conclusive results [76, 101, 102]. Interestingly, a similar increase in trypsin- and chymotrypsin-like activity of the proteasome is observed in human HD postmortem brains and in the HD94 mouse model, suggesting that immuno-proteasomes are induced in HD [63]. It is tempting to believe that this may reflect a protective response in order to degrade the accumulating polyQ fragments, especially since immuno-proteasomes also appear to be important for cellular homeostasis [103, 104]. Another described consequence of IFNγ induction, is increased protein translation via the mTOR pathway, resulting in the generation of defective, unfolded and oxidized proteins [105-107]. These Defective Ribosomal Products (DRiPs) become poly-ubiquitinated and tend to form aggresome-like induced structures (ALIS) as a cellular response to misfolded protein fragments in the cell. Therefore, simply inducing immuno-proteasomes by IFNγ to improve polyQ degradation might be counterintuitive, as the increase in DRiPs would only accelerate aggregation. However, in time the 19S cap dissociates from the 20S core and starts to form complexes with the newly formed immuno-proteasome 20S particles [104]. These newly formed complexes appear to be better capable in preventing protein accumulations since mice deficient in immuno-subunits showed higher amounts of ALIS after IFNγ induction, indicating that immuno-proteasomes may be preferred to deal with the clearance of ‘dangerous’ proteins and fragments. This is further supported by data showing that immuno-proteasomes and PA28αβ are also involved in the increased degradation of oxidized proteins after treatment of cells with hydrogen peroxide (H₂O₂) [103]. The remaining question is whether we can use these immuno-proteasomes in order to clean up the polyQ fragments that induce toxicity in HD (Fig. 3, route 3). The observed presence of immuno-subunits in HD94 mice has been shown to be a secondary effect due to inflammation [63, 108]. However, it remains unknown what would happen if immuno-proteasomes were present at an earlier stage of the disease.

**CONCLUDING REMARKS**

Since mHtt aggregates are mainly found in the nuclei of the affected neurons of human HD postmortem brain it seems favorable to increase the degradation capacities in the nucleus. The UPS appears to be a robust mechanism in polyQ expressing cells, as it can recover after a temporary impairment [109]. Although it is clear that the proteasome is involved in the degradation of mHtt, the role of proteasomes remains contradictory. It is unknown whether proteasomes are the good guys as they can efficiently degrade nuclear mHtt fragments, or the bad guys for generating toxic, aggregation-prone polyQ peptides, or even the ugly guys when they become clogged and impaired by the polyQ fragments. In all cases the modification of proteasome activity could stimulate them to improve clearance and prevent aggregation and toxicity of the polyQ fragments, not only in HD but also in related polyQ disorders. Introduction of different activators, exchanging the catalytic subunits or even using specific proteasome compounds that modulate proteasome activity might lead to improvement of the proteolytic cleavage of polyQ proteins. As proteins with an expanded polyQ stretch need to be soluble to enter the 20S core, a combination of proteasome activation and aggregate preventing compounds or chaperones could benefit the degradation process. It is known that particular heat shock proteins can decrease aggregation rates in polyQ models, especially two members of the Hsp40 family (DNAJb6 and DNAJB8) are promising candidates [110-113]. Alternatively, chemical compounds and aggregation-interfering peptides like QBP1 could improve the solubility of mHtt to optimize its targeting for proteasomal degradation [20, 114]. Thus far, the general idea is that proteasomes are negatively affected in HD and have a great contribution to the disease course. The actual situation may be less grim, since recent data suggest that proteasomes are not impaired which makes it an interesting therapeutic target.

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The Ubiquitin-Proteasome System in Huntington's Disease


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ABSTRACT

Several neurodegenerative disorders, including Huntington’s disease, are caused by expansion of the polyglutamine (polyQ) tract over 40 glutamines in the disease-related protein. Fragments of these proteins containing the expanded polyQ tract are thought to initiate aggregation and represent the toxic species. Although it is not clear how these toxic fragments are generated, in vitro data suggest that proteasomes are unable to digest polyQ tracts. To examine whether the resulting polyQ peptides could initiate aggregation in living cells, we mimicked proteasomal release of monomeric polyQ peptides. These peptides lack the commonly used starting methionine residue or any additional tag. Only expanded polyQ peptides appear peptidase-resistant, and their accumulation initiated the aggregation process. As observed in polyQ disorders, these aggregates subsequently sequestered proteasomes, ubiquitin and polyQ proteins, and recruited HSP70. The generated expanded polyQ peptides were toxic to neuronal cells. Our approach mimics proteasomal release of pure polyQ peptides in living cells, and represents a valuable tool to screen for proteins and compounds that affect aggregation and toxicity.

INTRODUCTION

Numerous neurodegenerative diseases are manifested by the accumulation and aggregation of intracellular proteins. These diseases include polyglutamine (polyQ) expansion disorders like Huntington’s disease (HD), Spinal Bulbar Muscular Atrophy (SBMA) and various spinocerebellar ataxia’s (SCAs). PolyQ disorders are dominantly inherited and caused by expansions of CAG repeats. Normally, the disease-related proteins involved contain sequences of 6-40 glutamine repeats, while expansion of these tracts to 40-300 repeats leads to disease. The age of onset of the disorder is inversely correlated with the repeat length of the polyQ tracts (reviewed by [1]).

The presence of proteolytic protein fragments harbouring a polyQ tract in aggregates [2-5] has led to the ‘toxic fragment hypothesis’, which states that proteolytic fragments of polyQ-expanded huntingtin [6], androgen receptor [7] or certain ataxins [8-10] initiate protein aggregation and induce neuronal toxicity. Full length polyQ proteins aggregate, but at a much slower rate than their proteolytic fragments [7]. These fragments can be generated by proteases like caspasases, aspartic endopeptidases, calpains and the proteasome [11-15]. Accumulation of these proteolytic fragments may therefore function as a nucleation centre that sequesters full-length polyQ proteins in time. The proteasome can degrade both wild-type and expanded forms of most polyQ proteins, as was demonstrated in cultured cells and animal models [16, 17]. Surprisingly, polyQ-expanded proteins are not degraded to completion by the proteasome both in vitro and in vivo [18, 19]. Venkatraman and colleagues showed that isolated proteasomes cannot digest polyQ tracts present within a protein, which will result in the release of polyQ peptides. While flanking amino acids may be removed by exo-peptidases, the polyQ tracts themselves will accumulate when not efficiently cleared by downstream peptidases.

RESULTS

PolyQ-expanded peptides accumulate and induce intracellular aggregates

To examine the fate of proteasomal-released polyQ-peptides in living cells, we generated fusion proteins of fluorescently-tagged Ub with polyQ peptides of wild-type and disease-related lengths. Upon expression, the C-terminal polyQ peptide will be efficiently released from GFP-Ub by immediate cleavage via ubiquitin C-terminal hydrolases [20]. As a result, the generated polyQ peptide does not contain a starting methionine residue, which may affect degradation properties due to similarities with the N-terminus of a full-length protein [23]. Also, no tags (such as fluorophores or antibody epitopes) were directly attached to the polyQ peptide. As Ub was fluorescently tagged, the fluorescence intensity reflected the amount of generated polyQ peptides. PolyQ peptides of 16, 65 or 112 glutamine residues were fused to GFP-Ub resulting in GFP-Ub-Q16, GFP-Ub-Q65 and GFP-Ub-Q112, respectively (Fig. 1A).

Expression of the different GFP-Ub-polyQ proteins and subsequent release of polyQ peptides were analyzed 48 hours after transfection. Western blot analysis demonstrated the presence of GFP-Ub (36kDa) separated from all polyQ proteins (Fig. 1B, left panel). In addition, a large Ub conjugate was present, as shown before for GFP-Ub [24]. No additional bands were detected that could represent uncleaved GFP-Ub-polyQ proteins. Efficient cleavage was also observed when the Western blot was analysed for Ub (Fig. 1B, right panel). These results indicate that all polyQ peptides were efficiently cleaved from the GFP-Ub protein. Subsequent immunoblotting against polyQ using the antibody 1C2 [25] showed that polyQ peptides were present in the GFP-Ub-Q65 and GFP-Ub-Q112 lanes (Fig. 1C). The mobility on SDS-PAGE of expanded polyQ peptides was different from their...
calculated molecular weights, as has been observed before for polyQ-containing proteins [18, 26]. Some additional high molecular weight bands were present, which may represent oligomeric polyQ structures as these bands are GFP and Ub negative (Fig. 1B). The absence of Q16 peptides in cells expressing GFP-Ub-Q16 indicates that small Q peptides are efficiently cleared from the cytoplasm. It is unlikely that small Q peptides are not recognized by the 1C2 antibody, since a Q16-GFP fusion protein was recognized by 1C2 with almost equal efficiency as expanded GFP-polyQ fusions (supplementary Fig. 1A). Accumulation of Q65 and Q112 peptides, but not of Q16 peptides, suggests that expanded polyQ peptides were not efficiently degraded in living cells. To our knowledge, these peptides represent the first group of peptides that are resistant to degradation.

Fig. 1. PolyQ-expanded peptides induce intracellular aggregates.

(A) Schematic representation of GFP-Ub-polyQ (Q16, Q65 and Q112) fusion proteins and the generation of polyQ peptides upon synthesis and cleavage by Ub C-terminal hydrolases. (B) Cytosolic cell lysates of HEK293T expressing the different GFP-Ub-polyQ fusions were immuno-blotted against GFP (left) or Ub (right) 48 hours after transfection. GFP-Ub migrated at the same height for all three fusion proteins, indicating efficient cleavage of polyQ from GFP-Ub. Transfection efficiencies were lower for expanded polyQ peptide constructs. (C) Subsequent staining with an antibody against polyQ (1C2) revealed only the presence of polyQ peptides in cells expressing GFP-Ub-Q65 and GFP-Ub-Q112, and not of GFP-Ub-Q16. The asterisk indicates potential oligomeric structures. (D) Confocal images of GFP-Ub and the various GFP-Ub-polyQ distribution in Mel JuSo cells. GFP-Ub-Q65 showed a Ub distribution similar to free GFP-Ub, whereas a high percentage of cells expressing GFP-Ub-Q65 and GFP-Ub-Q112 showed Ub redistribution into aggregates. Scale bar ~5μm. (E) Percentage of transfected HEK293T cells exhibiting fluorescent aggregate at 48 and 72 h after transfection of cells (data are mean ± SEM of 3 different experiments). The amount of aggregates in cells expressing expanded polyQ peptides increased both in time and with polyQ length. (F) GFP-Ub was present in a ring around the aggregate induced by GFP-Ub-Q112 (left panel) that had a fibrillar structure at the ultrastructural level (middle panel), similar to structures induced by non-cleavable GFP-Q65 (right panel). Scale bar ~1μm. (G) Filter retardation assay showed entrapment of aggregates in HEK293T cells expressing GFP-Ub-Q65, GFP-Ub-Q112 and httex1-Q103-GFP, after immunostaining using the 1C2 antibody. In contrast, GFP is only present when the non-cleavable fusion protein Htt-exon1-GFP is used. (H) Confocal images of cells expressing GFP-Ub or the various GFP-Ub-polyQ constructs after immunostaining using antibodies against polyQ (1C2). Mel JuSo cells expressing GFP-Ub or GFP-Ub-Q112 showed no polyQ staining. Cells expressing GFP-Ub-Q65 and GFP-Ub-Q112 showed cytoplasmic polyQ staining when no aggregates were present. The presence of aggregates depleted the cells of free polyQ peptides, preventing polyQ staining. The arrows indicate an aggregate. Scale bar ~5μm. (I) Protease K treatment dissolved the protein shells around the polyQ aggregate, resulting in labeling the outside of the aggregation core with the anti-polyQ antibody 1C2. Scale bar ~5μm. (J) The Q-binding peptide QBP1-CFP was redistributed into aggregates induced by RFP-Ub-Q112. The arrow indicates the presence of a visible aggregate by phase contrast. Scale bar ~5 μm.
Since proteolytic protein fragments containing polyQ tracts are more aggregation prone than the full-length protein, we examined whether the accumulation of Q65 and Q112 peptides initiated aggregate formation. We observed a similar intracellular distribution of GFP-Ub in cells transfected with either GFP-Ub or GFP-Ub-Q16. GFP-Ub was enriched in the nucleus but was also present in the cytoplasm and on vesicles (Fig. 1D), similar to the distribution of endogenous Ub [24, 27]. In contrast, expression of GFP-Ub-Q65 and GFP-Ub-Q112 resulted in the appearance of a distinct intracellular structure decorated with fluorescent Ub in a high percentage of the transfected cells, present in either the nucleus or cytoplasm. The number of cells containing these structures increased both in time and with polyQ length (Fig. 1E). To see whether the length-dependency of aggregate formation would also hold true for polyQ lengths nearly the threshold, we expressed GFP-Ub fused to polyQ peptides of 33 or 48 glutamine residues. Whereas GFP-Ub-Q33 showed no aggregates, cells expressing GFP-Ub-Q48 showed aggregates, although in a much lower percentage of cells than those expressing Q65 or Q112 peptides (supplementary Fig 1B). GFP-Ub fluorescence was usually present in a ring around a dark core indicating that Ub was recruited (Fig. 1F). At the ultrastructural level, this structure showed a radiating dense core similar to aggregates formed by non-cleavable GFP-polyQ fusion proteins (Fig. 1F) and expanded huntingtin [28]. In cells expressing Q65 and Q112 peptides, these dense structures were resistant to SDS and selectively trapped in a filter-retardation assay [29]. Immunostaining using 1C2 showed that the trapped structures contained polyQ peptides (Fig. 1G), similar to huntingtin exon-1 Q103 (htt exon-1 Q103-GFP) [29]. This suggests that expanded polyQ peptides induce intracellular SDS-resistant aggregates. Although htt exon-1 Q103-GFP is also positive for GFP, no GFP is present on filter trap with the GFP-Ub-polyQ constructs, indicating efficient cleavage of the GFP-Ub-polyQ fusion proteins (Fig. 1G). Also, analysis of the soluble and insoluble fraction of cell lysates showed no uncleaved GFP-Ub-Q112 fusion proteins in either fraction (supplementary Fig. 1C).

To confirm the presence of polyQ peptides in intracellular aggregates, we immunostained cells expressing Q16, Q65 or Q112 peptides with 1C2. As expected, no polyQ peptides were detected in cells expressing GFP-Ub or GFP-Ub-Q16 (Fig. 1H). However, cells transfected with GFP-Ub-Q65 or GFP-Ub-Q112 showed two patterns of polyQ staining, dependent on the presence of aggregates. When aggregates were not present, polyQ staining was mainly cytoplasmic, whereas GFP-Ub localization was predominantly nuclear. By contrast, cells containing polyQ peptide aggregates were not recognized by 1C2 (Fig. 1H, arrows indicate an aggregate). A similar difference in immunostaining was obtained using the anti-polyQ antibody MW1 [30] (supplementary Fig. 1D). The absence of polyQ staining in cells containing aggregates is likely due to the dense aggregate structure and its surrounding protein layers that may shield the polyQ core. Indeed, pretreatment with proteinase K degraded shielding proteins and resulted in positive immunostaining of polyQ peptide aggregate and its surrounding protein layers that may shield the polyQ core. Indeed, pretreatment with proteinase K degraded shielding proteins and resulted in positive immunostaining of polyQ peptide aggregate (Fig. 1I), as has been observed previously for huntingtin aggregates [28]. To further confirm that the aggregates contain polyQ peptides, we used a cyan fluorescent protein (CFP) tagged Q-binding peptide (QBP-3) which selectively binds to polyQ aggregates [31]. QBP-1 showed a cytoplasmic distribution pattern when expressed alone or together with RFP-Ub or RFP-Ub-Q16 (data not shown). However, cells harboring aggregates initiated by Q112 peptides showed binding of QBP-1 to aggregates (Fig. 1J). Taken together, these results indicate that expanded polyQ peptides are not efficiently degraded and subsequently initiate formation of aggregates that display all characteristics of disease-related polyQ aggregates.

PolyQ peptide aggregates recruit proteasomes, ubiquitin and chaperones

Aggregates formed by expanded polyQ proteins often sequester proteins involved in the ubiquitin proteasome system (UPS) but also chaperones [18, 21]. We examined whether aggregates induced by expanded polyQ peptides showed a similar sequestration of UPS components. GFP-Ub was present in a ring around the aggregates (Fig. 1F). Absence of Ub in the aggregate core can be explained by the lack of lysine residues in polyQ peptides, thereby excluding ubiquitination of the polyQ peptides. The presence of GFP-Ub around the core was not due to inefficient cleavage of GFP-Ub-polyQ, since no uncleaved GFP-Ub-polyQ fusions could be detected by SDS-PAGE (Fig. 1B and supplementary Fig. 1C) and filtertrap (Fig. 1G). In addition, co-expression of GFP-Ub with RFP-Ub-Q112 showed a similar sequestration of both fluorescently-tagged Ub proteins into aggregates (Fig. 2A), indicating efficient cleavage. This suggests that the presence of GFP-Ub is due to ubiquitination of sequestered proteins.

We examined whether proteasomes co-localized with polyQ aggregates in our model, by co-expressing the different RFP-Ub-polyQ constructs with GFP-tagged immuno-proteasomal subunit LMP2. LMP2 was efficiently incorporated into active proteasomes [32]. Notably, LMP2-GFP was present in the core of polyQ aggregates, suggesting that proteasomes were recruited to aggregates even before Ub sequestration (Fig. 2B). A similar recruitment was observed when using the constitutive proteasome subunit β7 (Fig. 2D). This finding most likely reflects a proteasomal attempt to degrade accumulating polyQ peptides. The sequestered proteasomes and Ub seemed irreversibly trapped, which was revealed when Fluorescence Recovery After Photobleaching (FRAP) [33] was applied to determine on/off rates of the sequestered molecules. Upon photobleaching of one half of an aggregate, no exchange between the sequestered proteasomes or Ub and the surroundings was observed (supplementary Fig. 2A). This indicates that the proteasome becomes immobilized, as has been previously observed [18].

We also examined whether chaperones such as HSP70 were interacting with polyQ aggregates, as has been observed in polyQ diseases [21, 34]. Upon co-transfection of the different RFP-Ub-polyQ fusion proteins with GFP-tagged HSP70, we observed an additional ring-like structure of HSP70-GFP around the Ub-positive aggregate (Fig. 2C). FRAP analysis revealed that HSP70 was not irreversibly trapped in the aggregate (supplementary Fig. 2A), consistent with previous observations [21, 34]. To compare the composition of aggregates initiated by Q112 peptides with aggregates formed by polyQ-expanded huntingtin exon-1, cells were transfected with either GFP-Ub-Q112 or htt exon-1 Q103-GFP together with β7-RFP-tagged proteasomes, and cells were subsequently immunostained for endogenous HSP70. Triple color analysis showed that the core of the aggregate was positive for proteasomes (red). This core was surrounded by Ub or htt exon-1 Q103 (green). Finally, HSP70 was present within the most outer layer of the aggregate (blue) (Fig. 2D). This suggests that various proteins associate at different stages or with different affinities during aggregate formation. The presence of GFP-Ub and htt exon-1 Q103 in a similar layer may suggest the recruitment of ubiquitinated proteins and polyQ proteins in this stage of aggregate formation. Since aggregates initiated by expanded polyQ peptides also contained Ub, proteasomes and chaperones as has been described before, our model faithfully mimics aggregate formation in polyQ diseases.
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

THREE

Fig. 2. PolyQ peptide aggregates recruit UPS components and chaperones.

Mel Juso cells were transfected with the indicated constructs and imaged 48 hours after transfection. (A) Co-expression of GFP-Ub and RFP-Ub derived from RFP-Ub-Q112 resulted in identical redistribution into aggregates. (B) Proteasomes labeled with LMP2-GFP colocalize with the core of aggregates induced by RFP-Ub-Q112, with RFP-Ub surrounding the core. LMP2-GFP was freely distributed in nucleus and cytoplasm of cells expressing RFP-Ub-Q16. (C) The chaperone HSP70-GFP was redistributed into aggregates induced by RFP-Ub-Q112, and formed an additional ring around the Ub-positive polyQ peptide aggregate. (D) Upon transfection with GFP-Ub-Q112 or httex1-Q103-GFP together with the proteasomal subunit β7-RFP, cells were immunostained for endogenous HSP70. The proteasome was within the aggregate core, surrounded by Ub and an additional ring of chaperones. Scale bar ~5 μm.

Sequestering of glutamine-containing proteins into polyQ peptide aggregates

The presence of httex1-Q103 in ring-like structures around the aggregate and not within the core (Fig. 2D) suggests recruitment of large polyQ fragments into aggregates in a later stage. To examine this hypothesis, we co-expressed RFP-Ub-Q112 and httex1-Q103-GFP. Indeed, we found that httex1-Q103-GFP was sequestered into aggregates induced by polyQ peptides (Fig. 3A). In addition, the aggregation rate of httex1-Q103-GFP was also dramatically increased when Q112 peptides were present (supplementary Fig. 2B), which suggests that polyQ peptides initiate aggregates that accelerate huntingtin aggregation. Similar results were obtained with truncated polyQ-expanded ataxin-3 (atx3-Q85-GFP) and the SBMA-related truncated androgen receptor with a Q84 repeat (AR-Q84-GFP) (data not shown).
Aggregates induced by disease-related polyQ proteins also sequester the wild-type protein expressed by the non-expanded allele [8, 35]. We examined whether polyQ peptide aggregates also sequester non-expanded, wild-type polyQ proteins. The non-expanded httex1-Q25-GFP remained freely distributed in cells that co-expressed either RFP-Ub or RFP-Ub-Q16 (supplementary Fig. 2B). In contrast, httex1-Q25-GFP was recruited into polyQ peptide aggregates when co-transfected with RFP-Ub-Q112 (Fig. 3B and 2B). A similar entrapment of wild-type truncated ataxin-3 (atax3-Q28-GFP) (supplementary Fig. 2C) and the truncated androgen receptor (AR-Q19-GFP) was observed (data not shown). This sequestration of wild-type polyQ proteins may therefore lead to loss of function. Sequestration of non-expanded polyQ proteins was not limited to disease-related proteins, as other polyQ proteins were recruited into aggregates initiated by polyQ peptides, including Q16-GFP (Fig. 3C), and also the Q-stretch containing transcription factor TBP1 when nuclear aggregates were present (Fig. 3D).

**PolyQ peptides induce aggregates and toxicity in neuronal cells**

To examine whether polyQ peptides also initiate aggregate formation in neuronal cells, we transiently transfected N2A neuroblastoma cells with the various GFP-Ub-polyQ constructs. N2A cells transfected with either GFP-Ub-Q65 or GFP-Ub-Q112 developed aggregates similar to those present in non-neuronal cells (Fig. 4A), whereas GFP-Ub-Q16 expressing cells showed an Ub distribution comparable to GFP-Ub alone. Since HD mostly affects striatal cells, we also used immortalized STHdh+/+ striatal cells [36] which similarly generated intracellular aggregates when transfected with GFP-Ub-Q65 or Q112 (Fig. 4A). Many cells rounded up after expression of expanded polyQ peptides, suggesting toxicity, although this did not correlate with the presence of GFP-Ub positive aggregates. To determine whether the expressed polyQ peptides were toxic, the viability of transfected N2A cells was tested using propidium iodide (PI). Expression of expanded polyQ peptides resulted in increased numbers of PI-positive cells (data not shown). However, hardly any double-positive cells were observed. This is presumably explained by the fact that uptake of PI into polyQ peptide expressing cells was often preceded by loss of GFP fluorescence (Fig. 4B) as observed previously [37]. Because loss of fluorescence seemed to be associated with cell death, we used another approach to quantify polyQ peptide induced toxicity. To determine changes in the number of GFP-Ub positive cells in time, we used FACS analysis and compared cell populations expressing the different GFP-Ub-polyQ proteins at 24 and 48 hours after transfection. There was no difference in GFP-Ub fluorescence between cells expressing either GFP-Ub or GFP-Q16 in time. However, a significant decrease in fluorescence was observed in cells expressing GFP-Ub-Q112 when compared to GFP-Ub or GFP-Ub-Q16 (p<0.05), indicating that expression of Q112 peptides induced cell death (Fig. 4C). GFP-Ub-Q65 had a mild, although not significant, effect on cell death. Taken together, these results showed that expanded polyQ peptides form aggregates and become toxic to neuronal cells.

**DISCUSSION**

Proteolytic fragments containing expanded polyQ tracts are more aggregation-prone than original full-length proteins, as has been shown for huntingtin [6], androgen receptor [7], ataxin-3 [8] and ataxin-7 [9]. Recently, it was also postulated that an expanded polyQ fragment was expressed in SCAB due to anti-sense transcription resulting in polyQ inclusions [38]. These data suggest that polyQ fragments may be fundamental in initiating aggregation. It has however been shown that expanded polyQ proteins are efficiently targeted to the proteasome [18], which can degrade entire proteins with the exception of polyQ tracts [19]. Degradation by the proteasome may result in the release of polyQ peptides, whose flaming amino acids may be removed by exo-peptidases. It is unknown whether the resulting pure polyQ peptides are rapidly degraded by peptidases. If resistant, their subsequent accumulation may initiate aggregation and toxicity as observed in polyQ disorders. In order to examine this toxic fragment hypothesis, we mimicked intracellular proteosomal polyQ peptide generation as closely as possible by fusing pure polyQ peptides to GFP-tagged Ub. While Ub-polyQ fusions have been used before, these polyQ fragments also included either GFP tags.
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

Expression of our constructs resulted in the efficient release of “naked” polyQ peptides due to immediate cleavage by Ub C-terminal hydrolases. This was shown both by SDS-PAGE that showed a band at equal height for GFP-Ub irrespective of the original construct (Fig. 1B), analysis of the filtertrap assay (Fig. 1G) and the insoluble fraction (supplementary Fig. 1C) and different intracellular locations of GFP-Ub and polyQ peptides (Fig. 1H). Since the released polyQ peptides do not contain a starting methionine or additional tags, they closely resemble peptide generation by the proteasome. All previous studies have relied on expression of polyQ fusions that did include such features, which can significantly alter the in vivo behavior of polyQ fragments. Starting methionines will lend the peptides resemblance to the N-terminus of proteins, possibly affecting the rate of degradation [23]. Fluorescent tags contain lysine residues, which can serve as targets for ubiquitination and subsequent degradation by the proteasome. The intracellular release of monomeric polyQ peptides is also closer to the in vivo situation than the addition of synthesized polyQ peptide aggregates to cells [42].

We showed that only polyQ peptides with Q repeat lengths similar to disease-related peptides accumulated in the cell and initiated aggregation. The characteristics of aggregates induced by expanded polyQ peptides were similar to aggregates initiated by expression of expanded polyQ-containing proteins [18, 21, 22, 28]. These characteristics include sequestration of proteasomes, ubiquitin and other polyQ containing proteins such as TBP, and the presence of HSP70. While previous studies only speculated on the effect of proteasomal release of polyQ peptides in living cells, we show here that ‘proteasomal-derived’ expanded polyQ peptides by themselves are sufficient to accumulate and initiate aggregation. Accumulation of expanded polyQ peptides is toxic to neuronal cells, but it remains to be established which particular step in aggregate formation is toxic. The toxicity seems to be induced by necrosis instead of apoptosis, as no apoptotic markers such as annexin-5 or activated caspases were detected (data not shown). The toxic species may be either small polyQ peptide oligomers or large polyQ aggregates. Further studies are required to determine whether the proteasome can indeed generate similar polyQ peptides from different polyQ proteins. If so, these released polyQ peptides may be the common feature of the different polyQ disorders.

Based on our findings, we propose a model in which expanded polyQ peptides are degradation-resistant, and their accumulation leads to intracellular polyQ aggregates (Fig. 5). Proteasomes are rapidly recruited into the polyQ core, possibly in a final attempt to degrade the expanded polyQ peptides. Subsequently, other proteins are sequestered and ubiquitinated, perhaps due to (partial) unfolding. These events also lead to the binding of chaperones like HSP70 that may recognize denatured proteins. All these events result in concentric ring-like structures formed around the aggregate (Fig. 5). Essential proteins are depleted from the cell, contributing to cellular dysfunction. We conclude that polyQ peptides may be fundamental in initiating aggregation and sequestration of different types of proteins including polyQ proteins. Whereas FRAP experiments indicated that UPS components were immobilized, we could not detect proteasomes or Ub in the SDS-insoluble fraction of cell lysates (data not shown) or by filtertrap (Fig. 1G). This suggests that the recruited UPS components can still be solubilized.

We were able to detect expanded polyQ peptides containing Q65 or Q112 on Western blot and by immunostaining in fixed cells, but we were unable to detect any Q16 peptides. These short polyQ peptides are most likely rapidly degraded by downstream peptidases like PSA [43] that can digest short polyQ peptides and perhaps also extended peptides with less efficiency. Alternatively, a technical explanation for this result might be poor staining by 1C2. It has been suggested that anti-polyQ antibodies do not detect the polyQ peptide itself, but interact with the secondary structure created by the expanded polyQ peptide [44]. Nonetheless, we showed that the 1C2 antibody was able to recognize a Q16 peptide fused to GFP with almost equal efficiency as GFP-Q65 and GFP-Q112 proteins. Similarly, the polyQ-antibody MW1 was able to detect a Q16-GFP fusion protein but no Q16 peptides derived from GFP-Ub-Q16 (data not shown). This shows our inability to detect Q16 peptides is not likely to be caused by the intrinsic inability of 1C2 to recognize this peptide species. Thus, the inability to detect any Q16 peptides in cells expressing GFP-Ub-Q16 is most likely due to rapid and efficient degradation of non-expanded polyQ peptides. During the preparation of this article, it has been suggested that isolated proteasomes are able to cleave multiple times within a short polyQ-containing peptide [45]. They argued that Venkatraman and colleagues [19] underestimated the amount of cleaved polyQ-frgments as a consequence of their mass-spectrometry methods. However, their conclusion was also based on other experiments such as Western blot analysis of polyQ protein products generated by proteasomes, and are in line with the conclusions by Holmberg and colleagues [18]. The observation by Pratt and Rechsteiner [45] was done in the presence of a mutated PA28γ subunit, which alters proteasomal access and
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

THREE

Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

THREE

specifiCity to peptides. In addition, although isolated proteasomes may be cleave short polyQ peptides, our observation that Q65 and Q112 peptides readily aggregate suggests that the proteasome cannot efficiently degrade expanded polyQ peptides and thus cannot prevent their accumulation.

PolyQ aggregation is commonly visualized using full-length or truncated polyQ-proteins that are GFP-tagged, which therefore represent proteins that require degradation by the proteasome. When such GFP-tagged proteins are degraded by the proteasome, this results in the release of non-fluorescent polyQ peptides that will initiate aggregation and subsequently sequester GFP-tagged fragments or full-length polyQ proteins in time. Visualization of aggregation using GFP-tagged polyQ proteins thus represents a later stage in aggregate formation and does not reveal much about the initiation of aggregation. Long- or short-lived polyQ proteins have been used to link degradation to aggregation kinetics, where long-lived GFP-polyQ [40, 46] and GFP-tagged polyQ-expanded htt fragment [39] fusion proteins were compared to short lived variants. In these studies proteasomal degradation of short-lived expanded polyQ proteins resulted in reduced formation of GFP positive aggregates compared to their long-lived counterparts. Strikingly, toxicity was higher in cells expressing the short-lived expanded htt fragment when compared to long-lived version [39]. Our model can explain this unexpected finding: short-lived polyQ proteins are more rapidly degraded than long-lived proteins, resulting in aggregation-prone and toxic polyQ peptides. However, such aggregates remain invisible as GFP fluorescence of the short-lived proteins is lost due to rapid breakdown, preventing its incorporation in the aggregates. Consequently, these results have likely led to an underestimation of the true number of aggregates formed by short-lived proteins in these studies. The increased toxicity was in fact presumably caused by higher levels of generated polyQ peptides. The reduced toxicity in GFP positive cells by Verhoef and colleagues [40] may similarly be explained by preferential loss of fluorescence by toxic fragments, since only toxicity of GFP-positive cells were analyzed.

Our method mimicking proteasomal release of polyQ peptides is also a valuable tool to investigate a number of important questions concerning the role of polyQ peptides in HD and related neurodegenerative disorders. It enables us to identify proteases or peptidases that can target intracellular polyQ peptides in vivo, providing a strategy to prevent accumulation of toxic polyQ peptides. Similarly, the role of alternative degradation pathways, such as autophagy, in clearance of polyQ aggregates can be investigated. Our approach may also be useful to screen for compounds that affect aggregation and decrease toxicity. We expect that the outcome of such studies using this tool that express polyQ peptides in living cells holds true for all expanded polyQ disorders.

MATERIALS AND METHODS

Plasmid Constructs. Ub was generated by PCR from GFP-Ub [24] with forward primer 5’-CGGAGC-TCAGATCGGATCTCGTGAAAG-3’ and reverse primer 5’-CTCGGCTCTACCACCTCTGAGGAGCAGG-3’ and ligated into EGFP-C1 (Clonetics). The resulting construct GFP-Ub was again generated by PCR with forward primer 5’-CGGAGGATCTCATAGAGCAGACAGGAGGAG-3’ and a reverse primer 5’-CGGGATCCCTCAGGAGGCCAGAGGAGGAGG-3’ and ligated into Ub-x-GFP-Q65/Q112 [40] where the Ub-x-GFP insert was replaced for GFP-Ub, resulting in GFP-Ub-Q65/Q112. This procedure was required to remove the restriction site PstI present between GFP and Ub, since PstI was also required for Ub-polyQ ligation. The usage of restriction sites required the presence of some flanking amino-acids, resulting in an N-terminal residue and a Glu-Thr-Ser-Pro-Arg sequence at the C-terminus. GFP was exchanged for mRFP to generate the different RFP-Ub-polyQ fusions. The alternative polyQ peptide lengths of Q33 and Q48 were generated by re-transformation of GFP-Ub-Q65, leading to altered polyQ lengths. Q16-GFP was generated by inserting a Q16 repeat (derived from Ub-M-GFP-Q16) in front of GFP. Htt exon-1 was kindly provided by Ron Kopito (Stanford University, USA), atx3 by Henry Paulson (University of Iowa, USA), AR by Paul Taylor (St. Jude Children’s Research Hospital, Memphis, USA), GFP-Ub, RFP-Ub, Ub-M-GFP-polyQ (used to express GFP-polyQ) and β7-RFP by Nico Dantuma (Karolinska Institute, Sweden), HSP70-GFP by Harm Kampina (UMC Groningen, The Netherlands), TBP1 by Rick Morimoto (Northwestern University, USA) and QBP1-CFP by Yoshitaka Nagai (Osaka University Graduate School of Medicine, Japan).

Transfections, cell-culture and toxicity assay. Human embryonic kidney cells (HEK293T) and Mel Juß fibroblast cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; GibCo) supplemented with 10 % FCS and penicillin/streptomycin/L-glutamine. The cells were transiently transfected with Fugene6 (Roche) and analyzed at indicated time-points after transfection. Mouse STNah1/2(Q7) cells (kindly provided by Marcy MacDonald) [36] and N2A neuroblastoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10 % FCS and penicillin/streptomycin/L-glutamine. Neuronal cells were transiently transfected with Lipofectamine 2000 (Invitrogen). Mouse STNah1/2(Q7) cells were incubated at 32°C. For toxicity measurements, N2A cells were analyzed by FACS LSRII for GFP fluorescence 24 or 48 hours after transfection, and the percentage of GFP-positive cells was quantified.

Western blot. Cytosolic extracts were generated by lysing cells with 0.1 % Triton X-100 for 30 minutes one ice, and the supernatant was used after spinning down the lysate. 20 µg of cytosolic protein lysates were separated by 18 % SDS-PAGE and transferred to Protran nitrocellulose membranes. Membranes were blocked in 5 % dry milk in TBS containing 0.3 % tween and probed with 1:1000 anti-GFP (Molecular Probes), 1:100 anti-Ub (SIGMA) or the anti-Polyglutamine 1C2 (MAB1574, Millipore). Polyclonal Horseradish Peroxidase (HRP) conjugated secondary antibodies, anti-rabbit (Sigma) or anti-mouse (DAKO) were used 1:10.000 to detect the primary antibodies via LumilightPLUS westernblotting substrate (Roche). Preparation of SDS-soluble and SDS–insoluble protein fractions was described before [47]. Briefly, cells were trypsinized, homogenized, and heated for 10 min at 99°C in sample buffer (70 mM Tris pH 6.8, 1.5 % SDS, 20 % glycerol) supplemented with 50 mM DTT 72 hours after transfection. Cell lysates were centrifuged for at least 30 minutes at 20,800 g at room temperature. Supernatants were used as SDS-soluble fraction to which 0.05 % bromophenol blue was added. Pellets represented SDS-insoluble fractions and were dissolved in 100 % formic acid, incubated 30 minutes at 37°C, lyophilized overnight in a speed vac (Eppendorf).
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

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Filter retardation assay. Filter retardation assay was performed as described before [29]. Briefly, 72 hours after transfection, HEK293T cells were lysed for 30 minutes on ice in Nondinet P-40 (NP-40) buffer (100 mM TrisHCl, pH 7.5, 300 mM NaCl, 2 % NP-40, 10 mM EDTA, pH 8.0, supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). After centrifugation 15 minutes at 20,800 g at 4°C, cell pellets were resuspended in benzonase buffer (1 mM MgCl$_2$, 50 mM Tris-HCl, pH 8.0) and incubated for 1 hour at 37°C with 250U benzonase (Merck). Reactions were stopped by adding 2x termination buffer (40 mM EDTA, 4 % SDS, 100 mM DTT). Aliquots of 30 μg protein extract were diluted into 2 % SDS buffer (2 % SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and filtered through a 0.2 μm cellulose acetate membrane (Schleicher and Schuell) pre-equilibrated in 2 % SDS buffer. Filters were washed twice with 0.1 % SDS buffer (0.1 % SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and subsequently blocked in 5 % nonfat milk (Protifar Plus, Nutricia) in TBS. Captured aggregates were detected by incubation with 1C2 antibody and further treated like western blots. Alternatively, GFP fluorescence of trapped aggregates was analysed by LAS3000.

Supplementary Fig. 1.

(A) The anti-polyQ antibody 1C2 recognizes both short (Q16-GFP and GFP-Q16) and expanded (GFP-Q65 and GFP-Q112) polyQ-tracts fused to GFP with almost equal efficiencies. (B) Whereas the expression of GFP-Ub-Q33 did not induce aggregates, the expression of GFP-Ub-Q48 led to aggregate formation in a low percentage of cells after 72 hours of transfection. (C) Whereas polyQ peptides derived from GFP-Ub-Q112 are present in both the soluble and insoluble fraction of transfected cells, these peptides are not positive for GFP, indicating efficient cleavage of GFP-Ub-Q112. (D) Cells transfected with GFP-Ub-Q112 showed only immunostaining with the anti-polyQ antibody MW1 when no aggregates were present, similar as observed with 1C2 (Fig. 1E). Scale bar ~5 μm.
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

THREE

Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity

THREE

REFERENCES


Supplementary Fig. 2.

(A) FRAP analysis of polyQ-induced aggregates show recovery of fluorescence upon photo-bleaching of HSP70, indicating a high on/off rate, whereas Ub and proteasomes are irreversibly sequestered. (B) The rate of aggregation of httex1-Q103-GFP was enhanced when co-expressed with RFP-Ub-Q112 (left panel). While httex1-Q25-GFP is freely distributed in cells co-expressing RFP-Ub or RFP-Ub-Q16, httex1-Q25-GFP was redistributed to aggregates initiated by RFP-Ub-Q112 (right panel). A representative graph is showing the percentage of cells containing huntingtin-positive aggregates at three time points after transfection. (C) Ataxin3-Q28-GFP distribution was affected by the presence of polyQ peptide aggregates, leading to sequestering into aggregates induced by RFP-Ub-Q112. Scale bar ~5 μm.
Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity


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THE DNAJB6 AND DNAJB8 CHAPERONES PREVENT INTRACELLULAR AGGREGATION OF POLYGLUTAMINE PEPTIDES.

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ABSTRACT

Fragments of proteins containing an expanded polyQ tract are thought to initiate aggregation and toxicity in at least nine neurodegenerative diseases including Huntington’s disease. Since proteasomes appear unable to digest the polyQ tract which can initiate intracellular protein aggregation, preventing polyQ peptide aggregation by chaperones should greatly improve polyQ clearance and prevent aggregate formation. Here we expressed polyQ peptides in cells and show that their intracellular aggregation is prevented by DNAJB6 and DNAJB8, members of the DNAJ (HSP40) chaperone family. In contrast, HSPA/HSP70 and DNAJB1, another member of the DNAJ chaperone family, did not prevent peptide-initiated aggregation. Intriguingly, DNAJB6 and DNAJB8 also affected the soluble levels of polyQ peptides, indicating that DNAJB6 and 8 inhibit polyQ peptide aggregation directly. Together with recent data showing that purified DNAJB6 can suppress fibrillation of polyQ peptides far more efficiently than polyQ expanded protein fragments in vitro, we conclude that the mechanism of DNAJB6 and 8 is suppression of polyQ protein aggregation by directly binding the polyQ tract.

INTRODUCTION

Polyglutamine (PolyQ) disorders are a group of dominantly inherited, progressive neurodegenerative disorders. These disorders are caused by expansion of the polyQ tract within coding regions of unrelated proteins. At least nine different polyQ disorders are known, including Huntington’s disease (HD) [1]. These disorders are characterized by atrophy of certain regions in the brain and the presence of intracellular aggregates that are thought to initiate disease, although neither the precise toxic species nor the cause of aggregation initiation is fully understood. Several studies showed that these aggregates contained not only the full length disease-related protein but also shortened fragments including the expanded polyQ tract [2-4]. Furthermore, it was shown that truncated forms of huntingtin (Htt), ataxin-3, ataxin-7, atrophin-1 and androgen receptor containing the expanded polyQ tract are more aggregation prone and enhance toxicity [5-11]. While proteasomes are able to degrade polyQ-containing proteins [12-15], they may not be able to cleave within polyQ tracts in vitro [16]. As a consequence, polyQ peptides may be released upon proteasomal degradation of expanded polyQ proteins. Similar polyQ peptides may directly be generated in related disorders such as SCA8 and Huntington’s disease like-2 (HDL2), which are thought to be initiated by polyQ peptides generated by antisense transcription that cause intranuclear inclusions [9, 17]. When mimicking polyQ peptide generation in living cells, we observed that expression of expanded polyQ peptides is sufficient to induce aggregation and toxicity in cells [18] and that expression of polyQ stretches alone cause toxicity in Drosophila [19].

Aggregates or inclusion bodies (IBs) initiated by expanded polyQ peptides as well as expanded polyQ-containing proteins are decorated with various proteins, such as components of the ubiquitin-proteasome system (UPS) and heat shock proteins (HSP) [20-26]. HSPs can function as molecular chaperones. Many of them are up-regulated under stress conditions, such as heat stress [27, 28], and have been shown to protect cells against heat-induced protein aggregation [29, 30]. Heat shock proteins can be classified into a number of families on the basis of their approximate molecular mass and preserved of conserved domains [31, 32]. The HSPA (HSP70) family consists of 11 members, whereas the DNAJ (HSP40) family consists of over 40 members in humans [32]. In a recent comparative screen of all members of the HSPA, HSPH (Hsp110) and DNAJ (sub)families, DNAJB6 and DNAJB8 were identified as the two most potent suppressors of aggregation and related toxicity of expanded polyQ proteins [33]. When examining the characteristics of these two chaperones in more detail, we now find that DNAJB6 and DNAJB8 can directly suppress aggregation of polyQ peptides generated in living cells. This action is largely dependent on the serine-rich region (SSF-SST) within the C-terminus, and less dependent of interaction with HSP70. Intriguingly, expression of DNAJB6 in the nucleus also represses aggregation in the cytoplasm, suggesting that polyQ peptides are kept solubilized in one cellular compartment by these chaperones and remain small enough to travel to the other compartment for subsequent degradation. The ability of DNAJB6 and DNAJB8 to reduce aggregation of expanded polyQ peptides prevent these polyQ peptides from acting as inducers of aggregation and improves their clearance.

RESULTS

DNAJB6b and DNAJB8 reduce aggregation of polyQ peptides

To determine the effect of chaperones on polyQ peptide aggregation in living cells, we used GFP-ubiquitin-polyQ (GFP-Ub-polyQ) constructs that are immediately cleaved upon expression into GFP-Ub and polyQ peptides by Ub C-terminal hydrolases (Fig. 1A) [34]. The resulting polyQ peptides lack any flanking amino acids such as a starting methionine, and only expanded polyQ peptides beyond 40 glutamines induced aggregation, as quantified by the sequestration of GFP-Ub into aggregates [18]. While approximately 60 % of cells expressing Q104 peptides contained aggregates after 72 hours, coexpression of HSPA1A (HSP70) had no significant effect on polyQ peptide aggregation, whereas DNAJB1 (HSP40) slightly reduced aggregation (Fig. 1B). In contrast, coexpression of DNAJB6b or DNAJB8 resulted in a dramatic decrease in polyQ peptide aggregation when scoring the number of polyQ-expressing cells having aggregates (Fig. 1B) or by filtertrap analysis (Fig. 1C) when similar levels of the different chaperones were expressed (Fig. 1D). In contrast, when endogenous DNAJB6 levels were decreased by siRNA (Fig. 1E, lower panel), aggregation of polyQ peptides increased as indicated by filtertrap analysis (Fig. 1E, upper panel).

To examine whether expression of DNAJB6b and DNAJB8 also reduced the amount of SDS-insoluble polyQ aggregates, cells expressing GFP-Ub-polyQ in combination with DNAJB6b or DNAJB8 were separated into SDS-soluble and SDS-insoluble fractions. Expression of Q104 peptides for 24 hours resulted in a specific polyQ-positive band in the soluble and various bands in the insoluble fractions (Figure 2A, arrows). Coexpression of DNAJB6b and DNAJB8 remarkably reduced the amount of polyQ peptides present in the SDS-insoluble fraction (Fig. 2A, right panel), which is in agreement with the reduced number of aggregates visualized in cells. Interestingly, the soluble fraction of
Q104 peptides increased, (Fig. 2A, left panel), suggesting that DNAJb6 and DNAJb8 keep polyQ peptides soluble. To examine whether the SDS-soluble fraction was a precursor of one of the insoluble polyQ peptide fractions, we separated fractions at different time-points after transfection. Both the soluble and insoluble fraction decreased when cells were treated with cycloheximide to block synthesis of new polyQ peptides during the last 16 hours, suggesting that the soluble fraction was not a precursor to a particular insoluble fraction (data not shown).

SDS-insoluble levels of Htt-exon1-Q103 were also decreased by coexpression of DNAJb6b or DNAJb8 (Fig. 2B, right panel). In contrast to polyQ peptides, the SDS-soluble Htt-exon1-Q103 protein levels were not affected (Fig. 2B, left panel). The observed decrease in insoluble Htt-exon-1 could, however, indicate that DNAJb6b and DNAJb8 can improve solubility and clearance of Htt-exon-1, as shown before [33, 35].

DNAJ6b and DNAJ8b are recruited into polyQ peptide aggregates

To examine the effect of DNAJb6b and DNAJb8 with polyQ peptides in more detail, polyQ peptides were tagged with the small tetracysteine (C4) motifFLNCPCGCMCP at the C-terminus for direct visualization. The membrane-permeable biarsenical dye ReA5H can bind this small tag and only then it becomes fluorescent [36]. To examine whether the C4-tag affected the behavior of polyQ
peptides, we transiently expressed GFP-Ub-polyQ and GFP-Ub-polyQ-C4 peptides. Expression of either C4-tagged or non-tagged polyQ peptides resulted in a similar percentage of fluorescent cells that contain aggregates after 72 hours (Fig. 3A). Similar to untagged Q104 peptides, DNAJB6b and DNAJB8 kept Q99-C4 SDS-soluble (Fig. 3B), and similar SDS-insoluble fractions were detected between cells expressing untagged Q104 or C4-tagged Q99 peptides (Fig. 3C). In cells without aggregates, both GFP-Ub and expanded polyQ peptides were present throughout the cytoplasm and nucleus (Figure 3D, upper panel). However, aggregation led to the redistribution of both GFP-Ub and ReAsH-labeled Q99-C4, with ReAsH-labeled Q99-C4 sequestered in the core of aggregates and GFP-Ub in a ring around the core (Fig. 3D, lower panel, also in the zoom-in). Only C4-tagged polyQ peptides of disease-related lengths accumulated and initiated aggregation, whereas short C4-tagged polyQ peptides were degraded, similarly to untagged polyQ peptides.

![Image](https://example.com/image.png)

Fig. 3. Tetracysteine (C4)-tagged polyQ peptides behave similarly as non-labelled polyQ peptides.

(A) Percentage of HEK293 cells containing aggregates 72 hours after transfection with GFP-Ub-Q16/Q104 or C4-tagged GFP-Ub-Q17/Q99 (data are mean ± SEM of three independent experiments). Similar amounts of aggregates were detected with either non-tagged or C4-tagged polyQ peptides. (B) SDS-soluble and SDS-insoluble fractions prepared at 24 hours after transfection of HEK 293 cells with Ub-Q99-C4 in combination with DNAJB6b and DNAJB8 and analyzed by Western blotting. Blots were stained for polyQ (C2) and actin. Arrows indicate specific Q104 peptide bands. (C) SDS-insoluble fractions of HEK293 cells expressing GFP-Ub-Q16/Q104 or C4-tagged GFP-Ub-Q17/Q99 prepared at 24 hours after transfection were stained for polyQ. (D) Confocal microscopy images of ReAsH labeled HeLa cells expressing C4-tagged GFP-Ub-Q17 or GFP-Ub-Q99 at 48 hours after transfection. ReAsH-positive cells were detected only when polyQ-expanded C4-tagged Q99 peptides were expressed (lower panel). The zoom-in shows a GFP-Ub-decorated aggregate. Scale bar: 10 µm.

**PolyQ peptides sequestered in aggregates strongly interact with DNAJB6b and DNAJB8**

To examine whether diffuse and aggregated polyQ peptides showed intermolecular interactions, we performed Fluorescence Resonance Energy Transfer (FRET) experiments. FRET enables sensitive evaluation of protein-protein interactions in living cells. Only when proteins are in close proximity (<10 nm) FRET can occur and be measured [37]. Fluorescence Lifetime Imaging Microscopy (FLIM) was used to measure FRET-FLIM, which is detected as a decrease in donor fluorescence lifetime [38]. HeLa cells expressing Ub-Q99-C4 were simultaneously labeled with equal amounts of FlAsH and ReAsH at 48 hours after transfection, leading to individual labeling of polyQ peptides with either FlAsH or ReAsH. When polyQ peptides were not clustered in aggregates, no reduction in lifetime of FlAsH was measured (Fig. 4A), suggesting that polyQ-polyQ peptide interactions hardly occurred when not sequestered in aggregates. In contrast, aggregated polyQ peptides showed a remarkable decrease in lifetimes of 39.6 % of FlAsH, which is indicative of FRET (Fig. 4A). Together, these data show that polyQ peptides are mainly monomeric when not present in aggregates, whereas aggregated polyQ peptides are immobile and only then strong polyQ-polyQ peptide interactions are observed.

The presence of GFP-Ub in aggregates most likely represents sequestered proteins that become ubiquitinated in time, whereas the polyQ peptides are not likely ubiquitinated as no lysine residues are present. Since expression of GFP-Ub-Q99-C4 results in an efficient separation of GFP-Ub and Q99-C4, cells expressing either GFP-Ub-Q99-C4 or GFP-Ub combined with Ub-Q99-C4 should hardly show interactions between polyQ peptides and GFP-Ub (Fig. 4B). The recruitment of proteasomes into the core of polyQ peptide-initiated aggregates suggests that proteasomes interact with the polyQ peptides. To substantiate this idea, cells were cotransfected with Ub-Q99-C4 and the GFP-tagged proteasomal component β7. Coexpression of ReAsH labeled Q99-C4 with the GFP-tagged proteasomal component β7 caused a decreased lifetime of GFP (Fig. 4B), indicating a direct interaction with the polyQ peptides. In the few cells that showed aggregates upon DNAJB6b or DNAJB8 coexpression, these chaperones were present in the core of aggregates (Fig. 4C). Therefore, we examined whether these chaperones also interact with aggregated polyQ peptides. Both GFP-tagged DNAJB6b and DNAJB8 interacted strongly with polyQ peptides as shown by the reduction in lifetime (Fig. 4D). In contrast, the recruitment of HSP70 into polyQ peptide-induced aggregates did not result in a significant reduced lifetime of GFP-tagged HSP70 (Fig. 4D), suggesting that HSP70 does not interact with polyQ peptides. Thus, most proteins that sequester in the core of aggregates, such as β7, DNAJB6b and DNAJB8, interacted with aggregated polyQ peptides. This suggests that in the rare event of aggregation when DNAJB6b and DNAJB8 are coexpressed, they are trapped into aggregates induced by polyQ peptides in an early stage, which may reflect a failed function of these chaperones.
The DNAJB6 and DNAJB8 chaperones prevent intracellular aggregation of polyglutamine peptides

Serine-rich region in DNAJB6 and DNAJB8 is essential for reduction of aggregation

The DNAJ family is defined by the presence of a J-domain that can regulate chaperone activity of the HSPA family by stimulating ATP hydrolysis (Fig. 5A) [39, 40]. The HPD sequence in the J-domain is essential for interaction with and accelerating the ATPase activity of HSPA [41]. The other domains of DNAJ family members are involved in recognition and binding of clients and are considered to be a main factor in driving the specificity of HSP70 machines [42]. Mutating His to Glu (H31Q) in the HPD motif results in an inactive J-domain [41] but this only partly impaired the ability of DNAJB6 and DNAJB8 to reduce polyQ peptide aggregation as scored by the amount of fluorescent cells having aggregates (Fig. 5B). The H31Q mutants also still reduced the appearance of polyQ peptides on filtertrap (Fig. 5C) as well as in the SDS-insoluble fraction (Fig. 5D). However, the SDS-soluble fraction was also decreased (Figure 5E), indicating that the J-domain is somewhat affecting DNAJB6 and DNAJB8 function, albeit that their main antiaggregation function is intact, consistent with was found before for preventing aggregation of Htt-exon-1 fragments [33].

In the C-terminal region of DNAJB6b and DNAJB8 a conserved serine-rich region (SSF-TST and SSF-SST, respectively) is present, which is absent in DNAJB1 or its related subfamily members (Fig. 5A) [43]. Deletion of this serine-rich region was found to be crucial for DNAJB6 and DNAJB8 to interact with histone deacetylases (HDACs) that regulate their function as suppressors of expanded polyQ protein aggregation [33]. Deletion of this serine-rich region also severely affected the ability of DNAJB6 and DNAJB8 to reduce polyQ peptide aggregation (Fig. 5B), resulting in more SDS-insoluble material on filtertrap (Fig. 5C) and Western blot (Fig. 5D), and a reduction in SDS-soluble levels (Fig. 5E). The defective chaperones were still recruited into polyQ peptide aggregates, but these sequestered chaperones lost their direct interaction with the labeled polyQ peptides once the serine-rich region was disabled (Fig. 5F). This suggests that the sequestration of both functional and disabled DNAJB6 and DNAJB8 may not be their primary way of interfering with aggregation, but rather reflect an inefficient attempt to prevent aggregate initiation and growth.

Since DNAJB6 and DNAJB8 strongly reduce aggregation of polyQ peptides and interact with polyQ peptides in the core of aggregates, these interactions may affect aggregation of polyQ peptides directly. To examine whether chaperones were able to reduce the strong interactions observed between polyQ peptides, we coexpressed Q99-C4 peptides in combination with DNAJB6, DNAJB8, HSP40 or HSP70. Double-labeling of C4-tagged Q99 peptides with FlAsH and ReAsH resulted in a high FRET-FLIM efficiency (Fig. 5G). Coexpression of DNAJB6b or DNAJB8 hardly affected interactions between aggregated polyQ peptides, indicating that the sequestration of DNAJB 6 or DNAJB8 does not interfere with peptide interactions within the aggregate. Similarly, HSP40 and HSP70 did also not affect the interactions between aggregated polyQ peptides.
The DNAJB6 and DNAJB8 chaperones prevent intracellular aggregation of polyglutamine peptides

DNAJB6b and the serine-rich region mutant (upper panel) and GFP-tagged DNAJB8 and the serine-rich region mutant (lower panel) in cells expressing ReaHt-labeled Ub-Q99-C4 at 48 hours after transfection shows high FRET-FLIM efficiencies between aggregated polyQ peptides and DNAJB6b or DNAJB8, but much less FRET-FLIM was detected between aggregated polyQ peptides and their serine-rich region mutant counterparts (data are mean ± SEM; two-tailed unpaired t-test; *** p<0.001; * p<0.05). Scale bar: 10 µm. (G) DNAJB6 or DNAJB8 do not reduce intermolecular interactions between aggregated polyQ peptides. Histogram represents average phase modulation lifetimes of FlAsH in HeLa cells expressing Ub-Q99-C4 together with indicated constructs and labeled with both FlAsH and ReaHt at 48 hours after transfection.

Nuclear DNAJB6a reduces aggregation in both cytoplasm and nucleus

The effect of DNAJB6b on SDS-soluble levels of polyQ peptides suggests that it may counteract very early oligomerization steps of these aggregation-prone peptides. To examine this phenomenon in more detail, we reasoned that only monomeric or small oligomers can shuttle freely through the nuclear pore by diffusion. If correct, the presence of DNAJB6 in the nucleus or cytoplasm only should then reduce aggregation in both compartments by keeping the polyQ peptides soluble. DNAJB6 has two isoforms. DNAJB6b is present in both the cytoplasm and nucleus, whereas DNAJB6a contains a putative nuclear localization signal and is, therefore, localized in the nucleus only (Fig. 6A) [33]. Despite the exclusive localization of DNAJB6a in the nucleus, expression of DNAJB6a was as effective as DNAJB6b and DNAJB8 in reducing polyQ-peptide aggregation (Fig. 6B). Interestingly, reduction in aggregate formation was not limited to the compartment were these chaperones were residing. Coexpression of DNAJB8 did not alter the ratio of nuclear and cytoplasmic aggregates after 48 hours, whereas coexpression of DNAJB6b resulted in a slight change in this ratio in favor of nuclear aggregates (Fig. 6B, upper panel). Expression of DNAJB6b and DNAJB8 resulted in even a higher percentage of aggregates present in nuclei after 72 hours (Fig. 6B, lower panel). The nuclear localization of DNAJB6a did not lead to the exclusive presence of aggregates in the cytoplasm, since the ratio of nuclear and cytoplasmic aggregates remained unaltered after expression of DNAJB6a (Fig. 6B). It is concluded that although DNAJB6a is present in the nucleus only, it does not reduce exclusively aggregation in the nuclear compartment.

Together, these data suggest that DNAJB6 and DNAJB8 are efficient in targeting earlier stages of aggregation, while the observed recruitment of DNAJB6 and DNAJB8 into aggregates reflects sequestration of these chaperones without any effect on improved clearance. To examine whether DNAJB6a, DNAJB6b and DNAJB8 were able to dissociate from the core of aggregates, we studied the mobility of the sequestered chaperones, using fluorescent recovery after photobleaching (FRAP) and Fluorescence Loss In Photobleaching (FLIP) [44, 45]. Fluorescent recovery was observed of HSPA1A, but not of DNAJB6a, DNAJB6b and DNAJB8 (Fig. 6C). When the entire cell was repeatedly bleached with exception of one fluorescent aggregate, fluorescence loss in the aggregate was measured of HSPA1A, whereas DNAJB6a, DNAJB6b and DNAJB8 present in the core of aggregates were hardly affected by bleaching (Fig. 6D). These data show that DNAJB6a, DNAJB6b, and DNAJB8 were sequestered irreversibly in the core of polyQ-induced aggregates, indicating that once the activity of these chaperones has failed to prevent aggregation, they are irreversibly trapped in the inert core of aggregates.
The DNAJB6 and DNAJB8 chaperones prevent intracellular aggregation of polyglutamine peptides

Intracellular polyQ clearance may be facilitated by HSP that prevent the formation of insoluble aggregates, allowing clearance pathways involving proteases and autophagic pathways to remove hazardous fragments. In this study, we examined mechanisms to prevent aggregation of polyQ fragments, focusing on expanded polyQ peptides that can initiate aggregation and toxicity. We found that the DNAJ family members DNAJB6 and DNAJB8 were efficient suppressors of polyQ peptide aggregation. Interestingly, detergent-soluble levels of mutant Htt-exon-1 were not affected by DNAJB6, whereas the level of soluble polyQ peptides was increased. Apparently, these chaperones prevent aggregation of polyQ peptides leading to improved clearance of polyQ peptides, while soluble Htt-exon-1 is not affected. The solubilized polyQ peptides might be more efficiently cleared than Htt-exon-1 fragments. Alternatively, aggregation of Htt-exon-1 might be initiated by smaller fragments derived from Htt-exon-1 that are subsequently targeted by DNAJB6. In contrast to polyQ peptides, fluorescently-tagged expanded polyQ proteins such as Htt-exon1-GFP, were not present in the core of aggregates, but sequestered in a ring around the aggregate [18, 25, 46-48]. This suggests that smaller fragments containing the polyQ tract initiate aggregation and that the original GFP-tagged polyQ proteins are sequestered in a later stage. Still, it remains to be resolved whether polyQ peptides are generated in cells that express polyQ-expanded proteins, similar to in vitro conditions [16]. This will, however, be a challenging task as the generated polyQ peptides will either be captured by DNAJB6 or DNAJB8 and/or degraded or initiate aggregation and subsequent sequestration of the original polyQ protein. To address the first option, we purified DNAJB6 from polyQ fragment expressing cells in order to detect putatively associated polyQ peptides. Although, we did find several peptide fragments associated with them, suggesting they indeed could be “peptide chaperones”, unfortunately polyQ peptides could not be detected due to the limitations of polyQ analysis by mass spectrometry (data not shown).

DNAJB6 and DNAJB8 are the first examples of HSPs that reduce aggregation of pure polyQ peptides. Our data are supported by recent observations by the group of Cecilia Emanuelsson, who showed that purified DNAJB6 and DNAJB8 can directly bind to and highly efficiently suppress polyQ peptide fibrillation initiated by soluble, pure polyQ peptides [49]. In fact, DNAJB6 and DNAJB8 were better at suppressing fibrillation when initiated by polyQ peptides than when initiated by Htt-exon-1 fragments. Given that in cells the two DNAJB proteins can prevent aggregation of Htt-exon-1 fragments [33] (this manuscript), this implies that although some HSPs (HSPA1 or DNAJB1) might prevent sequestration of polyQ proteins into aggregates, DNAJB6 and DNAJB8 reduce aggregation of expanded polyQ peptides. Thereby they prevent that polyQ peptides can act as nucleators of aggregation and subsequent sequestering of larger polyQ-containing fragments, such as Htt-exon1. Consistent with this model DNAJB6 and DNAJB8 were trapped in the core of aggregates together with polyQ peptides, whilst expanded polyQ proteins and HSPA1A or DNAJB1 were absent from the core. The recruitment of HSPA1A to the outer ring of aggregates may in fact be due to its recognition of sequestered, possibly unfolded proteins and its attempt to solubilize these proteins. Indeed, HSPA1A was, unlike DNAJB6 and DNAJB8 not trapped in these aggregates (reflecting failed function to prevent aggregate seeding) but showed a high degree of dynamics at the aggregate ring (Fig. 6C) [24]. Together, this indicates that only DNAJB6 and DNAJB8 may be able to directly prevent aggregation of polyQ proteins by directly interacting with the expanded polyQ repeat, while most other chaperones are mostly preventing the sequestration of other proteins in the aggregate. As a result, improving the activity or protein levels of these DNAJB chaperones might delay or even prevent the aggregation of polyQ fragments, enabling intracellular clearance pathways to target and degrade these fragments.

DNAJB6 and DNAJB8 may keep polyQ fragments soluble whereby they may be maintained in a degradation-competent state. It remains to be established whether these chaperones can also control their degradation by targeting them to particular proteases such as the proteasome, PSA or towards autophagosomes. Intriguingly, the ability of nuclear DNAJB6a to reduce polyQ peptide aggregation was not restricted to the nuclear compartment, as aggregation within the cytoplasm...
was reduced similarly. This suggests that DNAJB6a keeps polyQ peptides in a soluble intermediate, which allows these peptides to freely translocate between cytoplasm and nucleus. This is of importance as the autophagic machinery is present in the cytoplasmic compartment only [50, 51]. This may also explain our observation that the ratio of cytoplasmic versus nuclear aggregates decreased in time because cytoplasmic aggregates may be more efficiently cleared as compared to aggregates in the nucleus.

Deletion of the serine-rich region within DNAJB6 and DNAJB8 abolished the anti-aggregation properties of these chaperones. This suggests that this region is essential to recognize polyQ peptides and to inhibit their aggregation. An active J-domain was less important for DNAJB6b and DNAJB8 anti-aggregation properties, although it affected the soluble levels of polyQ peptides. Since the J-domain is known to interact with HSPA1A and stimulates its ATPase activity [39, 40], DNAJB6 and DNAJB8 do not seem to require HSPA1A to reduce polyQ peptide aggregation. Little is known about the function of the serine-rich region in DNAJB6 and DNAJB8, although this region is suggested to be involved in DNAJB6/8 oligomerization and histone deacetylase binding [33]. Also, purified DNAJB6 and DNAJB8 form large polydispersed oligomers which might be crucial for efficient polyQ peptide binding [49]. Previous studies showed that DNAJB6 is highly enriched in the central nervous system [35, 43] and colocalizes to Lewy bodies and aggregates induced by Htt-exon1 [35, 52]. However, expression of DNAJB8 is restricted to the testes [43]. Therefore, DNAJB6 may be the best candidate for potential therapeutic approaches in the fight against polyQ disorders, for example by inducing endogenous DNAJB6 expression. A combination of solubilizing polyQ peptides by expression of DNAJB6 and stimulating their subsequent degradation seems to be the most attractive alternative.

MATERIALS AND METHODS

DNA constructs. Generation of GFP-UB-polyQ constructs was described before [18], however, the initial polyQ peptides started with a Leu residue and a Glu-Thr-Ser-Pro-Arg sequence at the C-terminus. This Leu residue was changed into a Gin using Quickchange II site directed mutagenesis (Stratagene). At the C-terminus a stop-codon was introduced directly after the polyQ stretch using site directed mutagenesis, resulting in GFP-UB-Q16 and GFP-UB-Q104, respectively. To insert a C4-tag after the polyQ tract, a BamHI site was introduced at the N-terminus of polyQ peptides using GFP-UB-Q16/Q112 where Leu was already changed into a Gin. The C4-tag containing the following sequence FLNCCPGCCMEP [36] was obtained by annealing two encoding oligo primers with overhangs compatible with BamHI at the N-terminus and XbaI at the C-terminus. A C4-tag was inserted into GFP-UB-polyQ using BamHI and XbaI, thereby generating GFP-UB-Q17-C4 and GFP-UB-Q99-C4, respectively. Plasmids encoding DNAJB6a, DNAJB6b (including H31Q), and DNAJB8, (including H31Q, and Δ55S-55T) in pCDNA5 FR/TTO V5 vectors were described before [33], and were subsequent cloned into either pIRES-DsRed2 (Invitrogen) to generate untagged chaperones or into tagRFP (Evrogen) to generate chaperones N-terminally tagged with RFP. DNAJB6b ΔSSF-TST was generated by deleting amino acids 155–195 within DNAJB6b. Htt-exon1-Q103-GFP was kindly provided by Ron Kopito (Stanford University, Stanford, CA, USA), and HSPA1A-GFP by Prof. Harm Kampinga (University Medical Center Groningen, Groningen, the Netherlands).

Cell culture and transfection. HEK293 cells and the human melanoma cell line Mel Juso were cultured in Iscove’s Modified Eagle Medium (IMDM; Gibco) supplemented with 10 % FCS, 25 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM glutamine (Gibco). HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented as described above. HeLa and Mel Juso cells (0.15x10^6) were plated onto glass coverslips (24 mm; Fisher scientific) in a 6-well and were transiently transfected using FuGeneHD (Roche), respectively 24 hours after plating. HEK293 cells (0.1x10^6) were seeded in 6-well plates and transiently transfected with polyethylenimine (PEI; Polysciences) 24 hours after plating.

siRNA transfection. 24h after seeding cells were tarsfected with 50 nM control siRNA or DNAJB siRNA, repeated 48 h after seeding with 50 nM control siRNA or DNAJB6 siRNA together with 2.5 ug plasmid (protein expression for 48 h). Transfections were done with Lipofectamine 2000 (Invitrogen) and Dharmacon smartpool siRNA siGenome DNAJB6 (M-013020-00-0005) and non-targeting siRNA control siGENOME (D-001206-13-20).

Biarsenical labeling. HeLa cells were transfected with GFP-Ub-Q99-C4 and DNAJB6 or DNAJB8 and biarsenical labeling was performed 72 hours after transfection, as described before [36]. Briefly, 1 mM ReAsH was pre-incubated in 10 mM 1,2-ethanediithol (EDT; Sigma) in DMEM for 10 minutes. Cells were labeled with 1 µM ReAsH in 10 µM EDT in OptiMEM for 45 minutes at 37°C, 10 % CO₂ in the dark, and subsequently washed several times with 1 mM EDT in OptiMEM containing 10 % FCS during 30 minutes at room temperature to remove unbound dyes.

Confocal and FRET-FLIM microscopy. HEK293T cells were cotransfected with GFP-Ub-polyQ and various chaperones as indicated in figure legends and the percentage of aggregate-positive cells was determined after 72 hours. The number of cells containing aggregates in nucleus, cytoplasm or both compartments was determined 48 and 72 hours after transfection. Confocal microscopy images were obtained 48 hours after transfection using a Leica TCS SP2 confocal system equipped with an Ar/Kr laser with a 63x objective. For FLIP analysis, either a part of the cytoplasm or the whole cell except for the aggregate was photo-bleached at full laser power repeatedly. Fluorescence loss was measured either within the nucleus or within the non-bleached region containing the aggregate, respectively. For FRAP analysis, fluorescently-tagged chaperones trapped in polyQ peptide-induced aggregates were bleached at full laser power and fluorescence recovery was measured in time. FLIM was carried out after biarsenical labeling of living cells. To reduce interference of autofluorescence by DMEM, a special medium was added to cells (20 mM Hepes; pH 7.4, 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, and 20 mM glucose). FLIM was performed using the wide-field frequency domain approach on a home-build instrument [53] using a RF-modulated image intensifier (Lambert Instruments II18MD) coupled to a CCD camera (Photometrics HQ) as detector. A 40x objective was used for all measurements. The modulation
frequency was set to 75.1 MHz. Eighteen phase images with an exposure time of 20-200 ms were acquired in a random recording order to minimize artifacts due to photobleaching [54]. From the phase sequence an intensity (DC) image and the phase and modulation lifetime image were calculated using Matlab macros. From this data, the lifetime of individual cells was determined using ImageJ (http://rsb.info.nih.gov/ij/). Subsequently, average phase and modulation lifetimes (± s.d.) were calculated. The FRET efficiency E was calculated according to: E=(1–(τDA/τD))x100 % in which τDA is the fluorescence lifetime of the donor in presence of the acceptor (i.e. samples labeled with both FlAsH or GFP and ReAsH) and τD is the fluorescence lifetime of the donor (i.e. FlASH only or GFP only) in absence of the acceptor. Frequency domain FLIM yields a phase lifetime (τp) and a modulation lifetime (τm). Since τp is more sensitive than τm, FRET efficiency was calculated on the basis of τp.

Immunoblotting. Cells were trypsinized and lysed in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton-X100). Total cell lysates were boiled for 10 min at 99°C with 1x laemmli sample loading buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % glycerol, 6 % [i-mercaptoethanol, bromphenol blue) fractionated by SDS-PAGE gel electrophoresis and transferred to a PVDF membrane (0.45 µm pore size, Schleicher & Schuell). Membranes were blocked with 5 % milk, incubated with primary antibodies anti-polyQ 1C2 (1:1000, Millipore, MAB1574), polyclonal rabbit anti-GFP (1:1000, kindly provided by Prof. J. Neefjes, NKI, The Netherlands), anti-β-actin (1:1000, Santa Cruz, SC-130656) and subsequently incubated with secondary antibodies IRDye 680 or IRDye 800 (1:10,000, Li-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (Licorn). Soluble and insoluble fractionation was performed as described before [55]. Briefly, cells were lysed in 1x TEY buffer (70 mM Tris/HCl pH 6.8, 1.5 % SDS, 20 % glycerol). After sonification 50 mM DTT was added and samples were centrifuged at 14,000 rpm at RT. The pellet fraction was incubated with 100 % formic acid at 37°C for 40 min and evaporated by using a speedvac system (Eppendorf). 1x TEY buffer supplemented with 0.05 % bromophenol blue was added to the pellet and the soluble and insoluble fractions were loaded on a SDS-PAGE gel.

Filtertrap assay. The filter retardation assay was performed as described before [56]. Briefly, HEK293T cells expressing GFP-Ub-Q65 together with DNAJB6b or DNAJB8 for 72 hours incubated with the various inhibitors, were lysed for 30 minutes on ice in Nondinet P-40 (NP-40) buffer (100 mM TrisHCI, pH 7.5, 300 mM NaCl, 2 % NP-40, 10 mM EDTA, pH 8.0), supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). After 15 minutes centrifugation at 14,000 rpm at 4°C, cell pellets were resuspended in benzonase buffer (1 mM MgCl2, 50 mM Tris-HCl pH 8.0) and incubated for 1 hour at 37°C with 250 U benzonase (Merck). Reactions were stopped by adding 2x termination buffer (40 mM EDTA, 4 % SDS, 100 mM DTT). Aliquots of 30 µg protein extract were diluted into 2 % SDS buffer (2 % SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and filtered through a 2 µm cellulose acetate membrane (Schleicher and Schuell) pre-equilibrated in 2 % SDS buffer. Filters were washed twice with 0.1 % SDS buffer (0.1 % SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and subsequently blocked in 5 % dry milk in TBS. Captured aggregates were detected by incubation with IC2 antibody and further treated like Western blots.

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EXPANDED POLYGLUTAMINE-CONTAINING N-TERMINAL HUNTINGTIN FRAGMENTS ARE ENTIRELY DEGRADED BY MAMMALIAN PROTEASOMES

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Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

ABSTRACT

Huntington’s disease is a neurodegenerative disorder caused by an expanded polyglutamine (polyQ) repeat within the respective protein huntingtin (Htt). N-terminal fragments of the mutant Htt (mHtt) proteins containing the polyQ repeat are aggregation-prone and form intracellular inclusion bodies. Improving the clearance of mHtt fragments by intracellular degradation pathways is relevant to obviate toxic mHtt species and subsequent neurodegeneration. As the proteasomal degradation pathway has been the subject of controversy regarding the processing of expanded polyQ repeats, we examined whether the proteasome can efficiently degrade Htt-exon1 with an expanded polyQ stretch both in neuronal cells and in vitro. Upon targeting mHtt-exon1 to the proteasome, rapid and complete clearance of mHtt-exon1 was observed. Proteasomal degradation of mHtt-exon1 was devoid of polyQ peptides as partial cleavage products by incomplete proteolysis, indicating that mammalian proteasomes are capable of efficiently degrading expanded polyQ sequences without an inhibitory effect on the proteasomal activity.

INTRODUCTION

Huntington’s disease (HD) is a neuro-degenerative disorder caused by a polyglutamine (polyQ) repeat expansion in the respective protein huntingtin (Htt) [1, 2]. HD occurs when the polyQ tract exceeds a threshold of 35-40 glutamine residues in length with a strong inverse correlation between repeat length and age-of-onset of disease [3]. The polyQ expansion causes neuronal dysfunction through a toxic gain-of-function mechanism in both animal and cellular models independent of its protein context [4-7]. N-terminal fragments of mutant Htt (mHtt) protein containing the expanded polyQ tract are highly prone to aggregate and form intracellular inclusion bodies (IBs) as observed in human HD postmortem brain and in animal or cellular systems [8-12]. R6/2 mice expressing exon1 of the human mutant HD gene generate a neurological phenotype similar to human HD with an early onset of symptoms and a fast progression of the disease [13].

The two main intracellular pathways involved in protein degradation are the ubiquitin-proteasome system (UPS) and autophagy. Both pathways play a role in mHtt clearance [14, 15]. While the UPS is active in both the nucleus and the cytoplasm, it is merely capable of degrading unfolded monomeric Htt proteins [16-18]. The autophagic pathway is a cytoplasmic degradation machinery and targets soluble and aggregated Htt proteins for lysosomal destruction [19, 20]. Interestingly, disappearance of IBs and amelioration of disease phenotype are observed after shutdown of mHtt expression in a conditional HD mouse model, suggesting that autophagy can remove aggregated mHtt and HD may be reversible [21]. This data is supported by the fact that induction of autophagy decreases both aggregated and soluble mHtt, resulting in reduced toxicity in various models of HD [14]. However, cells lack autophagy in the nucleus and do not have the ability to eliminate nuclear aggregates efficiently, which may explain the high frequency of nuclear IBs formed by N-terminal mHtt fragments in human HD postmortem brain [8]. As proteasome activity diminishes with age, Zhou et al. showed that N-terminal mHtt fragments aggregate in the nucleus in association with the age-dependent decrease of proteasome activity in an HD knock-in mouse model [22]. Furthermore, levels of soluble and aggregated mHtt increase upon proteasomal inhibition in cell culture and HD mouse brain material [16-18]. Since IBs recruit proteins including Ubiquitin (Ub), many types of chaperones and whole proteasomes, this suggests that cells attempt to clear the aggregation-prone mHtt protein by the proteasomal pathway [17, 18, 23, 24]. In vitro studies suggest that proteasomes are not capable of cleaving within the expanded polyQ repeat, whereas a partial proteolytic product is released lackinganking sequences of the polyQ tract [25, 26]. However, activating the proteasome by the mutant proteasome activator PA28γ (K188E) appears to improve in vitro degradation of peptides containing 10 glutamines with cleavage after each of the glutamines [27].

In this study, we investigated whether the proteasome can degrade mHtt-exon1 with an expanded polyQ stretch, both in vitro and in neuronal cells by targeting mHtt-exon1 exclusively to the proteasome. We show that mHtt-exon1 is subsequently fully degraded by the cellular UPS, thereby preventing mHtt-exon1 accumulation and aggregation. The proteasomal degradation of soluble mHtt-exon1 does not lead to the release of a polyQ peptide as partial cleavage product by incomplete proteolysis. Furthermore, mammalian proteasomes are capable of degrading expanded polyQ sequences, and proteasomal activity is not affected by the presence of mHtt-exon1.

RESULTS

mHtt-exon1 is degraded by macroautophagy and the proteasome

To determine whether macroautophagy and the proteasome are involved in mHtt-exon1 degradation, mHtt-exon1-97Q was expressed for 24 hours in Neuro-2a cells treated with the proteasomal inhibitor Epoxyomycin or the autophagy inhibitor 3-MA for the last 16 hours of expression. Cells were harvested for soluble and insoluble fractionation, and filtertrap assay to evaluate both monomeric and aggregated mHtt-exon1 levels using the 1C2 antibody that recognises polyQ sequences. Whereas autophagy inhibition increased the level of mHtt-exon1 aggregates detected in the insoluble fraction (arrow) and on filtertrap (Fig. 1A), proteasome inhibition showed no obvious effect on the amount of monomeric and aggregated mHtt-exon1 in Neuro-2a cells. Furthermore, protein level of wildtype (wt) Htt-exon1-2SQ expressed in Neuro-2a cells was elevated upon inhibition of the autophagic degradation by 3-MA (Fig. 1B). To verify the effect of 3-MA as an inhibitor of macroautophagy, Neuro-2a cells were treated for 16 hours with 3-MA in combination with the inhibitor BafA1 or DMSO as control. Western blot analysis clearly shows an effect of 3-MA on the endogenous LC3-II level, indicating an inhibition of the formation of LC3-II-positive autophagosomes by 3-MA (Fig. 1C). Assessing the effect of Epoxyomycin and 3-MA on the proteasomal catalytic activity in treated Neuro-2a cells, endogenous proteasomes were labeled in the cell lysates with a proteasome activity-based probe where the catalytical activities were...
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes. Visualised by in-gel fluorescence (Fig. 1D). In addition, the degradation of fluorogenic proteasomal AMC-substrates added to Neuro-2a cell lysates was analysed (Fig. 1E). While Epoxomicin reduces the caspase-like, trypsin-like and chymotrypsin-like proteasomal activities, referred to as β1, β2, and β5, 3-MA had no effect on the proteasomal activity nor on the level of polyubiquitinated material. To exclude that 3-MA treatment inhibits the proteasomal degradation of substrates at a level upstream of the proteasome catalytic activity the degradation of the proteasome reporter Ub-G76V-GFP in Neuro-2a cells was analysed. Epoxomicin but not 3-MA increased the protein level of the proteasome reporter substrate showing that 3-MA inhibits autophagic but not proteasomal degradation of proteins (Fig. 1F). While proteasomal inhibition did not lead to an increase of Htt-exon1 protein levels, the impairment of the ubiquitin proteasome system might be compensated by an upregulation of autophagy (reviewed in [28]). To examine the role of the proteasome in mHtt-exon1 degradation in more detail, Htt-exon1-97Q-C4 was expressed in wt and Atg5-deficient mouse embryonic fibroblasts (MEFs) [29] and treated with Epoxomicin. The short C4-tag with 12 amino acids binds the biarsenical dye ReAsH and the Htt protein becomes fluorescent [30]. The Atg5-Atg12 protein conjugation is essential for the formation of LC3-II-positive autophagosomes, with LC3-II being a key marker for autophagosomes [31]. Atg5-/- MEFs deficient of LC3-II-positive autophagosomes showed an increase of mHtt-exon1 aggregates after proteasomal inhibition compared to wt MEFs, indicating that soluble mHtt-exon1 can indeed be targeted by the proteasome when autophagy is impeded (Fig. 2A, B). To exclude that Atg5-/- MEFs compensate with an increase of proteasomal catalytic activity compared to wt MEFs, cellular proteasomes were labeled in the cell lysates with a proteasome activity-based probe where the catalytical activities were visualised by in-gel fluorescence (Fig. 2C). Together, this data indicates that mHtt-exon1 can be degraded by both macroautophagy and the proteasome.

**Targeting mHtt-exon1 to the proteasome**

Previous studies showed inefficient in vitro proteasomal degradation of fusion proteins containing an expanded polyQ stretch, leading to the conclusion that eukaryotic proteasomes fail to cleave within expanded polyQ sequences [25, 26]. However, it remained unclear whether expanded polyQ stretches embedded in native polyQ protein sequences like mHtt-exon1 are also inefficiently degraded by proteasomes in living cells. In case of remaining polyQ stretches as partial cleavage products by incomplete proteolysis these fragments would accumulate within the cell and starting to aggregate as previously shown in living cells expressing pure expanded polyQ peptides without flanking sequences [23].

To examine whether intracellular proteasomes are able to fully degrade mHtt and also cleave within the expanded polyQ tract, we prevented degradation of mHtt by the autophagic pathway and targeted the mHtt-exon1 protein with a specific degradation signal to the proteasomal pathway. To generate a mHtt-exon1 protein, which is exclusively destructed via the proteasomal degradation pathway and independent of macroautophagic clearance within the cell, degron signals for the ubiquitin-dependent and ubiquitin-independent proteasomal destruction were fused to the

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Fig. 1. Intracellular degradation of Htt-exon1.

(A) Soluble and insoluble fractionation of Neuro-2a cell lysate after transient transfection of Htt-exon1-97Q or an empty vector as control. Six hours after transfection cells were treated for 16 hours with DMSO, Epoxomicin or 3-MA and harvested. Soluble Htt and formic acid-dissolved and non-dissolved (arrow) Htt aggregates were detected on western blot by the 1C2 antibody. SDS-insoluble Htt aggregates were analysed by filtertrap assay in doublets. β-actin was used as a loading control. Quantification of soluble and insoluble mHtt protein levels; *p<0.05 (n=3). (B) Western blot analysis of Neuro-2a cells after transient transfection of Htt-exon1-25Q or an empty vector as control. Six hours after transfection cells were treated for 16 hours with DMSO, Epoxomicin or 3-MA and harvested. Soluble Htt was detected on western blot by the 1C2 antibody. β-actin was used as a loading control. Quantification of soluble wt Htt protein levels *p<0.05 (n=3). (C) Neuro-2a cells were treated for 16 hours with 3-MA in combination with BafA1 or DMSO as control (last 4 hours) before harvest. Endogenous LC3-I and -II levels were detected by
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

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antibody 3B5H10, suggesting that Ub-R-KK-Htt-exon1-97Q is fully degraded by the proteasome.

tracts missing the flanking sequences were not detected on western blot with the specific polyQ of the Ub-R-KK-Htt-exon1-97Q protein by the proteasome consisting of putative intact polyQ proteasome before it can accumulate and aggregate within the cell. Smaller partial digest products in inhibitory Epoxomicin but not the autophagy inhibitor 3-MA resulted in an accumulation of Ub-Htt-exon1-97Q protein is efficiently targeted to the UPS, as treatment with the proteasomal aggregated mHtt-exon1 detected on filtertrap. Due to the N-degron signal the soluble monomeric Ub-R-KK-Htt-exon1-97Q has a low abundance of soluble protein level on western blot and no the inhibitors Epoxomicin and 3-MA. In contrast to Htt-exon1-97Q, the "N-end rule" Htt protein Htt-exon1-97Q-CL1 expressed in rabbit reticulocyte lysate is not efficiently targeted by the proteasome. This data show that the N-degron signal in Ub-R-KK-Htt-exon1 targets soluble mHtt-exon1 exclusively to the proteasomal pathway independent of macroautophagy, and represents an adequate substrate suitable for studying proteasomal degradation of polyQ-expanded mHtt-exon1 within the cell.

Next, we generated a mHtt-exon1 fusion protein flanked on its N-terminus by an "N-end rule" degradation signal, termed N-degron, composed of an ubiquitin moiety which is removed upon translation by deubiquitinases. This exposes the destabilising amino acid arginine (R) followed by a 40 amino acid region consisting of two lysines for polyubiquitination as a target signal for the proteasomal degradation [34] (Fig. 3C). To test the effect of the specific C-terminal degrons on mHtt-exon1 degradation and protein aggregation Neuro-2a cells were transfected with the Htt-exon1-97Q constructs, expressed for 24 hours and treated with the inhibitors Epoxomicin and 3-MA. Despite these degron signals, mHtt-exon1 with a CL1 or a MODC degron were still a target for macroautophagy, similar to mHtt-exon1 without an additional degron signal (Fig. 3B). Only the autophagy inhibitor 3-MA increased the amount of aggregated mHtt-exon1 in the SDS-insoluble fraction detected by the filtertrap analysis, whereas, neither the CL1 degron nor the MODC degron targeted mHtt-exon1 protein exclusively towards proteasomal degradation. Fusion of the CL1 or MODC degron to the N-terminus instead of the C-terminus of mHtt-exon1 also did not change the degradation pattern compared to mHtt-exon1 without an additional degron signal (data not shown).

Simultaneous inhibition of both the proteasome and autophagy by Epoxomicin and 3-MA does not reveal a partial digest product of the Ub-R-KK-Htt-exon1-97Q protein by the proteasome that would be subsequently targeted by macroautophagy (data not shown). Next, the number of Neuro-2a cells containing mHtt-exon1 aggregates upon expression of Ub-R-KK-Htt-exon1-97Q or Htt-exon1-97Q, with a C4-tag was determined. The amount of mHtt-exon1 aggregates in transfected cells confirms the filtertrap data from Fig. 3D, as almost no aggregates of Ub-R-KK-Htt-exon1-97Q-C4 are detectable compared to Htt-exon1-97Q-C4 (Fig. 3E). Addition of the inhibitors Epoxomicin or 3-MA for the last 16 hours of expression shows an increase of mHtt-exon1 aggregation, which was mainly due to inhibition of autophagy, whereas mutant Ub-R-KK-Htt-exon1 aggregation only increases upon inhibition of the proteasomal function. Similarly, in vitro transcription and translation of mHtt-exon1 with the N-degron signal and the CL1 degron in rabbit reticulocyte lysate reveals a rapid degradation of the short-lived Ub-R-KK-Htt-exon1-97Q protein by the proteasome compared to Htt-exon1-97Q-CL1 protein after treatment with MG132, showing that the disappearance of the Ub-R-KK-Htt-exon1-97Q protein and its polyubiquitinated species is dependent on proteasomal function (Fig. 3F). Like in Neuro-2a cells Htt-exon1-97Q-CL1 expressed in rabbit reticulocyte lysate is not efficiently targeted by the proteasome. This data show that the N-degron signal in Ub-R-KK-Htt-exon1 targets soluble mHtt-exon1 exclusively to the proteasomal pathway independent of macroautophagy, and represents an adequate substrate suitable for studying proteasomal degradation of polyQ-expanded mHtt-exon1 within the cell.

mHtt-exon1 protein. C-terminal degrons such as the 16 amino acids-long ubiquitin-dependent CL1 degron and the ubiquitin-independent C-terminal PEST sequence from the mouse ornithine decarboxylase (MODC) are known to reduce the half-life of GFP by introduction of these additional proteolytic signals [24, 32, 33]. These degron signals were fused to the C-terminus of mHtt-exon1 (Fig. 3A). To evaluate the effect of the specific C-terminal degrons on mHtt-exon1 degradation and protein aggregation Neuro-2a cells were transfected with the Htt-exon1-97Q constructs, expressed for 24 hours and treated with the inhibitors Epoxomicin and 3-MA. Despite these degron signals, mHtt-exon1 with a CL1 or a MODC degron were still a target for macroautophagy, similar to mHtt-exon1 without an additional degron signal (Fig. 3B). Only the autophagy inhibitor 3-MA increased the amount of aggregated mHtt-exon1 in the SDS-insoluble fraction detected by the filtertrap analysis, whereas, neither the CL1 degron nor the MODC degron targeted mHtt-exon1 protein exclusively towards proteasomal degradation. Fusion of the CL1 or MODC degron to the N-terminus instead of the C-terminus of mHtt-exon1 also did not change the degradation pattern compared to mHtt-exon1 without an additional degron signal (data not shown).

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**Efficient proteasomal degradation of short-lived mHtt**

Expression of the Ub-R-KK-Htt-exon1-97Q protein reveals a direct clearance of the full mHtt-exon1 protein by the proteasome, since no expanded polyQ stretches as partial proteolytic products were detectable on western blot by the polyQ antibody 3B5H10 (Fig. 3D). To prove that this particular antibody is able to detect pure polyQ tracts independent of the flanking Htt sequences by western blot analysis, we expressed the constructs GFP-Ub-Q112 and as a control GFP-Ub in Neuro-2a cells and analysed the polyQ peptides generated after N-terminal GFP-Ub hydrolysis by cellular deubiquitinases [23]. The specific polyQ antibody 3B5H10 is able to detect polyQ peptides with a size around 30 kDa (arrow) and higher molecular species, which may represent polyQ oligomers (asterisks) (Fig. 4A). To examine whether a possible proteasomal polyQ product derived from mHtt-exon1 is more highly prone to aggregate than the mutant Ub-R-KK-Htt-exon1 protein and therefore not detectable on the soluble level by western blotting, a soluble and insoluble fractionation of transfected Neuro-2a cell lysate treated with Epoxomicin was performed. When proteins from the soluble and insoluble fractions were stained on a western blot with the polyQ antibody 3B5H10, no additional polyQ fragments generated by the cellular proteasomes were detectable besides the low level of the proteasomal substrate Ub-R-KK-Htt-exon1-97Q which increases in both fractions as monomeric and poly-ubiquitinated forms after Epoxomicin treatment (asterisk) (Fig. 4B). To exclude the possibility that polyQ peptides generated by the proteasome accumulate within the cell and form formic acid-insensitive aggregates, we coexpressed wtHtt-exon1-25Q protein as an aggregation reporter for filtertrap analysis and fluorescence microscopy. Untagged Htt-exon1-25Q coexpressed with GFP-Ub-Q112 in Neuro-2a cells co-aggregates with Q112 peptides detectable on filtertrap assay by the Htt specific antibody N18 (Fig. 4C). Similarly, confocal microscopy of fixed Neuro-2a cells coexpressing non-fluorescent Ub-Q112 and the reporter Htt-exon1-25Q-GFP show nuclear and cytoplasmic inclusion bodies formed by polyQ peptides that co-sequester wtHtt-exon1 proteins (Fig. 4D). In contrast, coexpression of the short-lived mHtt-exon1 variant Ub-R-KK-Htt-exon1-97Q and the reporter Htt-exon125Q-GFP reveals no detectable aggregation on filtertrap by the antibody GFP except for Epoxomicin treated cells (Fig. 4E). In addition, coexpression of the aggregation reporter Htt-exon1-25Q-GFP with Htt-exon1-97Q-C4 or its short-lived variant Ub-R-KK-Htt-exon1-97Q-C4 only shows ReAsH and GFP co-stained aggregates in cells expressing Htt-exon1-97Q-C4, whereas the reporter’s GFP signal in cells coexpressing Ub-R-KK-Htt-exon1-97Q-C4 remains diffusely distributed in the cytoplasm (Fig. 4F). These results support that short-lived mHtt-exon1 neither aggregates nor is proteolytically degraded into pure polyQ peptides that would co-sequester the reporter protein Htt-exon1-25Q-GFP.

Next, we investigated whether targeting mHtt-exon1-97Q to the UPS not only reduces levels of soluble and insoluble mHtt-exon1 but would also reduce mHtt-exon1 induced toxicity as measured by cellular uptake of propidium iodide (PI). When Htt-exon1-97Q and its short-lived variant Ub-R-KK-Htt-exon1-97Q were expressed in Neuro-2a cells for 48 hours and stained with PI, mHtt-exon1-97Q increased PI-staining of the Neuro-2a cells, whereas fusion of the N-degron signal to mHtt-exon1 did not lead to mHtt-induced toxicity (Fig. 4G).
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes.

**Fig. 4. Efficient proteasomal degradation of the short-lived Htt-exon1-97Q protein.**

(A) Transient transfection of Neuro-2a cells with constructs encoding GFP-Ub-Q112 and GFP-Ub as control. Within the cells removal of N-terminal GFP-Ub by ubiquitinases releasing pure polyQ peptides (arrow) and its oligomeric species (asterisks) detectable with the polyQ antibody on western blot. β-actin was used as a loading control. (B) Soluble and insoluble fractionation of Neuro-2a cells after transient transfection of Ub-R-KK-Htt-exon1-97Q. Six hours after transfection cells were treated for 16 hours with Epoxomicin and DMSO as control and harvested for western blot analysis. Expanded polyQ-containing Htt was detectable by the polyQ antibody on western blot. β-actin was used as a loading control. (C) Filtertrap analysis of Neuro-2a cells after transient transfection of Ub-R-KK-Htt-exon1-97Q and Htt-exon1-25Q-GFP or the empty vector as control. Six hours after transfection cells were treated for 16 hours with Epoxomicin and DMSO as control and harvested for filtertrap analysis (in doublets) with the GFP antibody. (D)Transient cotransfection of Neuro-2a cells with Ub-Q112 and Htt-exon1-25Q-GFP. Within the cells ubiquitinases cleave of the N-terminal Ub releasing polyQ peptides which are prone to aggregate. After 24 hours cells were fixed and aggregates of Q112-peptides co-sequestering the reporter protein Htt-exon1-25Q-GFP were detected by immunofluorescence. The nucleus is stained by Dapi. Scale bar: 10 µm. (E) Filtertrap analysis of Neuro-2a cells after transient transfection of Ub-R-KK-Htt-exon1-97Q and Htt-exon1-25Q-GFP or the empty vector as control. Six hours after transfection cells were treated for 16 hours with Epoxomicin and DMSO as control and harvested for filtertrap analysis (in doublets) with the GFP antibody. (F) Transient cotransfection of Neuro-2a cells with Ub-Q112 and Htt-exon1-25Q-GFP and either Htt-exon1-97Q-C4 or Ub-R-KK-Htt-exon1-97Q-C4 for 48 hours. Htt-exon1-97Q-C4 aggregates stained with RedH co-sequester wt Htt, whereas Ub-R-KK-Htt-exon1-97Q-C4 neither aggregates nor is proteolytically degraded into pure polyQ peptides co-sequestering the reporter protein Htt-exon1-25Q-GFP. The nucleus is stained with Dapi. Scale bar: 10 µm. (G) Cellular uptake of propidium iodide (PI) in control cells transfected with an empty vector and cells expressing mutant Htt with or without the N-end rule degron signal; ***p<0.001 (n=3). (H) Soluble and insoluble fractionation of TPPII-/- MEF cell lysate after transient transfection of Ub-R-KK-Htt-exon1-97Q or the empty vector as control. Six hours after transfection cells expressing Ub-R-KK-Htt-exon1-97Q were treated for 16 hours with DMSO, PAQ22/Bestatin, Phenanthroline or Epoxomicin. Polyubiquitinated and monomeric (arrow) short-lived mutant Htt was detected on western blot by the polyQ antibody. β-actin was used as a loading control.

Previous in vitro studies suggested that in the case of expanded polyQ-containing proteins, eukaryotic proteasomes cannot cleave within the polyQ sequences releasing pure polyQ stretches without flanking sequences [25, 26]. Subsequently, these polyQ fragments may be degraded by cytosolic peptidases before they start to aggregate. Peptides released by the proteasome are rapidly degraded into amino acids by various peptidases [35-39] like tripeptidyl peptide II (TPPII), which can also target peptides longer than 15 amino acids [40, 41]. The proposed cellular function of TPPII is an exo-and endopeptidase activity downstream of the proteasome. However, the proteolytic processing of proteasomal products has TPPII in common with other peptidases like puromycin-sensitive aminopeptidase (PSA). PSA may target released polyQ peptides as it was found to be capable of degrading short polyQ peptides [42]. Recently, Menzies et al. showed that PSA promotes autophagy independent of its cytosolic peptidase function leading to enhanced clearance of aggregation-prone proteins [43].

To examine whether TPPII and PSA may target potential expanded polyQ peptides released by the proteasome upon degradation of Ub-R-KK-Htt-exon1-97Q, we expressed the short-lived mHtt-exon1 in TPPII-/- MEF cells treated with a combination of the PSA inhibitors PAQ22 and Bestatin or Phenanthroline (Fig. 4H). As a control transfected MEFs were treated with Epoxomicin. Western blot analysis of the soluble and insoluble fraction of lysates shows the detection of the Ub-R-KK-Htt-exon1-97Q protein only after proteasomal inhibition (arrow), while no additional smaller polyQ-containing fragment is detectable by the polyQ antibody 3B5H10 in TPPII-/- MEF cells treated with inhibitors for PSA. This suggests that TPPII and PSA do not have an impact on the degradation of putative expanded polyQ stretches downstream of the proteasome, and indicates that short-lived mHtt-exon1 is efficiently targeted for proteasomal degradation, whereas no release of toxic, aggregation-prone polyQ peptides by the proteasome occurs.
Mammalian 20S proteasomes are able to degrade expanded polyQ sequences \textit{in vitro}

The previous results with the efficient cellular mHtt degradation, independent of peptidases like TPP1 or PSA, predict that purified mammalian 20S proteasomes are capable of degrading purified mutant Htt protein completely. To test this, both mHtt-exon1-97Q and wtHtt-exon1-25Q proteins were expressed in Neuro-2a cells and subsequently purified by immunoprecipitation via an HA-agarose followed by dialysis. Purified open-gated mammalian 20S proteasomes were incubated with purified mHtt-exon1 or wtHtt-exon1 for 12 or 24 hours before the reaction was stopped by adding sample loading buffer. The digests were analyzed on a western blot with the Htt specific antibodies N18 against the Htt N-terminus, polyQ 385H10 against the polyQ tract and HA antibody against the C-terminus. Within 24 hours both mHtt-exon1 (Fig. 5A) and wtHtt-exon1 (Fig. 5B) were almost fully degraded with no remaining polyQ fragment as a partial proteolytic proteasomal product lacking the flanking N-terminal and C-terminal sequences. Addition of MG132 prevents the degradation of Htt-exon1 by the 20S proteasome completely. As the mHtt-exon1 protein is almost completely degraded within 12 hours incubation time, we followed the \textit{in vitro} degradation kinetics for the time points 0, 3, 6 and 16 hours to analyze earlier events (Fig. 5C). While mHtt-exon1 diminishes in time as detected by western blot analysis using the polyQ antibody 385H10, marginal smaller proteasomal cleavage fragments appear within the first 3 hours of digest as putative proteolytic intermediates. After 16 hours of digest only \textasciitilde{}10 % of the purified mHtt-exon1 protein staining remains.

Next, we examined whether any partial processing products may aggregate within the 16 hours of digest, and would therefore not be detectable as soluble fragments on western blot. However, analysis of the formic acid-treated insoluble fraction of the \textit{in vitro} digest reveals no additional aggregation-prone polyQ fragments (Fig. 5D). To exclude the possibility that glutamine residues in the polyQ-stretch are deamidated to glutamate prior the \textit{in vitro} digest, resulting in unintended protein degradation by the caspase-like activity of the proteasome, purified mHtt-exon1 was incubated with proteinase K, an enzyme that is capable of cleaving after glutamate but not glutamine. Incubation of mHtt-exon1 with proteinase K generates a proteolytic product within 2 hours of incubation with a size of \textasciitilde{}27 kDa, which is the expected size of a pure 97Q peptide without the flanking Htt N-terminal and C-terminal sequences due to removal by proteinase K (Fig. 5E). Since glutamine deamidation in the mHtt-exon1 protein would result in various smaller fragments of different sizes after proteinase K digest, the appearance of a specific monomeric protein band (arrow) and a formic acid-soluble oligomeric species (asterisk) detectable by the polyQ antibody 385H10 upon proteinase K digest but not proteasomal degradation, suggests that the mHtt-exon1 protein is completely degraded by the 20S proteasome.

While mHtt-exon1 and wtHtt-exon1 are efficiently degraded by the proteasome, we analysed whether the expanded polyQ stretch affect \textit{in vitro} proteasome activity, as impairment of proteasomes by polyQ proteins has been suggested before [25, 26]. To test this, we monitored the chymotrypsin-like activity of purified 20S proteasome after incubation with mHtt-exon1 and wtHtt-exon1.
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

- Anti-Htt antibody polyQ. The amount of added 20S proteasome was shown with an anti-alpha2 antibody used as a loading control. Quantification of the remaining full-length purified Htt-exon1-97Q-H4 protein stained by the polyQ antibody within 16 hours digest by the 20S proteasome. (D) After incubation of purified mHtt with 20S proteasome aggregated proteins were separated from soluble protein material by centrifugation and solubilized at time point 16 hours with formic acid (insoluble fraction) and subjected to SDS-PAGE and western blot analysis using anti-Htt antibody polyQ. (E) In vitro degradation of the mutant Htt protein with proteasine K. Purified mHtt was incubated with proteasine K at 37°C for the time points indicated. After digest aggregated proteins were detected by high speed centrifugation and solubilized by formic acid (insoluble fraction). The proteins were subjected to SDS-PAGE and western blot analysis using anti-Htt antibody polyQ. The pure polyQ fragment (arrow) and a soluble oligomeric intermediate (asterix) generated by the proteasine K digest were detected by the polyQ antibody (F). Chymotrypsin-like activity of the purified mammalian 20S proteasome monitored by Suc-LLVY-AMC digestion 16 hours after incubation of proteasomes with purified wt or mHtt treated with or without MG132 at 37°C (n=3).

wtHtt-exon1. The expansion of the polyQ tract in the Htt-exon1 protein had no effect on the proteasomal activity as measured with degradation of the proteasomal substrate Suc-LLVY-AMC in time (Fig. 5F). This supports the data that mammalian proteasomes are capable of degrading expanded polyQ sequences while main proteasomal activity is not affected by the presence of mHtt-exon1.

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Detection of proteasome-mediated cleavage products from Htt-exon1 by mass spectrometry

As an additional step to evaluate cleavage of mHtt-exon1 by the mammalian 20S proteasome, in vitro digests were analysed by mass spectrometry (MS). Peptides generated by the proteasome within 16 hours digest of mHtt-exon1 and wtHtt-exon1 were separated and identified by LC-MS (Table 1). As result of the analysis, we specified a number of N-terminal and C-terminal Htt peptides as proteasomal cleavage products that are represented in a schemata where the identified Htt peptides are summarised and marked in green (N-terminal peptides) and red (C-terminal peptides) (Fig. 6). The pattern of the Htt peptides suggests that there are one or more initial cleavage events in the purified Htt-exon1 protein, thereby generating longer peptides that may represent intermediate products that are sequentially trimmed at the C-terminus to smaller peptides detected by MS as 8-23 amino acid long peptides. Undetected proteasomal cleavage products exclusively consisting of only glutamine residues would be underestimated by MS due to their poor ionization [27].

To exclude the possibility that mHtt-exon1 degradation is attributed to a contaminating protease potentially co-purified with the 20S proteasome or the mHtt-exon1 protein, we identified co-purified proteins by MS analysis of peptides generated by trypsin cleavage (Table 2). However, no co-purified contaminating protease could be detected, indicating that Htt-exon1 was exclusively degraded by the proteasome. Furthermore, MS analysis of the Htt-exon1 in vitro digest including the proteasomal inhibitor MG132 or merely without the 20S proteasome shows no Htt peptide generation (data not shown). Interestingly, some chaperones were identified by MS analysis which have been co-immunoprecipitated with mHtt-exon1 and could facilitate unfolding and degradation both in living cells and in vitro.

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Table 1. Mass spectrometric indentification of 20S mammalian proteasome-mediated degradation products from Htt-exon1-97Q-H4 and Htt-exon1-25Q-H4

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<th># detection events</th>
<th>Intensity</th>
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Our data shows that mHtt-exon1 is a substrate for both macroautophagy and the proteasome. While treatment of cells with the proteasome inhibitor did not show an increase in monomeric and aggregated mHtt-exon1 levels, this may be explained by the subsequent induction of macroautophagy upon UPS inhibition [28]. A clear contribution of Htt-exon1 proteasomal degradation could be visualised when macroautophagy was impaired, such as in Atg5−/− MEF cells. To analyse whether the cellular proteasome is capable of clearing within an expanded polyQ stretch considering the native Htt-exon1 protein context, we targeted the mHtt-exon1 protein to the proteasomal pathway in ubiquitin-dependent and -independent manners by the fusion of mHtt-exon1 to various degron-signals. We show that the mHtt-exon1 short-lived variant with the N-degron signal (Ub-R-KK) is efficiently cleared by the proteasome before aggregation can occur, independent of macroautophagy. This data clearly indicates that the eukaryotic UPS can degrade mHtt-exon1 completely, including the expanded polyQ sequence. Fusion of pure polyQ sequences to GFP with an N-terminal degradation signal also decreased soluble and insoluble GFP-polyQ protein levels in transfected cells [44, 45]. However, expression of the GFP-polyQ protein carrying the Ub-R degron signal still led to the formation of aggregates within cells, suggesting that this degron signal is not as efficient as the Ub-R-KK degron signal used in our study where no aggregation of short-lived mHtt-exon1 was detectable. In contrast to previous studies [25,51], we analysed the proteasomal degradation of mHtt-exon1 without a fusion to large fluorescent proteins, like GFP, and in addition we determined the role of polyQ clearance by macroautophagy and putative cytoplasmic peptidases next to the proteasomal degradation.

Intriguingly, C-terminal degrons such as the CL1 degron and the C-terminal PEST sequence from MODC are known to reduce the half-life of GFP [24, 32, 33], and a previous study showed that the degron-signal of ODC converts mHtt with a length of 163 amino acids to an instable protein [46]. However, our data using mHtt-exon1-CL1 and mHtt-exon1-MODC show that these proteins are not proper proteasomal substrates, suggesting that the protein context next to the specific degron signal plays an important role in substrate recognition and its subsequent proteasomal degradation. It also underscores the importance of targeting mHtt fragments to the proteasome, which is most likely dependent on an appropriate ubiquitination of the specific substrate similar to the N-end rule. Improving the targeting of monomeric mutant Htt fragments to the nuclear and cytoplasmic proteasomal degradation pathway before aggregation occurs is certainly a very important issue that should be addressed in follow-up studies. Enhancing the processes of efficient ubiquitination to target nuclear and cytoplasmic mutant Htt to the proteasomal pathway might be a potential therapeutic approach, but requires the identification of involved E2/E3 ligases or deubiquitinating enzymes and whether their activity can be specifically triggered.

Rapid degradation of mHtt-exon1 with an N-degron by cellular proteasomes might occur through several sequential rounds in ubiquitin-dependent and -independent manners. First, polyubiquitinated mHtt-exon1 gets recognized, deubiquitinated and unfolded by the 19S regulatory particle, followed by entry of the unfolded mHtt-exon1 into the hollow cavity of the 20S core
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

particle. Here Htt fragments of different lengths are generated as proteolytic products containing the full or shortened polyQ tracts. Second, some of these generated Htt fragments may either again become ubiquitinated and processed into smaller peptides by the 26S proteasome, or third, an ubiquitin-independent proteasomal way is responsible for the degradation of the released unfolded Htt fragments into smaller peptides that are subsequently recycled into amino acids by cytoplasmic peptidases [47]. The ubiquitin-independent proteasomal processing of Htt-exon1 or subsequent Htt peptides might be performed by 20S proteasomes associated with activating caps. While the 26S proteasome is composed of a 19S cap and a 20S catalytic core particle, the 19S cap can be replaced by 11S activators, termed PA28αβ or PA28γ [48-51]. PA28α and PA28β form a heteroheptameric ring, and PA28γ forms a homoheptameric ring [52-55]. PA28αβ or PA28γ activators dock on the 20S proteasome, recognise unfolded peptides and proteins in an ATP-independent manner and stimulate the proteasomal catalytic activity [56-58]. Alternatively, 11S activators function in the context of hybrid proteasomes where a 20S proteasome is capped by one 11S activator and on the opposite site by a 19S cap [58]. Interestingly, in vitro studies revealed that the mutant PA28γ (K188E) cap increases the activity of all three 20S catalytic subunits and proteasomal degradation of short polyQ peptides is 10-fold more compared to wt PA28γ, suggesting a beneficial effect of activators towards polyQ stretch degradation [27, 59].

Importantly, the in vitro data in our study were obtained with mHtt-exon1 without the specific N-terminal degron, showing that also non-modified mHtt-exon1 can be degraded entirely by 20S proteasomes. The in vitro digests confirm our observations in living cells that the proteasome efficiently degrades the expanded polyQ repeat. Our results are in contrast with a study reporting the release of pure polyQ sequences during in vitro proteasomal degradation of a polyQ-fusion protein due to inefficient degradation [26]. This former in vitro study implicates that enhancing the proteasomal degradation of expanded-polyQ proteins is not valid as a therapeutic approach for polyQ diseases. In this study, we show for the first time that mammalian proteasomes can entirely degrade mutant Htt fragments in living cells, and that both cellular and in vitro proteasomal destruction of mHtt-exon1 was devoid of long polyQ peptides as partial cleavage products. Subsequently, reducing the amount of monomeric mHtt by accelerating the mHtt proteasomal destruction obviates the accumulation of toxic Htt fragments and finally represents a therapeutic strategy for HD.

However, the discrepancy between the different in vitro results could be a consequence of distinct substrate or proteasome purification for the degradation assay. Nevertheless, Pratt et al. showed by MS that activation of the proteasome by the mutant proteasome activator PA28γ (K188E) appears to improve in vitro degradation of peptides containing 10 glutamines with cleavage after each of the 10 glutamines, indicating that the proteasome is capable of digesting short polyQ sequences [27]. Our MS data together with the western blot analysis of the in vitro degraded Htt-exon1 gives a first glimpse how the proteasome may mechanistically degrade Htt fragments independent of the length of the polyQ stretch. We identified N- and C-terminal Htt peptides that appear to be cleaved off from the polyQ/polyP tract as initial cleavage products, and in addition peptides resulting from C-terminal single amino acid trimming of these Htt cleavage products, suggesting a subsequent shortening of the intermediate Htt peptides (Table 1). Since proteasomal cleavage products consisting of only glutamine or proline residues would be underestimated by mass spectrometry due to their poor ionization pure polyQ peptides cannot be identified by mass spectrometry. Therefore, additional western blot analysis with the specific polyQ antibody clearly reveals a complete degradation of the polyQ tract by the proteasome but not by protease K, which cannot cleave after glutamine residues.

Although cellular Htt-exon1-97Q is aggregation-prone and forms Htt-positive IBs in Neuro-2a cells, no additional mHtt-exon1 aggregation occurred upon purification and during the incubation time of several hours at 37°C, indicating that this purified protein mainly persists in a probably monomeric unfolded state suitable for processing by the open-gated 20S proteasome. A reason for the maintenance of the mHtt native monomeric form might be explained by the mHtt-exon1 co-purified proteins identified by MS. Our data show that mHtt-exon1 is associated with a number of chaperones that may keep purified mHtt-exon1 in a monomeric state (Table 2). We identified HSPA1 (HSP70), HSPAB (HSC70), HSPA9 (mHSP70), HSP90 and subunits of the chaperonin TRIC (CCT) in the purified Htt-exon1 protein solution. Molecular chaperones are important modulators of polyQ-expanded protein aggregation in the cell and several reports evaluated the effects of chaperone function on polyQ aggregation and toxicity in vivo and in vitro [60-64]. Overexpression of HSP70 suppresses polyQ-induced neuropathology in a Spinocerebellar ataxia 1 (SCA1) mouse model and in a SCA3 fly model [65], and in vitro experiments demonstrated that HSP70 and its co-chaperone HSP40 suppressed the assembly of mHtt into amyloid-like fibrils [61]. Furthermore, a recent study reported that mHtt interacts with HSP90 and cell treatment with a selective HSP90 inhibitor enhanced mHtt clearance by the UPS [66]. The role of TRIC in polyQ aggregation was addressed showing that the ring-shaped, hetero-oligomeric chaperonin TRIC inhibits mHtt-exon1 aggregation in yeast and in cell culture [67, 68]. In a follow up study, Tam et al. reported that TRIC binds to the N17 domain of Htt thereby stabilizing the monomeric conformation by acting as a ‘cap’ preventing aggregation [69]. The latter might be a reasonable explanation why purified mHtt-exon1 from Neuro-2a cells is kept in a soluble monomeric form.

Although mHtt-exon1 is polyubiquitinated within the cell, this post-translational modification is not a sufficient signal for fast proteasomal destruction when compared to the mHtt-exon1 with an N-degron signal that is targeted for proteasomal clearance via the K48-ubiquitin-linkage [34]. Since the level of polyubiquitinated mHtt-exon1 without a specific degron signal is not increased after proteasomal inhibition, this suggests that either the ubiquitin-linkage pattern is different compared to short-lived Htt-exon1, or that mHtt-exon1 mainly becomes polyubiquitinated upon aggregation, and this Htt species cannot be cleared by the proteasome (manuscript in preparation). Previously, a selective degradation of phosphorylated Htt was proposed, which involved both the lysosomal and proteasomal pathway [15]. Overexpression of the kinase IKK increased phosphorylation and reduced polyubiquitination of Htt-exon1 in transfected S114A cells, indicating that phosphorylation of the Htt N-terminus influences the post-translational modifications of neighbouring lysine residues, such as ubiquitination, and consequently the half-life of Htt.
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

To avoid undesired posttranslational modifications independent of Htt-exon1, we expressed an Htt-exon1 construct with a stop codon after amino acid 90 with no additional tag for immunoblotting, and for imaging of IBs and quantification we made use of a short tetracysteine tag (C4-tag) instead of a GFP fusion protein. This tag is lacking lysine residues for putative additional ubiquitination compared to GFP to exclude an impact of GFP posttranslational modifications on Htt clearance since it was shown that Ub-R-GFP as a proteasomal reporter is ubiquitinated after removal of the N-terminal ubiquitin moiety and rapidly degraded by the proteasome [33, 70].

In the aggregation process from soluble monomeric Htt to large IBs a variety of intermediate species have been described [71, 72]. The role of the different Htt species in the HD pathology is controversial, as a protective role for IBs has been suggested in striatal cells transfected with Htt by sequestration of toxic soluble mutant Htt species and thereby reducing neuronal death [73]. Whether monomeric or oligomeric forms represent toxic species is currently still under debate, although evidence supports a more toxic role for soluble polyQ oligomers than polyQ monomers [74]. However, there is a strong correlation between detection of mutant Htt monomers and small oligomers by the polyQ antibody 3B5H10, and prediction of neuronal toxicity [75]. Consequently, decreasing the level of monomeric mHtt by accelerating the mHtt proteasomal degradation obviates the accumulation of toxic species within the cell and represents a beneficial therapeutic strategy for HD.

MATERIALS AND METHODS

Constructs. Htt-exon1-97Q constructs with C-terminal sequences encoding the CL1 degron and the C-terminus of the mouse ornithine decarboxylase (MODC), respectively, were generated by introducing dsDNA oligos with a final stop codon. For the generation of the Ub-R-KK-Htt-exon1-97Q construct the N-terminal degron signal sequence was cut out as a XhoI/NcoI fragment followed by klenow fill from the plasmid RISQ (kindly provided by M.D. Kaytor, Emory University, USA) [76] and cloned into a 5′XhoI site after blunt ending the vector containing an Htt-exon1-97Q sequence. The N-terminal degron signal sequence from the lacI gene encodes an ubiquitin followed by a stretch of 40 amino acids consisting of two lysines for polyubiquitination as a target signal for the proteasomal degradation [77]. The N-terminal ubiquitin is removed by cellular ubiquitinases with a subsequent exposure of a stabilizing amino acid (R). Ub-R-KK-Htt-exon1-97Q-C4 was generated by introducing a tetracysteine C4-tag sequence (FLNCCP-GCCMEP) with a stop codon in a 3′BamHI site. Htt-exon1-25/97Q-H4 constructs were generated by cloning the Htt-exon1-25/97Q sequence with a 5′XhoI and 3′BamHI site into a vector encoding a C-terminal H4-tag (MA-Hi5-His-HA, kindly provided by J. Steffan, University of California, USA). The Htt-exon1-25Q-GFP construct was kindly provided by R. Kopito (Stanford University, USA). GFP-Ub, GFP-Ub-G112 and Ub-G112 were generated as described previously [23].Ub-G76V-GFP was a kindly provided by N. Dantuma (Karolinska Institute, Stockholm, Sweden).

Cell culture, transfection and electroporation. Atg5+/+ MEF, Atg5−/− MEF cells (kindly provided by N. Mizushima, Tokyo Medical and Dental University, Japan), Tpp1+/− MEF cells (kindly provided by K. Rock, University of Massachusetts Medical School, USA) and Neuro-2a cells were maintained in DMEM (Invitrogen) supplemented with 10 % fetal calf serum, 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5 % atmospheric CO₂. Neuro-2a cells were transfected with PEI (poly-ethylenimine) according to the manufacturer’s instructions (Polysciences Europe). Atg5+/− MEF, Atg5−/− MEF and Tpp1+/− MEF cells were electroporated with DNA constructs using the Neon Electroporation System (Invitrogen) according to the manufacturer’s instructions. For inhibitor studies cells were treated 6 hours after transfection or electroporation with various inhibitors for additional 16 hours: 5 mM 3-MA (3-methyladenine), 50 nM Epoxomicin, 0.5 μM Bestatin, 100 μM 1,10 Phenanthroline (all purchased from Sigma-Aldrich), 1 μM PAQ22 (SOPACHEM) or additional 4 hours: 200 nM Bafilomycin A1 (BafA1, Sigma-Aldrich), and DMSO as control. Loss of Neuro-2a cell membrane integrity was detected by uptake of propidium iodide (PI, Sigma-Aldrich). Transfected cells were cultured for 48 hours in total and for the last 4 hours treated with 500 nM Staurosporine (Sigma-Aldrich) prior staining cells with PI (5 μg/ml) and Hoechst 33342 (5 μg/ml, Invitrogen). Quantification of percent PI-positive cells was performed by fluorescent microscopy, where a minimum of three fields per condition were counted.

Soluble and insoluble fractionation. Cells were harvested and lysed in 1x TEX buffer (70 mM Tris/HCl pH 6.8, 1,5 % SDS, 20 % glycerol). After sonification (Soniprep150, Sanyo) 50 mM DTT was added fresh and samples were centrifuged at 14.000 rpm at RT. The pelleted SDS insoluble fraction was incubated with 100 % formic acid at 37°C for 40 min followed by evaporation of the formic acid using a speedvac system (Eppendorf). 1x TEX buffer supplemented with 0.05 % bromophenol blue was added to the pellet and the soluble and insoluble fractions were loaded on a SDS-PAGE gel.

Proteasome activity labeling. Cells were harvested in TSDG buffer (10 mM Tris/HCl pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA and 8 % glycerol) and lysed by three consecutive freeze-thaw cycles with liquid nitrogen. After high speed centrifugation (15 min at 14.000 rpm and 4°C) the total protein concentration of the clarified lysate was determined by a Bradford protein assay. Proteasomes were labeled in the cell lysate with 0.5 μM activity-based probe Bodipy-epoxomicin for 1 hour at 37°C (kindly provided by H. Overkleeft, Institute of Chemistry, Leiden, The Netherlands) [78]. Sample loading buffer was added to 10 μg lysate and the samples were boiled for 3 minutes and loaded on a 12.5 % SDS gel with subsequent in-gel fluorescence imaging using a Typhoon imager (GE Healthcare) with the S808BP30 filter to detect the Bodipy-epoxomicin probe.

Western blot analysis and filtertrap assay. Cells were harvested in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton-X100, 20mM NEM, supplemented with complete mini protease inhibitor cocktail (Roche)). Total cell lysates were boiled for 10 min at 99°C with 1x laemml sample loading buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % glycerol, 6 % β-mercaptoethanol, brom phenol blue) fractionated by SDS-PAGE gel electrophoresis and transferred to a PVDF

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102

103
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

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membrane (0.45 μm pore size, Schleicher & Schuell). Western blot membranes were blocked with 5 % milk, incubated with primary antibodies anti-Htt 1C2 (1:1000, Millipore, MAB1574), anti-polyQ (1:1000, Sigma-Aldrich 3858H10), anti-Htt N18 (1:1000, Enzo, BML- PW0595-0100), anti-HA (1:1000, Sigma-Aldrich, H3663), polyclonal rabbit anti-GFP (1:1000, kindly provided by J. Neefjes, NKI, The Netherlands), anti-α-actin (1:1000, Santa Cruz, SC-130656), anti-alpha2 (1:1000, abcam, ab22666), anti-p62 (1:500, Clonagen), anti-LC3 (1:500, Abcam, 48394) and anti-ubiquitin (1:100, Sigma-Aldrich, U5379), and subsequently incubated with secondary antibodies IRDye 680 or IRDye 800 (1:10.000; LI-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (LiCor). Filtertraps assay was performed with the pellet obtained after high speed centrifugation of the cell lysate (15 min at 14.000 rpm at 4°C). Pellet with aggregates was resuspended in benzonase buffer (1 mM MgCl2, 50 mM Tris/HCl pH 8.0) and incubated for 1 hour at 37°C with 125 U Benzonase (Merck). Reaction was stopped by adding 2x termination buffer (40 mM EDTA, 4 % SDS, 100 mM DTT fresh). Samples with 50 μg protein extract diluted in 2 % SDS buffer were filtered through a 0.2 μm pore size cellulose acetate membrane (Schleicher & Schuell), pre-equilibrated in 2 % SDS wash buffer (2 % SDS, 150 mM NaCl, 10 mM Tris/HCl pH 8.0) and spotted on the membrane in doublets. Filters were washed twice with 0.1 % SDS buffer (0.1 % SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and blocked with 5 % milk for further treatment like western blot membranes.

ReASh staining. For imaging, cells were seeded on coverslips and transfected with the plasmids. Cells were cultured for 24 hours, rinsed with pre-warmed 1x PBS buffer and labeled for 30 min with pre-warmed DMEM containing 1 μM ReASh (kindly provided by H. Overkleeft, University Leiden, The Netherlands) and 10 μM 1,2-ethanediol EDT (Sigma-Aldrich) at 37°C. After staining cells were washed 5 times for 15 min each washing step in pre-warmed DMEM containing 1 mM EDT and 10 % FCS. Finally, cells were washed twice with 1x PBS, fixed with 4 % PFA solution and mounted on objective glasses using Vectashield (Vector laboratories).

Fluorescence microscopy. Cells were transfected or electroporated with the indicated DNA constructs 24-48 hours prior to imaging and fluorescent cells were scored for the percent of cells with aggregates and the percent PI-positive cells using an inverted fluorescence microscope (Leica DMR). For imaging, transfected cells were fixed on coverslips and images were obtained using a confocal microscope equipped with an Ar/Kr laser and a 63x objective (Leica TCS SP2).

Htt protein and 205 proteasome purification. Neuro-2a cells were transfected with the Htt exon1-25/97Q-H4 construct and 48 hours after expression harvested in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton-X100, 20mM NEM, supplemented with complete mini protease inhibitor cocktail (Roche). Centrifugation at 14,000 rpm at 4 C for 15 min was performed to remove cell debris and protein aggregates. Cell lysates were ultracentrifuged for 2 hours using EView Red Anti-HA Affinity Gel (Sigma-Aldrich) to purify Htt-exon1-25/97Q-H4. Proteins were eluted for 5 min at RT with 0.1 M Glycine pH 2.5 and immediately neutralised with 1.5 M Tris/HCl pH 8.8 to avoid deamidation of proteins. The protein eluate was dialysed against 50 mM Tris/HCl pH 7.5 for 2 hours at 4 C with an additional exchange of buffer after one hour.

205 proteasomes (kindly provided by B. Dahllmann, Charité, Germany) were purified from human erythrocytes as described previously [79].

In vitro degradation assays. To analyse the in vitro proteasomal degradation of purified Htt-exon1-25/97Q-H4 protein by the 20S mammalian proteasome 50 pmol purified Htt protein was incubated with 1.4 pmol mammalian 205 proteasomes in 1x 205 buffer (10 mM Tris/HCl pH 7.4, 30 mM NaCl, 1 mM MgCl2, 400 μM fresh DTT) in a total volume of 15 μl treated with or without 40 μM MG132 for 37 C for the indicated time points. Reactions were stopped by adding 6x sample loading buffer. Aggregated Htt was spun down at 14,000 rpm for 15 min and treated as an insoluble fraction. To assess the effect of the inhibitors 3-MA and Epoxomicin on the enzymatic activity of the proteasome in Neuro-2a cells 5 mM 3MA, 50 mM Epoxomicin and DMSO as control were added to living Neuro-2a cells, respectively, incubated for 16 hours and subsequent lysed in KMH buffer (110 mM KAc, 2 mM MgAc and 20 mM HEPES, pH 7.2) containing 100 μM digitonin. The cytoplasmic fraction was obtained after centrifugation (15 min at 14,000 rpm) and protein concentration was determined by Bradford protein assay. The assay was performed with 30 μg total cytoplasmic protein and fluorogenic substrate with an end-concentration of 100 μM Suc-LVY-AMC, 150 μM Ac-RLR-AMC and 150 μM Ac-GPLD-AMC (all purchased from Enzo). To assess the effects of purified Htt proteins with a normal and an expanded polyQ stretch on the enzymatic activity of purified mammalian 205 proteasome in vitro chymotrypsin-like activity of the proteasome was monitored by Suc-LVY-AMC digestion after incubation of 1.4 pmol proteasomes with 50 pmol purified wt or mHtt treated with or without 40 μM MG132 for 16 hours at 37°C. Experiments were performed in triplicates and generation of free AMC was measured at 37°C with the spectro-photometric plate reader FLUOstar Optima (BMG Labtec.).

In vitro transcription and translation. Htt proteins were transcribed and translated in vitro with a TNT T7-coupled reticulocyte lysate system according to the manufacturer’s instructions (Promega). The in vitro protein expression level and protein degradation by the proteasome was determined by adding 100 μM MG132 to reticulocyte cell lysate prior protein expression and subsequent western blot analysis.

Proteinase K treatment. Purified mHtt protein (1 μg) was incubated with 100 μg/ml proteinase K (Invitrogen) at 37 C in 50 mM Tris/HCl pH 7.4 reaction buffer for the indicated time points. After digest aggregates were captured by centrifugation (14,000 rpm for 15 min) and solubilized by formic acid (insoluble fraction). The proteinase K digest was stopped by adding 6x sample loading buffer and proteins were subjected to SDS-PAGE.

Mass spectrometry. Reactions of purified Htt-exon1-25/97Q-H4 with mammalian 205 proteasomes after an incubation time of 16 hours at 37 C were analysed for Htt peptide generation. As control Htt-exon1-25/97Q-H4 only and mammalian 205 proteasomes only were analysed. A <3kDa peptide pool fraction was separated using an Amicon Ultra-0.5 ml Centrifugal Filter 3K device (Millipore) for subsequent mass spectrometry analysis. After adjusting the pH of the <3kDa fractions to 3.0 (using 10 % trifluoroacetic acid (TFA)), samples were desalted via C18-Stage Tip purification as described previously [79].
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

Statistical analysis. All values were obtained from three independent repeated experiments and expressed as mean ± SD. Statistical analysis was performed using Student’s t-test. p < 0.05 was considered statistically significant.

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[80]. Peptide samples were analysed by B. Florea (University of Leiden, The Netherlands) on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo). The column was packed with BioSphere C18 5 µm 120 Å from Nanoseparations (Nieuwkoop, The Netherlands). Instrument settings and measuring parameters were used as described by Florea et al [78]. The resulting raw data were analysed by MaxQuant software (version 1.2.2.5, [81]. Statistical analysis was performed using Andromeda search algorithm against a manually generated fasta-file containing the sequence of Htt-exon1-25/97Q-H4. Search parameters: Enzyme: Trypsin, max. miscleavages: 40. To determine Htt-exon1-97Q·H4 and 20S proteasome co-precipitated proteins, the 3 kDa filter supernatant (≥3kDa fraction) was diluted in 100 µL 50 mM NH4HCO3 (ABC) buffer to 3.0. Samples were analysed using the same protocol as for the <3kDa fractions. Search was performed instead against ipi.HUMAN.v3.68 database. Search parameters: Enzyme: Trypsin, max. miscleavages: 2.
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ENLIGHTENING PROTEASOMES: METHODS TO VISUALIZE THEIR INTRACELLULAR DISTRIBUTION, ACTIVITY AND INTERACTIONS

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SIX Enlightening Proteasomes: Methods to Visualize their Intracellular Distribution, Activity and Interactions

ABSTRACT

The ubiquitin proteasome pathway is the major protein degradation pathway in the cell responsible for turnover of short-lived and misfolded proteins. The proteasome is involved in many cellular processes and malfunctioning is linked with various diseases. Its activity can be modified by exchanging catalytic subunits or by the interaction with various activators. We have established methods to study proteasome functioning in the cell, using non-invasive fluorescently-tagged proteasome subunits that are incorporated in mature complexes, proteasome activity probes and small fluorophores. These fluorescent tools are used in microscopy and biochemistry to study proteasome localization, activity and interactions with activators.

INTRODUCTION

The proteasome is the main protease of the cell and is essential for many cellular processes. Besides clearing damaged, misfolded and aged proteins in order to maintain homeostasis, the proteasome also has an important regulatory function in various cellular processes such as transcription and cell cycle control [1-6]. Proper proteasomal function is thus essential for cellular vitality [7]. The proteasome is also a key player in antigen processing, as it generates peptides which are further processed for antigen presentation or even by splicing, thereby contributing directly to antigen presentation by MHC class I and the immune responses [8, 9]. Due to their involvement in multiple pathways, it is not surprising that proteasomes play a role in the pathogenesis of many diseases, either as primary cause or as a secondary response [5, 10-12]. Additionally, proteasome inhibitors have shown to be effective as therapeutic agents against cancer and stroke and as immune regulator [13-15].

Intracellular distribution of proteasomes and their activity are often studied using fluorescently-tagged proteasome subunits and proteasome substrates, respectively [16]. Fluorescently-tagged proteasomes can be applied to living cells, which enables the study of proteasome dynamics and intracellular distributions in cells. However, their multi-subunit complexity that is also modified under various physiological conditions has to be taken into account. The basic core complex, called the 20S proteasome, is a cylindrical shaped complex of four stacked rings, each containing 7 different subunits [17, 18]. The two outer rings are each composed of seven α-subunits (α1-α7 or PSMA1-7) which form the backbone during complex formation [19]. These α-subunits regulate substrate delivery as their hydrophobic loops close the 20S barrel and prevent random entry of substrates. Protein entry can only be established after gate opening by proteasome activators such as the 19S cap or PA28, after which substrates can enter the interior of the 20S core for degradation [20]. Activation of proteasomes for substrate entry is predominantly executed by the 19S cap, which recognizes, unfolds and shuttles protein substrates into the 20S core [21, 22]. The inner two rings of the 20S barrel consist of the subunits β1-β7 (PSMB1-7), with each β-ring including the 3 catalytic subunits β1, β2 and β5. When complex formation is completed, the pro-peptides of these catalytic β-subunits are auto-catalytically removed to expose an N-terminal threonine residue as reactive nucleophile that can cleave peptide bonds within substrate proteins inside the 20S barrel [18, 23, 24]. These subunits have distinct activities, with caspase activity or peptidyl-glutamyl peptidase-like activity for β1 that prefers cleavage behind acidic residues, trypsin-like activity for β2 that prefers cleavage behind basic residues and chymotrypsin-like activity for β5 that prefers cleavage behind hydrophobic residues [25, 26].

Proteasomal activity is altered by incorporation of alternative catalytic subunits that are induced by cytokines such as interferon γ (IFNγ). IFNγ induces expression of various components of the MHC class I pathway, including the transcription of immunosubunits that are incorporated in so-called immunoproteasome [27-29]. After exposure to IFNγ, the 3 catalytic immunosubunits β1i (LMP2), β2i (MECL-1) and β5i (LMP7) replace the constitutive subunits β1, β2 and β5, respectively, in de novo proteasomes. Thereby changing the general cleavage pattern of substrates and generating different epitopes for antigen presentation [30-33]. In addition to immunosubunits, IFNγ also induces the proteasome activator PA28αβ. Activation by PA28αβ specifically enhances production of peptides with a sufficient length for MHC class I presentation and increases the efficiency of the proteasome to generate these peptides [35-37]. Importantly, the immunoproteasome not only encounters intracellular infections when induced by IFNγ, but is also induced upon oxidative stress for a broader role in cellular homeostasis [38-41].

Changes in proteasomal conformation, intracellular localization and activity provide invaluable information to understand proteasomal functioning in (patho)physiological intracellular processes. We show various methods to determine the efficiency of incorporation of GFP-tagged α- and β-subunits into proteasomal complexes, using native PAGE and photobleaching experiments. We also describe methods to visualize intracellular distribution patterns of proteasomes and determine changes in proteasome activity in intact cells, cell lysates and upon electrophoresis. Finally, we describe methods to analyze interactions between the 20S core and PA28 using FLIM.

RESULTS

Visualizing the proteasome using fluorescent tags

Analysis of incorporation efficiency of fluorescently-tagged proteasomal subunits. Noninvasive tags such as green fluorescent protein (GFP) have enabled visualization of proteasomal subunits to study proteasomal distribution and dynamics in living cells. When fluorescently-labeled proteasomes are studied in cells it is important to ensure that the tagged subunits are efficiently incorporated in the proteasomal complex because large fractions of fast-diffusing, non-incorporated fluorescent subunits interfere with distribution and kinetic studies. GFP-tagged β1i was the first fluorescently-labeled subunit to be incorporated in the proteasome [42, 43]. Afterwards, several other fluorescent subunits were used to visualize proteasomes in cells [16]. Incorporation of subunits in proteasomal...
complexes can be determined by immunoprecipitation using antibodies against different subunits to pull down proteasome complexes, and the determination of the remaining pool of fluorescent subunits that were not incorporated in these complexes. Alternatively, centrifugation of sucrose gradients, and subsequent immunoblotting of GFP can be used to separate non-incorporated GFP-tagged subunits from assembled proteasome complexes [42, 43]. A relatively simple technique is complex separation by native gradient PAGE and can be applied to visualize incorporation of fluorescent proteins like GFP. Since proteins are not denatured upon electrophoresis they can therefore be visualized directly.

To determine differences in incorporation efficiencies between various GFP-tagged α- and β-subunits of the 20S proteasome, we used C-terminal tagged α3-GFP (PSMA4), α7-GFP (PSMA3), β7-GFP (PSMA4), β3i-GFP (LMP2 or PSMB9), β5i-GFP (LMP7 or PSMB8) and β2i-GFP (MECL-1 or PSMB10) (Fig. 1). Only the exposed C-terminus of these subunits is suitable for tagging since the N-terminus of the α-subunits is involved in gating of the 20S barrel, whereas the N-termini of the catalytically-active β-subunits possess the catalytic nucleophile after removal of the pro-sequence [19]. All tagged subunits were expressed and were incorporated into proteasomal complexes, but their incorporation efficiencies differ. Following transient transfection of HeLa cells for 24 or 48 hours with the various GFP-tagged subunits, cell lysates were subjected to a 4-12 % gradient native gel in order to separate proteasomal complexes from non-incorporated subunits or pre-mature inactive complexes. Note that large complexes, such as the 19S-capped 26S proteasome, are difficult to pull down proteasome complexes, and the determination of the remaining pool of fluorescent subunits that were not incorporated in these complexes. Alternatively, centrifugation of sucrose gradients, and subsequent immunoblotting of GFP can be used to separate non-incorporated GFP-tagged subunits from assembled proteasome complexes [42, 43]. A relatively simple technique is complex separation by native gradient PAGE and can be applied to visualize incorporation of fluorescent proteins like GFP. Since proteins are not denatured upon electrophoresis they can therefore be visualized directly.

This data shows that GFP fluorescence does not necessarily represent intracellular distribution of active proteasomes, since the fluorescent pre-complexes do not represent mature active proteasomes. Various studies have used β3i-GFP to determine proteasome localization since it was shown that this subunit is incorporated into active proteasomes. However, at 48 hours of expression, half of the fluorescent β3i-GFP is present in pre-complexes. Stable expression of subunits does not guarantee efficient incorporation into active proteasomal complexes either, since the production of new subunits generates a background signal. However, prolonged expression and lower expression levels improve incorporation into complete proteasome complexes. This data shows that visualization of β3i is the most representative for mature proteasomes. The efficient incorporation of β3i may be explained by the strong interaction with the proteasome maturation protein (POMP), a chaperone in proteasome assembly [30, 44]. Alternatively, α7-GFP shows efficient incorporation and can be used as well.

Photobleaching techniques to visualize proteasome complex formation. GFP and related fluorophores are used to visualize distribution patterns of a protein of interest in living or fixed cells. Photobleaching techniques can be used to study the dynamics of fluorescent proteins in cells. Depletion of fluorescence in a selected intracellular region and the fluorescence recovery afterwards, provides information on the mobility of tagged proteins. The most frequently used technique is detection of fluorescence recovery after photobleaching (FRAP), where a small region is briefly illuminated with high laserpower and the recovery of fluorescence is monitored in time [45, 46]. While irreversibly photobleached proteins diffuse out of the monitored region, mobile fluorescent proteins from surrounding regions diffuse into the bleached area. The rate and level of recovery are directly linked to the diffusion speed and mobility of the proteins, respectively. Alternatively, a region of the cell can be photobleached for a prolonged period, and in time the fluorescence loss in photobleaching (FLIP) can be monitored in another region of the cell in time. This provides information on the accessibility between these two regions for proteins, for example due to diffusion between nucleus and cytoplasm, or between different organelles.

When studying proteasomal complex formation, the nuclear pore complex can be used as a molecular sieve to distinguish large proteasomal complexes from smaller pre-complexes. The nuclear pore complexes allow free diffusion of proteins up to 60-110 kDa and prevents passive
Fig 2. Incorporation of GFP-tagged proteasomal subunits. (A) Efficiency of subunit incorporation at different timepoints after transfection. Various GFP-tagged α- and β-subunits were transiently expressed for 24 h or 48 h to visualize incorporation into 20S complexes. Proteasomal complexes were separated from smaller complexes and subunits on 4-12% gradient native gels. GFP-labeled subunits were identified by fluorescent scanning of the wet gel slab (left panel) and proteasome complexes were identified by blotting for the α2-subunit after transfer to a nitrocellulose membrane (right panel). The various subunits showed differences in incorporation, and longer exposure increased incorporation. (B) Incorporation of transient and stably expressed proteasome subunits. The α-subunits α3-GFP and α7-GFP were expressed in U2OS cells and β1i and β7i were expressed in HeLa cells, either stably or transiently for 24 h and 96 h. GFP-tagged subunits were identified using fluorescence scanning and 20S complexes were detected by blotting with anti-α2 antibodies. When compared to transient expression for 96 h, stable expression of subunits did not further improve incorporation. (C) Fluorescence loss in photobleaching (FLIP) to distinguish non-incorporated from incorporated GFP-tagged subunits. By photobleaching the entire cytoplasm also the small GFP-tagged subunits that freely diffuse between the nucleus and cytoplasm are photobleached, while GFP-tagged proteasomes in the nucleus remain fluorescent. (D) Quantification of GFP-tagged proteasome subunits that remain in the nucleus upon photobleaching the cytoplasm. Cells either transiently transfected for 48 h or stably transfected with the indicated constructs were analyzed for free diffusion of non-incorporated subunits by photobleaching the entire cytoplasm, and quantifying the decrease in fluorescence in the nucleus. The percentage shown is the remaining fluorescence in the nucleus (mean ± SD), representing large GFP-tagged proteasome complexes. Nuclear fluorescence of β1i-GFP and to a lesser extent α3-GFP decreases in time, indicating a substantial non-incorporated pool of GFP-tagged subunits. Scale bar = 5 μm.

Alternative fluorescence-labeling strategies to follow proteasome kinetics in time. While fluorophores like GFP can be efficiently used to visualize the distribution and dynamics of proteins in cells, it is more difficult to follow the dynamics of a specific pool of proteasomes that are synthesized in a defined period of time. A fluorescence pulse-chase experiment allows the labeling of a subset of proteasomes to be followed in time, like radio-active pulse-chase experiments allow following the synthesis and post-translational modifications of proteins. The development of the tetracysteine (C4) motif made it possible to tag proteins of interest with the C4 motif and label the proteins at a given time-point with cell-permeable dyes [49, 50]. The C4 motif can be genetically built in a protein and is then specifically recognized by the biresinental dyes FLiAsh and ReAsh. Upon binding to the C4-tag, these dyes become green and red fluorescent, respectively. An additional advantage of these tags is their limited size. GFP has a molecular weight of 27 kDa and the C4-tag is only 12 amino acids or 1.3 kDa, causing less interference with the biological behavior of the protein [51].

Since the C4-tagged protein can be fluorescently labeled at any specific timepoint, it can be used as a tool to study cellular dynamics. When C4-tagged proteins are expressed, proteins can be labeled with FLiAsh or ReAsh, allowing labeling of two pools of the same protein that were synthesized at different time points. Therefore, the method can be used to study protein turn-over or protein exchange at particular intracellular sites in the cell, such as gap junctions [52] or intracellular aggregates that recruit proteins including proteasomes (manuscript submitted).
Aggregation induced by polyglutamine (polyQ) expanded fragments in cells is a hallmark of Huntington’s disease [53]. Proteasomes are recruited into these aggregates and FRAP analysis suggested that these proteasomes are irreversibly present in aggregates [54]. However, imaging FRAP usually is a relatively brief procedure, which does not allow the detection of slow dynamics. We examined whether proteasomes are indeed irreversibly recruited into aggregates, similarly to the sequestration of the aggregation-prone polyQ peptides themselves. We expressed C4-tagged polyQ99 peptides which were subsequently labeled with ReAsH. Following a chase of 8 or 20 h, newly synthesized Q99-C4 peptides were labeled with FlAsH (Fig. 3A). Confocal imaging showed a sequential recruitment into aggregates, with the initial ReAsH-labeled Q99-C4 being present in the aggregate (red) and the newly synthesized FlAsH-labeled pool sequestered around the aggregate (green). To study the dynamics of proteasome recruitment into polyQ aggregates, we expressed untagged Q99 fragments to induce aggregation and co-expressed the proteasomal subunit β7-C4. After similar staining conditions, a reversible recruitment of proteasomes into aggregates was observed since the old and newly-synthesized pool of proteasomes exchanged and remained at the outer shell of the aggregate (Fig. 3B).

Specific proteasome labeling by both ReAsH and FlAsH was confirmed by native gel analysis, showing that the FlAsH- and ReAsH-labeled proteins run at the same height as proteasomal complexes that are immunostained by an antibody against the proteasomal α2-subunits (Fig. 3C). Fluorescent proteasome labeling was absent when cycloheximide was added during the chase period. This example illustrates the benefits of the C4 tag when performing fluorescent pulse-chase experiments, including recruitment of proteasome complexes to specific intracellular processes and the turnover of proteasome complexes. In addition, these dyes can be used for both living cell analysis, especially when proteasome dynamics are too slow to visualize using FRAP analysis, and in vitro analysis by comparing different pools of proteasomes on gel.

Visualizing proteasome activity

Fluorophores such as GFP and FlAsH are ideal to visualize proteasome distribution and dynamics, but cannot demonstrate activity of proteasomes. Changes in proteasomal activity are related to various diseases. Increased proteasomal activity was shown in muscle wasting diseases, while downregulation of proteasomal activity was observed in a wide range of diseases, particularly in neurodegenerative diseases [11, 55-57]. Proteasomal activity also decreases during aging, which may contribute to various late onset disorders [58, 59]. In addition, changes in proteasome composition can induce changes in activity, like the incorporation of catalytically-active immunosubunits induced by cytokines. Both ubiquitin-independent fluorogenic peptides and ubiquitin-dependent fluorescent reporters have been valuable tools in studies of proteasomal activity, but cannot be used to visualize the intracellular localization of active proteasomes [60-63]. The more recently developed activity-based probes (ABPs) are often used to detect alterations in proteasomal activity in gels and can also be used to visualize proteasome activity in living cells [64-66]. ABPs are small molecules that consist of a proteasome inhibitor linked to a small fluorophore. Fluorescence labeling of proteasomes occurs through the nucleophilic attack of the proteasomal threonine that binds the inhibitor, leading to a covalent bond between the labeled warhead of the ABP and the proteasomal active site (Fig. 4A). Unlike fluorescently-labeled tags such as GFP, these ABPs only label completely assembled and active proteasomal complexes. Here we show various methods to determine proteasome activity by using ABP labeling in living cells, in lysates or in gels.
Proteasomal activity labeling in living cells. We used two types of proteasomal activity probes which have been described for the detection of alterations in proteasomal activity. The first type of probe has a vinyl sulphone warhead coupled to a Bodipy fluorophore, which we will refer to as ABP1 (green fluorophore) and ABP3 (red fluorophore) [67, 68]. The second activity probe has an epoxomicin-based warhead that is also labeled with the Bodipy fluorophore and was recently used to identify β5t, a proteasomal subunit present in the thymus [66, 69]. This probe is further referred to as ABP2 (green fluorophore) and ABP4 (red fluorophore). To visualize and compare their distribution in living cells, we incubated U2OS cells with the four probes, ABP1-4, and compared activity labeling by confocal microscopy (Fig. 4B). All ABPs showed a proteasomal distribution pattern similar to GFP-tagged proteasomes, but some nonspecific perinuclear staining was observed as well, which remained present when proteasomes were inhibited by epoxomycin (Fig 4B). Both ABP1 and ABP3 showed vesicular staining, which was much less apparent with ABP2, whereas ABP4 showed endoplasmic reticulum background staining. When cells were incubated with the cytokine IFNγ in order to induce expression of immunoproteasomes, no obvious changes in active proteasome distribution patterns were observed (Fig. 4B). Quantification of proteasome activity upon IFNγ treatment showed that only ABP2 labeling increases significantly in fluorescence levels from 51.2 ± 2 arbitrary units (a.u.) to 93 ± 1.9 a.u. in IFNγ-treated cells (average ± SEM, n=30).

The various ABPs can be used to visualize intracellular proteasomal activity, but can also be used to examine recruitment of active proteasomes to particular sites in cells, such as DNA repair foci or protein misfolding-aggregates. Various studies have proposed that proteasomes recruited into aggregates become clogged, for example by the polyQ fragments that initiate aggregation. When cells were transfected with polyQ-expanded mCherry-Huntingtin (Q74), which initiates aggregation, and GFP-β5i to visualize proteasomal distribution patterns, recruitment into aggregates by fluorescently-tagged proteasomes was detected (Fig. 4C). Similarly, when cells were transfected with mCherry-Huntingtin (Q74) followed by incubation with ABP2, activity labeling was also present in the aggregates with a similar localization as β5i-GFP in the aggregates (Fig. 4C). This example demonstrates the use of ABPs in combination with fluorescently-tagged proteasomes to compare proteasomal distribution and activity under specific experimental conditions.

Proteasome activity labeling in cell lysates. The various ABPs can also be used to detect proteasome activity in gels, either by adding the ABP to living cells prior to cell lysis or to lysates, although differences in labeling occur between the two methods [67]. The activity of the three ABP-labeled β-subunits can then be visualized and quantified by scanning the wet gel slab for fluorescence. Lysates of cells cultured in the presence or absence of IFNγ were labeled with the four different ABPs and subsequently analyzed for differences in labeling on SDS-PAGE gels (Fig. 5A). In contrast to ABP1 and ABP3 that labeled all three catalytic sites equally, ABP2 and ABP4 preferred labeling of the β5 active site which is characteristic for epoxomicin [70]. None of the probes distinguishes β1 and β5 from their immunosubunit counterparts β1i and β5i, which run at identical heights.

While analysis of proteasome activity by SDS-PAGE is an easy method to visualize the overall activity of individual subunits, this method has two drawbacks: a shift in activities from β1 and activity of individual subunits, this method has two drawbacks: a shift in activities from β1 and


**Fig. 5. Proteasome activity labeling in cell lysates.**

(A) Visualizing proteasome activity on SDS-PAGE. HEK293 cells were grown in the presence or absence of IFNγ to induce immunosubunits. After 72 h cells were harvested, cell lysates were incubated with 0.5 μM of the activity probes, subjected to a 12 % SDS-PAGE gel and scanned for fluorescence. The vinyl sulfone based probes ABP1 and ABP3 give a better read-out for the activity of all 3 constitutive catalytic sites. (B, C) Activity labeling of individual subunits versus proteasome complexes. Control cell lysates, lysates pre-incubated with MG132 and lysates of cells overexpressing PA28ββ were incubated with ABP4 and loaded on SDS-PAGE (left) or 3-12 % native gels (middle and right). Wet gel slabs were scanned for activity labeling and intensities were determined using AlphaEase software. After transfer to membranes, anti-α2 antibodies were used to identify proteasome complexes and PA28αβ antibodies were used to show PA28 over-expression. Expression of PA28ββ induced a shift in proteasome activity towards PA28-capped proteasomes. Increases in proteasome activity was more profound when measuring individual complexes than measuring activity changes of individual subunits. (N=3, mean ± SEM, two tailed unpaired t-test; **p<0.01; ***p<0.001). (D) Visualizing activity of constitutive and immunosubunits on 2D gels. Cell lysates of control HeLa cells and IFNγ-stimulated HeLa cells were subjected to 2D analysis, showing the activities of the household and induced immunosubunits (Fig. 5D). Interestingly, most subunits show various spots which may represent post-translational modifications that affect their isoelectric point.

**Activity labeling in gels.** Proteasome activity can also be visualized and quantified using immunography, where proteasomes are separated using native-PAGE gels to keep them active as complexes. Subsequent incubation of the gel in a buffer containing ABPs or fluorogenic peptide substrates generates locally a fluorescent signal that can be quantified as a measure of activity (Fig. 6A). When using ABPs as substrates, cell lysates are pre-incubated with either ABP4, a proteasomal inhibitor, or DMSO as untreated sample. Proteasomal complexes are separated on 3-12 % gradient native gels and subsequently incubated in a ABP containing buffer (Fig. 6B). Addition of the ABP prior to electrophoresis or afterwards give different results. Addition of the ABP before electrophoresis shows all proteasomal complexes, including the 20S core complexes (Fig. 6B). Whereas addition of the ABP after electrophoresis labeled only activated complexes such as the 26S and PA28-capped proteasomes and not the latent 20S complex (Fig. 6B). This data suggests that the ABP can only enter activated proteasomal complexes, and that a fraction of the complexes dissociate during sample preparation and separation (72). When the activity of specific complexes has to be determined, it is thus more representative to label proteasomal complexes after electrophoresis than labeling prior to electrophoresis. Immunoblotting of the α2-subunit to identify the different proteasomal complexes confirmed the presence of latent 20S in all samples.

In addition to ABPs, also quenched substrates can be used in this method. These substrates include the commonly used proteasome substrates to detect the caspase, trypsin-like and chymotrypsin-like activities, but also quenched peptides with specific sequences. These quenched peptides β5 to β1 and β5i, respectively, cannot be detected and alterations in activity due to proteasome capping by 19S or PA28 are difficult to detect since all proteasomal complexes are separated into the individual active subunits. When cell lysates of non-transfected cells are compared to lysates of cells over-expressing PA28ββ, no significant increase in activity is observed upon incubation with ABP4 and analysis on SDS-PAGE (Fig. 5B). However, when the same lysates are loaded on 3-12 % native gradient gels, a shift to PA28-capped proteasome and hybrid proteasome activity could be observed, and a reduction in 20S proteasome activity was measured while the activity of double capped 26S proteasomes (30S) remained unchanged (Fig. 5B and 5C).

While separation of proteasomal complexes to distinguish activity of these specific complexes gives a better representation of the overall proteasomal activity changes, it does not solve the problem of the β-subunit activities that run at identical heights. This can be solved by using ABPs that only bind one catalytic site specifically [66]. Alternatively, all catalytic activities can be separated and visualized by 2D gel analysis. Using this method, subunits are not only separated by size, but also on the basis of differences in isoelectric points. 2D analysis has been intensely used to identify proteasomal subunits [71]. However, when the proteasomal subunits are labeled with ABPs, proteasomes do not have to be purified because only the active, incorporated subunits will appear after scanning for fluorescence. Identification of all catalytic subunits was demonstrated when control HeLa cells or IFNγ-stimulated HeLa cells were subjected to 2D analysis, showing the activities of the household and induced immunosubunits (Fig. 5D). Interestingly, most subunits show various spots which may represent post-translational modifications that affect their isoelectric point.
become fluorescent upon degradation due to separation of the quencher and fluorophore that are coupled to different residues (Fig. 6A). To examine whether proteasomes can cleave between glutamine residues, we developed a quenched peptide containing 8 glutamine residues that are flanked by a quencher and a fluorophore, and some non-degradable D-amino acids at the termini to prevent aminopeptidase activity. When cell lysates were separated on a native gel and incubated with the quenched Q8-peptides (Fig. 6C), fluorescence appeared at the same molecular weight levels as ABP labeled proteasomes. These bands were not present in the lane containing lysates pretreated with proteasome inhibitor, thus confirming specific degradation of the Q8-peptide by proteasomes. These results show that proteasomes are able to cleave within polyQ sequences, and illustrate how designed quenched peptides can be used to detect proteasome activity and specificity. Alterations in activity and cleavage specificity due to proteasomal modifications and induction of immunoproteasomes can be tested with the use of these methods.

Visualizing proteasome capping by PA28

To date, 4 different caps have been identified to activate the proteasome, the 19S cap, PA28αβ, PA28γ and PA200 [20, 72]. The 19S cap has been intensively investigated, but the exact functions of PA28 and PA200 remain elusive. Visualization of proteasomal capping by these proteasome activators allows examination of complex formation in cells, but it requires labeling of the 20S proteasomal core and the proteasome activator with different fluorophores. Importantly, tagging of both protein complexes should not interfere with their interactions. Here we describe how to optimize interaction studies between the 20S core and proteasome activators in living cells by using the PA28αβ activator cap.

20S capping by fluorescently tagged PA28αβ. The heptameric PA28αβ cap (Fig. 1) is a ring-like structure that is abundantly present in immune tissues and its expression is upregulated by IFNγ, which indicates a role in antigen processing although its function may not be limited to that. To
explore its function, studying localization of PA28αβ and the interactions with the 20S proteasome in cells may be valuable. When tagging PA28 subunits for visualization, it is important to place the fluorescent tag at the N-terminus since the C-terminus are important for 20S binding [73]. To determine whether the fluorescent GFP tag at the N-terminus interferes with either PA28 ring formation or interaction with the 20S proteasome, GFP-PA28α, GFP-PA28β, both or GFP-PA28γ were expressed in the presence or absence of ATP and complex formation was analyzed using 4-12% native gradient gels (Fig. 7A). Detection of fluorescence identified ABP labeled 20S proteasomes (Fig. 7A) and capping by PA28 was determined by co-localization of GFP fluorescence. Multimeric complexes below ABP labeled complexes suggest the formation of PA28 ring structures, while only small fractions of GFP-tagged PA28αβ, PA28α and PA28γ co-localize with active proteasomes (Fig. 7A). Indeed, PA28α alone has been reported to activate the proteasomes however with low affinity [34, 74]. Still, the fractions of GFP-tagged subunits that interact with 20S proteasomes is very low compared to the non-interacting subunits.

To test whether the relatively low levels of interactions were due to the GFP tag, we compared proteasomal capping between untagged, GFP-tagged and C4-tagged PA28, which were only one of the subunits (α or β) was tagged. Analysis of ABP labeling showed that expression of either untagged PA28α or PA28β alone did not cause a significant shift towards PA28-capped complexes, while co-expression of both untagged PA28 subunits induced a full shift from 20S to PA28-capped complexes (Fig. 7B). When using the small C4 tag, which was stained with FlAsH, a shift in proteasomal activity is observed, with most C4-tagged PA28 in complex with the proteasome (Fig. 7B). The shift in proteasomal activity is also observed with GFP-tagged PA28, although no sharp band is visible due to the formation of PA28 rings with varying numbers of GFP tags and therefore different molecular weights (Fig 7B). Both C4 and GFP can thus be used to tag PA28αβ and allow complex formation with the 20S core, although a fraction of uncapped 20S complexes remains.

Since untagged PA28αβ showed the most efficient shift in proteasome activity towards PA28-capped proteasomes, we further optimized 20S capping by transfecting different ratios of GFP-tagged and untagged PA28. This results in conditions where only one PA28 subunit in the ring structure is tagged to complexes were all PA28 subunits are tagged (Fig. 8A). ABP labeling indicates that fewer tags per PA28 ring improved activation of the proteasomes, since the largest shift in proteasome activity was observed when only 25-50% of PA28α is GFP-tagged and PA28β is untagged. Quantification of activity labeling and GFP fluorescence shows that GFP fluorescence of capped 20S complexes is highest when all PA28α subunits are tagged, which is relevant for microscopical experiments. Similar effects were observed when PA28 was tagged with C4 (Fig. 8B). Together this shows that tagging the N-terminus of PA28 does not obstruct assembly of the PA28αβ caps, but interfere with 20S binding and thereby activation of the 20S core complex, independently of the size of the tag (Fig. 8B). A lower ratio of tagged/untagged subunits improves capping, but sufficient fluorescence has to remain to allow imaging of a limited amount of tagged subunits that were present in the cap.

Visualizing PA28 proteasome interactions in living cells. Tagging of PA28 with fluorophores can be useful to study its intracellular localization, but to visualize its interactions with proteasomes
in living cells is even more informative. Interactions between protein complexes like PA28αβ and the 20S core can be measured using advanced fluorescence microscopy techniques such as fluorescence resonance energy transfer (FRET) and fluorescent lifetime imaging microscopy (FLIM).

FRET can be detected between two fluorophores when these are in a close proximity, and the energy of the so-called donor fluorophore is transferred to the acceptor fluorophore. With FLIM, the decay rate of a briefly excited fluorophore is measured, and when the excited fluorophore is close to an acceptor, a decrease in the lifetime of the donor fluorophore occurs. With FRET-FLIM, the detected lifetime of the donor is a value for proximity and thus interaction [75, 76]. In order to visualize PA28-20S interactions by FRET-FLIM, both a PA28 subunit and an α-subunit of the 20S core need to be fluorescently tagged with a donor fluorophore on one side and an acceptor fluorophore on the other side of interaction. To determine whether the presence of a fluorophore in the α-ring of the

**Fig. 8.** Optimizing proteasome capping by using different ratios of tagged and untagged PA28.

(A) A decrease in the ratio tagged-untagged PA28 increases capping. HeLa cells were transfected with different ratios of GFP-tagged and untagged PA28αβ as indicated, with either untagged PA28αβ, both PA28α and PA28β tagged, and untagged PA28β in combination with different ratios of tagged and untagged PA28αβ. Cell lysates were incubated with ABP4 and subjected to a 4-12% gradient native gel. Fluorescent scanning revealed increased activity labeling of PA28 čapped proteasomes (upper panel) when the ratio of fluorescently-tagged PA28 subunits were reduced (middle panel). The graph in the lower panel represents the fluorescent intensity of each band in percentages of the total fluorescence. (B) A decrease in C4-tags results in increased activity labeling of PA28 capped proteasomes (upper panel) when the ratio of fluorescently-tagged PA28 subunits were reduced (middle panel). The graph in the lower panel represents the fluorescent intensity of each band in percentages of the total fluorescence.

**Fig. 9.** Measuring proteasome-PA28 interactions by FRET-FLIM.

(A) Exchanging fluorophores between PA28 and 20S. To analyze interaction when proteasomes and PA28 are fluorescently labeled, the 20S subunit α3 was tagged with either GFP (and PA28 with C4, left lanes) or tagged with C4 (and PA28 with GFP, right lanes). Both combinations resulted in PA28-capped proteasomes but less capped complexes were formed when PA28 was tagged with GFP. (B) PA28 as acceptor molecule improves interaction measurements. The cells were stained with ReAsH after 48 h of expression. The graph represents phase lifetimes (Tϕ). The first and third bar display the donor lifetime in the presence of nonspecific ReAsH staining and the second and fourth bar displays the donor lifetime in the presence of the acceptor. When switching donor (GFP) and acceptor (C4-ReAsH) between the protosomal subunit α3 and PA28αβ, the most significant drop in lifetime is observed when PA28α-GFP is used as donor. (C) Better incorporation does not necessarily improve interaction. a7 was used both as donor and acceptor fluorophore. While using the a-ring subunit as acceptor again gave more efficient FRET efficiencies, the measured interactions were less efficient than interactions measured with the α3-subunit. The tables summarize the average phase shift (Tϕ) in ns) and the FRET efficiency (E_FRET) based in the average phase shift. (mean ± SEM; two tailed unpaired t-test; **p<0.001; *p<0.01; *p<0.05).
20S proteasome interferes with PA28 capping, we visualized the lysates of HeLa cells transfected with both donor and acceptor subunits on native gels (Fig. 9A). Since the subunits in the α-ring of the 20S complex are closest to the cap, we used a ReAsH-labeled C4-tagged α3-subunit and GFP-tagged PA28 as donor for interaction measurements, or vice versa. All 20S complexes were capped with PA28 when using C4-PA28 and α3-GFP, while a relatively large pool of uncapped 20S remained when GFP-PA28 was used in combination with α3-C4. Though, since most of the GFP-tagged PA28 is in complex with proteasomes it can still be used for FLIM measurements. When either PA28α or PA28β were tagged, differences in complex formation were not observed (as also shown in Fig. 7B), indicating that for complex formation itself a particular PA28 subunit is not preferred for labeling.

Since both FRET combinations showed incorporation of the tagged proteins into the PA28 cap and the 20S core on native gels, we tested whether both combinations were functional in living cells. GFP lifetime was measured with α3-GFP or GFP-PA28α as donor fluorophore (Fig. 9B). Higher FRET efficiencies were detected when PA28α was tagged with the donor fluorophore when compared to α3-GFP as donor, implicating that the PA28 cap should be used as donor molecule in interaction studies. Together, this shows that determination of interactions on native gels cannot predict the best combinations of FRET-FLIM fluorophores. α3-GFP as donor showed better interactions with C4-labeled PA28 caps on native gels (Fig. 9A), indicating a higher energy transfer. However, FLIM measurements showed otherwise. Additionally, we tested whether α7 is a better subunit to measure interactions, since it is more efficiently incorporated into 20S proteasomes than α3 (Fig. 2). α7 was used both as a GFP-tagged donor or as an C4-tagged acceptor and fluorescent lifetimes were measured in living cells (Fig. 9C). It was shown that better incorporation of α7 did not improve lifetime measurements. This interaction studies show that it is important to determine how fluorescent tags can affect complex formation, and that swapping fluorophores or using different ratios of tagged and untagged subunits have to be tested. Changing the position of the tags or reducing the amount of tags by co-transfection of untagged proteins can be a useful strategy to optimize interaction studies in living cells.

CONCLUSIONS

The fluorescent toolbox can be used to detect proteasome distribution and dynamics in living cells but also to detect proteasomal activity and even complex formation with proteasome activators. However, each technique has its limitations. One needs to check the level of subunit incorporation, overlapping activities of different subunits or complexes formation when analyzing proteasome activities, but also during the challenges of optimizing FRET studies in proteasome complex formation or interactions with other proteins. Since the proteasome is such an essential player in many processes in cells, it is important to study both its functions and functionalities in relation to disease and aging. In addition, proteasomes are considered to be therapeutic targets in age-related diseases such as neurodegenerative disorders and cancer [59, 77-80]. Since the discovery of the proteasome much has been learned, however many questions also remain to be solved. Once synthesized, the proteasome is not a static complex and modifications, changes in activators and expression of cytokines affect proteasomal intracellular functioning. Exchange of proteasome-activating caps affect protein turnover but also specific cellular processes like cell cycle regulation, splicing, transcription and DNA repair [37, 72, 81-86]. The existence of proteasomal subtypes other than the constitutive or immuno 20S has been shown as well [87-90], and this variation in 20S proteasomes is found in both tissues and cellular compartments and results in different cleavage specificities. Finally, proteasomal functioning is affected by various posttranslational modifications like glycosylation and phosphorylation, which in turn affect activation and localization of proteasomes [91-93]. To improve our insight into the consequences of these modifications in cells, it is important to have robust tools. We have discussed various methods to determine localization, composition and activity of proteasomes that are useful to explore the role of proteasomes in health and disease.

MATERIAL AND METHODS

DNA constructs. α3-GFPn1 and β7-mRFP were kindly provided by Prof. N.P. Dantuma (Karolinski Institute, Stockholm, Sweden). by annealing the forward primer 5’CCGGATTTCTTAATGTTGTCCGTGGTGTATGGAACCTTAAATCT-3’ and the reverse primer 5’CTAGTAAAGGTTGTCCATGACAACGAGACACCATGAAAGAATAAATA-3’ which results in a C4-tag with flanking restriction sites for AgeI and XbaI. GFP was removed using the same restriction enzymes and the annealed tag was inserted. β7-GFP was generated by obtaining β7 via PCR from the mRFP backbone using the following primers, Forward 5’CCGGATTTCTTAATGTTGTCCGTGGTGTATGGAACCTTAAATCT-3’ and the reverse primer 5’GGGCTTTAAAGGACCTTATGATG-3’ and reverse 5’GGGCTTTAAAGGACCTTATGATG-3’. The PCR product was cloned into an eGFPn1 vector using Apal and EcoRI. 5’-C4 was generated by annealing the forward primer SCCTCTTAATTGTAGCCCTTTCTCTGCTTGGTGTAGGAACTT-3’ and the reverse primer 5’CGAGGAATTCAGCCACACTGAGGGTCCATCCCGAAG-3’. The PCR product was cloned into an eGFPn1 vector using EcoRI and BamHI. A PCR product of PA28α was obtained by using the following primers forward 5’CGAGGAATTCAGCCACACTGAGGGTCCATCCCGAAG-3’ and the reverse primer 5’AAAGGATCCGGGCTCAATAGCATCTTCTCTTGG-3’. The PCR product was cloned into an eGFPn1 vector using EcoRI and BamHI. A PCR product of PA28β was obtained by using the following primers forward 5’GTATCCGGAAAGGAAGCTTTGTTGGGTTCCCTGAGG-3’ and the reverse primer 5’GAGGATTCCTGATACATCGTATGGATCTTTCTTTTACC-3’. The PCR product was cloned into an eGFPn1 vector using Xhol and EcoRI. PA28β was obtained via the SalI and EcoRI restriction sites and cloned into an eGFPn2 vector. C4-tags were generated by annealing the following primers forward 5’GGGCTTTAAAGGACCTTATGATG-3’ and the reverse primer 5’TGGAGGAATTCAGCCACACTGAGGGTCCATCCCGAAG-3’.
GF was removed by Agel and Xhol en the C4-tag was inserted. β1i, β2i and β5i were kindly provided by Prof. P.M. Kloetzel (Charité Universitätsmedizin Berlin, Germany). PCR products were obtained with the following primers; β1i forward 5'-CTCGAATTCCACCATGTCGGGCGGGGAGCA-3' and reverse 5'-AGAACGCTTGCTCATACATAGAATTTGGAGTC-3'; β2i forward 5'-CTCGAATTCCACCATGTCGGGCGGGGAGCA-3' and reverse 5'-AGAACGCTTGCTCATACATAGAATTTGGAGTC-3'; β5i forward 5'-CTCGAATTCCACCATGTCGGGCGGGGAGCA-3' and reverse 5'-AGAACGCTTGCTCATACATAGAATTTGGAGTC-3'. PCR products were cut with EcoRI and AgeI end and reverse inserted into eGFp1 backbone. Only β2i did not have a restriction site at the 5'-end, therefore the AgeI site in the backbone was blunted. Cherry-mHtt(Q74) was kindly provided by Prof. A.L. Goldberg (Harvard Medical School, USA).

Cell culture and transfection. β1i-GFP and β5i-GFP were transfected in HeLa cells with jetPEI and kept on G418 selection, 750 mg/ml ( Gibco/Invitrogen, Breda, The Netherlands) in DMEM supplemented with 10 % fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine ( Gibco/Invitrogen, Breda, The Netherlands). a3-GFP and a7-GFP in U2OS (kindly provided by Dr. O. Coux, CRBM Institute, Montpellier, France) were kept in DMEM supplemented with 10 % FCS and penicillin/streptomycin/L-glutamine ( Gibco/Invitrogen, Breda, The Netherlands). HeLa, U2OS and HEK293 cells were all cultured in DMEM supplemented with 10 % fetal calf serum and penicillin/streptomycin/L-glutamine ( Gibco/Invitrogen, Breda, The Netherlands). All cell lines were kept at 37°C in a 5 % CO2 atmosphere. HeLa, U2OS and HEK293 cells were transfected with jetPEI as described by the manufacturer (Polyplus transfection). For confocal microscopy imaging cells were harvested in TSDG buffer (10 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8 % glycerol) and lysed by 3 freeze/thaw cycles at 37°C for 8 h in the presence or absence of 50 μM cycloheximide (Sigma, St. Louis, MO, USA) or for 20 h in DMEM supplemented with 20 % fetal calf serum. After the chase, newly synthesized proteins were labeled with FLAsH by the same procedure. Following the washing steps, cells were harvested in trypsin, washed in PBS, resuspended in lysis buffer (50 mM Tris pH 7.4, 250 mM Sucrose, 50 mM MgCl2, 5 mM DTT, 2 mM ATP) and lysed by 3 freeze/thaw cycles in liquid nitrogen. After centrifugation (15 min, 20.817 x g) native sample buffer (20 mM Tris pH 8.0, 50 % glycerol, 0.1 % bromophenol blue) was added and the total supernatant was directly added to a 3-12 % NativePAGE Novex Bis-Tris Gel (Invitrogen, Life Technologies Europe BV, Bieswijk, The Netherlands). Electrophoresis was performed at 150V for 3 h. Fluorescent detection was done on a Trio Typhoon (GE Healthcare) using the 610 BP 30 filter to detect ReASh and the 520 BP 40 filter for FLAsH detection. Proteins were transferred and blotted as described above. For confocal imaging cells were fixed with 4 % paraformaldehyde (EMS, Hatfield, PA, USA) in 1x PBS ( Gibco/Invitrogen, Breda, The Netherlands) after washing steps and embedded in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were examined using a Leica TCS SP8 confocal microscope equipped with UV (405nm), Argon (488nm) and a white light laser (e.g. for ReASh excitation) and 40x or 63x objective (Leica Microsystems, Mannheim, Germany). For the described fluorescence loss in photobleaching (FLIP) experiments, an averaged image was obtained prior to Photobleaching, followed by 25 scans with 100 % Argon laserpower and FRAP booster enabled, and the entire cytoplasm selected as region of interest (and the entire nucleus excluded). During, immediately after bleaching and by a following time-lapse the fluorescence of the nucleus was monitored, and the remaining fluorescence in perspective to the cytoplasm was quantified and defined as the ‘immonile fraction’ (too large proteins to diffuse passively into the cytoplasm).

Activity labeling in living cells. U2OS cells were incubated for 1 h at 37°C in serum free medium supplemented with 0.5 μM ABP1 ( green BodipyFL-Ahx-15VS) [67], ABP2 ( green Bodipy-epoxomicin, LW68) (generated by Prof. HS. Overkleeft, Leiden Institute of Chemistry and Netherlands Proteomics Centre, The Netherlands), ABP3 (red BodipyTM-R-Ahx-15VS) [68] and ABP4 (red Bodipy-Cy3- epoxomicin, MVB003)[69]. Cells were subsequently washed 3 times in PBS ( Gibco/Invitrogen, Breda, The Netherlands) and imaged using a Leica TCS SP8 X confocal microscope equipped with white light laser and stage incubator (Leica Microsystems, Mannheim, Germany). To determine nonspecific binding cells were preincubated with 1 μM ( Sigma, St. Louis, MO, USA) for 1 h. 100 U/ml IFNγ (Roche diagnostics, Mannheim, Germany) was added to medium to activate proteasomes. Quantifications were done by means of Leica LAS AF light software (Leica Microsystems, Mannheim, Germany).

Activity labeling in lysates. HEK293 cells were harvested in TSDG buffer (10 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8 % glycerol) and lysed by 3 freeze/thaw cycles in liquid nitrogen. After centrifugation (15 min, 20.817 x g) the concentration of the supernatant was determined by Bradford protein assay ( Sigma, St. Louis, MO, USA) for 1 h. 100 U/ml IFNγ (Roche diagnostics, Mannheim, Germany) was added to medium to activate proteasomes. Quantifications were done by means of Leica LAS AF light software (Leica Microsystems, Mannheim, Germany).

Biarsenical labeling, confocal imaging and photobleaching. At 48 h after transfection, HeLa cells were stained as described by Martin et al. [49] to stain the pre-existing pool of C4-tagged proteins. Briefly, 1 mM ReASh which was pre-incubated in 10mM 1,2- ethanedithiol ( EDT, Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide (DMSO) for 10 min. Subsequently, cells were washed using PBS ( Gibco/Invitrogen, Breda, The Netherlands) and incubated for 45 min at 37°C with 1 μM ReASh in Optitrem ( Gibco/Invitrogen, Breda, The Netherlands), followed by 4 washes at RT in wash medium ( complete DMEM medium with 1 mM EDT). Subsequently, cells were incubated at 37°C for 8 h in the presence or absence of 50 μM cycloheximide ( Sigma, St. Louis, MO, USA) or for 20 h in DMEM supplemented with 20 % fetal calf serum. After the chase, newly synthesized proteins were labeled with FLAsH by the same procedure. Following the washing steps, cells were harvested in trypsin, washed in PBS, resuspended in lysis buffer ( 50 mM Tris pH 7.4, 250 mM Sucrose, 50 mM MgCl2, 5 mM DTT, 2 mM ATP) and lysed by 3 freeze/thaw cycles in liquid nitrogen. After centrifugation (15 min, 20.817 x g) native sample buffer ( 20 mM Tris pH 8.0, 50 % glycerol, 0.1 % bromophenol blue) was added and the total supernatant was directly added to a 3-12 % NativePAGE Novex Bis-Tris Gel ( Invitrogen, Life Technologies Europe BV, Bieswijk, The Netherlands). Electrophoresis was performed at 150V for 3 h. Fluorescent detection was done on a Trio Typhoon ( GE Healthcare) using the 610 BP 30 filter to detect ReASh and the 520 BP 40 filter for FLAsH detection. Proteins were transferred and blotted as described above. For confocal imaging cells were fixed with 4 % paraformaldehyde (EMS, Hatfield, PA, USA) in 1x PBS ( Gibco/Invitrogen, Breda, The Netherlands) after washing steps and embedded in Vectashield containing DAPI ( Vector Laboratories, Burlingame, CA, USA). Samples were examined using a Leica TCS SP8 confocal microscope equipped with UV (405nm), Argon (488nm) and a white light laser ( e.g. for ReASh excitation) and 40x or 63x objective ( Leica Microsystems, Mannheim, Germany). For the described fluorescence loss in photobleaching ( FLIP) experiments, an averaged image was obtained prior to Photobleaching, followed by 25 scans with 100 % Argon laserpower and FRAP booster enabled, and the entire cytoplasm selected as region of interest ( and the entire nucleus excluded). During, immediately after bleaching and by a following time-lapse the fluorescence of the nucleus was monitored, and the remaining fluorescence in perspective to the cytoplasm was quantified and defined as the ‘immonile fraction’ ( too large proteins to diffuse passively into the cytoplasm).
Tris pH 8.0, 50 % glycerol, 0.1 % bromophenol blue) was added to 25 μg lysate. The samples were loaded on a 4-12 % Criterion XT Precast Bis-Tris gel (Biorad, Hercules, CA, USA) and ran for 3 h to 4 h at 180 V. Alternatively, 3-12 % NativePAGE Novex Bis-Tris Gels (Invitrogen, Life Technologies Europe BV, Bleiswijk, The Netherlands) were used to identify 26S capped proteasomes. For the detection of individual subunits on SDS-PAGE, 10 μg lysated was incubated with 0.5 μM ABP1, ABP2, ABP3 and ABP4. After 1 h incubation at 37°C, 6x sample buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % glycerol, 6 % b-mercaptoethanol) was added, samples were boiled and loaded on a 12 % SDS-PAGE. Fluorescence imaging was performed on a Trio Thyphoon (GE Healthcare) using the 580 BP 30 filter to detect the ABP1 and ABP2, and the 520 BP 40 filter was used for detection of ABP3 and ABP4.

**Activity labeling in 2D.** A confluent 10 cm plate of HeLa cells was harvest in 500 μl proteasome-activity buffer (50 mM TRIS pH 7.5, 50 mM Sucrose, 50 mM MgCl2, 1 mM DTT, 1 mM ATP), Lysis by 3 cycles of freezing/thawing in liquid nitrogen. Protein concentration were determined using Bradford (Serva, Heidelberg, Germany), 500 μg final protein concentration was taken and incubated with 0.5 μM TRIS pH 4.8 for 1 h at 37°C. TCA precipitation was preformed to reduce the sample volume and the protein pellet was solved in 125 μl Urea buffer (7.7 M Urea, 2.2 M Thiourea, 4 % CHAPS, 30 mM TRIS pH 9.8) with 0.5 % hydroxethyl-disulfide (destreak reagent, GE healthcare) and 2 % IPG buffer (pH 3-10 NL, GE Healthcare) freshly added. Sample was loaded on an Immobilon drystrip (pH 3-10 NL, GE Healthcare) and incubated o/n at room temperature. IEF was performed on a Protean IEF Cell (Biorad, Hercules, CA, USA) using the following program; 0.1 min 50 V, 30 min 200 V; 30 min 200 V,30 min 400 V, 30 min 400 V, 30 min 600 V, 60 min 3500 V, 240 min 3500 V, 10 min 200 V. After focusing, the strips were incubated for 0.5 h in equilibration buffer (50 mM TRIS pH 8.8, 6 M Urea, 30 % (v/v) Glycerol, 20 % (w/v) SDS, BPB) with 10 mg/ml fresh DTT. Subsequently, the strips were directly transferred in equilibrium buffer with 25 mg/ml IAA and incubated for 0.5 h. The strips were recovered, loaded on top of a 12 % SDS-PAGE gel and fixed in agarose sealing solution (15 % v/v glycerol, 1 % agarose, 1x Leammli electrophoresis buffer, BPB). Electrophoresis was performed at 30 mA per gel. Fluorescent detection was done on a Trio Thyphoon (GE Healthcare) S80 BP 30 filter to detect ABP4.

**Activity measurements in gel.** HEK293 cells were harvested in TSDG buffer (10 mM Tris pH7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8 % glycerol ), lysed by three freeze/thaw cycles in liquid nitrogen and protein levels were determined by a Bradford assay (Serva, Heidelberg, Germany), 500 μg final protein concentration was taken and incubated with 0.5 μM ABP4, 0.5 μM Epoxomicin (Sigma, St. Louis, MO, USA) or similar amounts of DMSO for 1 h at 37°C. Samples were loaded on a 3-12 % NativePAGE Novex Bis-Tris Gels (Invitrogen, Life Technologies Europe BV, Bleiswijk, Netherlands) and run at 150V for 3 h. For in gel proteasome labeling, the gel was first scanned for fluorescence on a Trio Thyphoon (GE Healthcare) using the 580 BP 30 filter. Following, the wet gel slab was incubated for 20 min in 20 ml Overlay buffer (20 mM Tris pH 7.5, 5 mM MgCl2, 1 mM ATP) and 0.25 μM ABP4. After three times 10 min washing in Destain buffer (5 % acetic acid, 20 % MeOH), the gel was scanned again for fluorescence to detect additional labeling. To detect substrate cleavage in gel, the wet gel slab was directly after electrophoresis incubated in overlay buffer with 400 nM of the quenched peptides. Fluorescent intensities were measured on a Typhoon FLIM imager (GE Healthsciences) using the 580 BP 30 filter. The wet gel slabs were transferred to PVDF membrane (Millipore, Bedford, MA, USA) and Western blotting was preformed as described above.

**FLIM analysis.** FLIM was carried out after biarsenical labeling of C4-tagged proteasome subunits in living cells as described above. Living cells were imaged in microscopy medium to reduce background fluorescence (20 mM Hapes; pH 7.4, 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2 and 20 mM glucose). FLIM was performed using the wide-field frequency domain approach on a home-build instrument [96] using a RF-modulated image intensifier (Lambert Instruments II18MD) coupled to a CCD camera (Photometrics HQ) as detector. A 40x objective was used for all measurements. The modulation frequency was set to 75.1 MHz. Eighteen phase images with an exposure time of 20-200 ms were acquired in a random recording order. From the phase sequence, an intensity image and the phase and modulation lifetimes were calculated using Matlab macros. The lifetimes were calculated of individual cells. Subsequently, average phase lifetimes (τ s.d.) were calculated. The FRET efficiency E was calculated according to: E=1-(τp/(τc))x100 % in which τp is the fluorescence lifetime of the donor in presence of the acceptor (i.e. samples with both GFP and CS labeled with ReAsh) and τc is the fluorescence lifetime of the donor (i.e. GFP with background ReAsh staining) in absence of the acceptor. Frequency domain FLIM yields a phase lifetime (τs) and a modulation lifetime (τr). Since τr is more sensitive than τs, FRET efficiency was calculated on the basis of τr.

**REFERENCES**


DYNAMIC RECRUITMENT OF ACTIVE PROTEASOMES INTO POLYGLUTAMINE INITIATED INCLUSION BODIES

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ABSTRACT

Neurodegenerative disorders such as Huntington’s disease are hallmarked by neuronal intracellular inclusion body formation. A controversial topic in these protein misfolding disorders is the observed irreversible sequestration of proteasomes into inclusion bodies. These proteasomes may become clogged by the aggregated protein fragments, leading to impairment of the ubiquitin-proteasome system. Here, we show by fluorescence pulse-chase experiments in living cells that proteasomes are dynamically and reversibly recruited into inclusion bodies. As these recruited proteasomes remain catalytically active and accessible for substrates, our results challenge the concept of proteasome sequestration and impairment in Huntington’s disease, and support the reported absence of proteasome impairment in mouse models for Huntington’s disease.

INTRODUCTION

Huntington’s disease (HD) is caused by a polyglutamine (polyQ) expansion in the disease-related huntingtin (Htt) protein, and is hallmarked by inclusion body (IB) formation in neurons [1]. N-terminal fragments of the mutant Htt (mHtt) protein containing the expanded polyQ repeat are thought to initiate IBs that subsequently sequester components of the ubiquitin-proteasome system (UPS) [1, 2]. The observed proteasome sequestration into IBs is considered to contribute to HD pathology due to disruption of proper UPS function [3, 4]. Fluorescence recovery after photobleaching (FRAP) experiments suggest an irreversible sequestration of fluorescently-tagged proteasomes into IBs [5]. In addition, in vitro data suggests that proteasomes cannot cleave within polyQ repeats in polyQ-expanded proteins. As a consequence, pure polyQ fragments are released in the cell that may subsequently act as initiators of aggregation [6, 7]. UPS impairment which is observed in both cell cultures and HD patient material may indeed be caused due to sequestration of proteasomes into IBs initiated by expanded polyQ fragments [6, 8, 9]. However, robust proteasome impairment has not been detected in HD mouse models after IB formation [10, 11]. To examine whether proteasomes are indeed irreversibly sequestered into polyQ IBs, we visualized dynamics of proteasomes and polyQ fragment in IBs by fluorescent pulse-chase experiments. In addition, we used proteasome activity-based probes to visualize proteasome accessibility and activity in IBs of individual cells and in brains of HD mice.

RESULTS

Proteasomes directly interact with polyQ peptides when recruited into IBs

To examine the distribution patterns of intracellular proteasomes within polyQ IBs, we initiated aggregation using the N-terminal exon1 fragment of polyQ-expanded mHtt (mHtt(Q97)exon1), which represents the N-terminal cleavage product that is found in brain tissue of HD patients [1, 12]. In addition, pure polyQ peptides, representing products downstream of the proteasome, were used as initiators of aggregation, since it is considered that proteasomes are unable to degrade the polyQ tract [6, 7]. The Ub-Q99 constructs release Q99 peptides directly upon synthesis, as ubiquitin is cleaved off by ubiquitin C-terminal hydrolases [13]. The GFP-tagged proteasomal subunit PSMB4 (B7) was used to visualize proteasomes in living cells. This subunit is efficiently incorporated in both 20S and 26S proteasomes, as shown by complex separation on native gel and subsequent analysis of fluorescence (Supplementary Fig. S1). Recruitment of proteasomes into mHtt initiated IBs was observed when the PSMB4-GFP was co-expressed with tetracysteine-tagged mHtt (mHtt(Q97) exon1-C4). mHtt(Q97)exon1-C4 was labeled with the biarsenical fluorophore ReAsH [14] and was found to be present in the core of IBs. However, dependent on IB size, proteasomes were differently distributed (Fig. 1A). Proteasomes colocalized with mHtt(Q97)exon1-C4 in smaller IBs (Fig. 1A, upper panel), but were located in the outer layers of the larger IBs, forming a ring-like structure (Fig. 1A, lower panel). A similar redistribution of proteasomes was observed in cells stably expressing a7-GFP, this efficiently incorporated subunit was used as an alternative proteasomal subunit (data not shown). When C4-tagged polyQ peptides were used (Ub-Q99-C4), a similar proteasomal distribution pattern was observed (Fig. 1B). These findings suggest that proteasomes are not irreversibly trapped, but remain at the periphery of expanding IBs.

To determine whether these proteasomes interact directly with polyQ peptides, both in the core and also in the periphery, we used fluorescence lifetime imaging microscopy (FLIM) to study proteasome-polyQ interactions. Previously, we have shown by wide-field FRET-FLIM microscopy that GFP-tagged proteasomes, ubiquitin and the chaperone HSP70 were all recruited into polyQ aggregates but only GFP-tagged proteasomes directly interacted with polyQ fragments. Whereas GFP-ubiquitin and HSP70-GFP only showed moderate reductions in fluorescence lifetime [15]. By using confocal FRET-FLIM, we could distinguish between protein interactions in de core of the IB and at the periphery of IBs. Co-expression of ReAsH-labeled UbQ99-C4 and GFP-tagged PSMB4 showed a significant decrease in GFP donor lifetime compared to the negative control, both when proteasomes were present in the core or in the ring-like structures of IBs (Fig. 1C). This indicates that while chaperone HSP70 does not act on polyQ peptides in IBs, proteasomes continuously interact with polyQ fragments both at early stages of IB formation as well as during later stages.
Proteasomes are dynamically recruited into polyQ initiated IBs

To further determine proteasome dynamics in IBs, we performed a fluorescence pulse-chase experiment. Since FRAP studies did not show fluorescence recovery of proteasomes within IBs over a time span of minutes [5], we studied protein distribution over a time span of hours. Therefore, we first labeled the pre-existing pool of the C4-tagged proteins with red fluorescence using ReAHS. After a subsequent chase period of 8 or 20 hours, to allow the synthesis of new C4-tagged proteins, the cells were incubated with FlAHS to label the newly-synthesized proteins with green fluorescence. Pulse-chase labeling of mHtt(Q97)exon1-C4 (Fig. 2A) or Ub-Q99-C4 (Fig. 2E) showed a sequential sequestration of these polyQ fragments, as the newly synthesized mHtt(Q97)exon1-C4 or polyQ-C4 peptides were recruited to the outer layers of the IBs whereas the initial polyQ fragments were exclusively found in the core of the IB, both after an 8 and 20 hours chase period. The difference in distribution was not due to uneven penetration of FlAHS and ReAHS, as both dyes efficiently label the core of Q99-C4 aggregates [15]. In contrast, C4-tagged proteasomes (PSMB4-C4) showed a different distribution pattern in IBs that were initiated by co-expression of untagged mHtt(Q97)exon1 (Fig. 2C) or polyQ-peptides (Fig. 2F). After an 8 hours chase phase, there was a partial overlap between the pre-existing and newly-synthesized proteasomes. After a 20 hours chase period, a complete overlap in localization of both proteasome pools was observed in ring-like structures. Indicating that the initial ReAHS-labeled pool exchanged with the newly-synthesized FlAHS-labeled pool of proteasomes. These findings indicate that proteasomes are dynamic within IBs. Fluorescence intensity plots of cross sections of IBs show the difference in localization of the initial pool (red) and the chased pool (green) of mHtt(Q97)exon1-C4 or proteasome subunit PSMB4-C4 in the IB (Fig. 2B and Fig. 2D, respectively). The difference in recruitment between proteasomes and mHtt was also shown biochemically. Proteasomes were present only in the SDS-soluble fraction of cells expressing mHtt(Q97)exon1, whereas polyQ proteins were present in both the SDS-soluble and SDS-insoluble fraction (Supplementary Fig. S2). Together, these findings indicate that proteasomes are not irreversibly trapped in mHtt IBs, but are dynamically recruited albeit with relatively low exchange rates.

Proteasomes recruited into IBs are still catalytically active and accessible

The dynamic recruitment of proteasomes into IBs and the direct interaction between proteasomes and polyQ fragments does not exclude that proteasomes may become catalytically impaired due to clogging by polyQ fragments, as suggested before [5, 6]. To examine whether proteasomes are active and accessible for substrates in IBs, we added activity based probe 1 (ABP1) (BodipyFl-Ahx3L3VS) [16] to living cells. The ABP covalently labels all three catalytic subunits of proteasomes if the active sites are accessible, and allows their detection via fluorescence. When cells expressing mHtt(Q97)exon1-C4 or Ub-Q99-C4 were incubated with the ABP1, proteasomes in both the smaller and larger IBs were labeled (Fig. 3A). The activity labeling also overlapped with proteasomes that were visualized via RFP-tagged PSMB4, indicating that there were no proteasomes present in the dense core that were not labeled and thus catalytically inactive (Fig. 3B). To further examine whether the activity-labeled proteasomes are also accessible to substrates, we preformed fymography to identify only those proteasome complexes that are labeled by the activity probe (Fig. 3C). All proteasome complexes were labeled by ABP1 when cell lysates were incubated with the probe prior to native gel electrophoresis (left panel). However, uncapped 205 proteasomes were not labeled with ABP1 when the probe was added after separation of the different proteasome complexes by electrophoresis (right panel). This indicates that proteasome labeling with ABP1 only occurs when proteasomes are in complex with either 195 or PA28 activating caps. Since the probe
IBs in mHtt transgenic mice brains contain active proteasomes

To translate our finding to an in vivo model, we incubated brain sections of R6/2 transgenic mice that express mHtt(Q200) [17] with ABP1 in order to confirm the presence of active proteasomes in neuronal IBs. Both the cerebral cortex and striatum in R6/2 mice have the most mHtt-induced IBs, and immunohistochemistry was used to identify the mHtt-containing IBs in neuronal cells in the cerebral cortex (Fig. 4). Co-localization of N18 (mHtt), NeuN (neuronal nuclei marker) and ABP1 confirmed the presence of active proteasomes within IBs in mouse brains, with 93 % (±6 %, SD) of the nuclear inclusions efficiently labeled with the proteasome activity probe, and 87 % (±5 %, SD) of the cytoplasmic inclusion bodies. To confirm the specificity of proteasome activity labeling in mouse brain tissue we pre-incubated brain slices with proteasome inhibitor epoxomycin, resulting in a decrease of fluorescence labeling (data not shown).

DISCUSSION

When we visualized the dynamics of proteasomes over a time span of hours, an exchange of proteasomes was observed (Fig. 2C). This is in contrast to earlier Fluorescence Recovery After Photo-bleaching (FRAP) experiments in living cells that did not show recovery of fluorescently-tagged proteasomes within a time span of minutes [5]. This indicates that proteasomes have a slow off-rate into IBs, which was also suggested by the differences in distribution of fluorescently-tagged proteasomes in aggregates (Fig. 1A). Our data is in correspondence with the assumed protective role of IB formation, which was shown when aggregating GFP-tagged mHtt fragments in striatal neurons were imaged in time by automated fluorescence microscopy [18]. IB formation improved cell survival when compared to cells that showed only a diffuse mHtt distribution but no aggregates. Similarly, IB formation also coincided with less proteasomal impairment, which was detected using a short-lived UPS reporter [19]. A drop in proteasome activity was only observed just before IBs were formed, indicating that IB formation would indeed be a protective mechanism to sequester toxic mHtt species in the cell that may otherwise impair the UPS.

The observed recruitment of proteasomes into IBs appears to be independent of ubiquitination of the aggregated polyQ fragments. Aggregates induced by mHtt [20] but also by pure polyQ peptides [7] are initially devoid of ubiquitin, while proteasomes are directly recruited when small aggregates are formed. Although we show that the recruited proteasomes remain catalytically active and needs access to the 20S core via these activator caps, activity labeling in IBs can therefore only occur when 20S proteasomes are activated and thus accessible for protein substrates, representing functional proteasomes. In addition, subsequent analysis by SDS gel electrophoresis under reducing conditions demonstrated that all three proteasomal catalytic subunits remained equally active in cells with mHtt(Q97)exon1-C4 or Ub-Q99-C4 initiated IBs (Supplementary Fig. S3).
Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies

Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies

Our observation that proteasomes which are recruited into IBs remain active and accessible seems in contrast to earlier reports that suggest that proteasomes can become impaired due to unsuccessful attempts to degrade polyQ proteins. When examining polyQ protein degradation by proteasomes in vitro and in living cells, it was suggested that proteasomes may actually be unable to degrade the polyQ repeat present in the proteins. The undigested, expanded polyQ peptide derived from a degraded mHtt fragment may be unable to diffuse out of the proteasome, resulting in proteasomal impairment. A stable interaction between fluorescently-tagged proteasomes and fluorescent mHtt fragments was indeed observed by FRET microscopy [5], although this could also be due to a reversible interaction when proteasomes act on mHtt in IBs, as shown by FLIM microscopy (Fig. 1C). Importantly, our fluorescent pulse-chase experiments show that these interactions are reversible since there is an exchange of proteasomes at the periphery if IBs, in contrast to mHtt and polyQ peptides that are irreversibly trapped in IBs.

PolyQ fragments that enter the proteasome appear to be efficiently degraded, since less aggregation is observed when polyQ proteins like mHtt are targeted towards the proteasome [21]. This indicates that the proteasome can indeed deal with polyQ proteins, which is in line with our results that proteasomes recruited into IBs remain catalytically active and accessible for substrates. The dynamic recruitment of active proteasomes to IBs may also occur in other protein-folding diseases such as Parkinson’s disease and ALS. Therefore, up-regulation of the UPS may be a potential therapeutic target to slow down disease progression.

Fresh frozen brain sections of R6/2 (mHtt(Q200)) transgenic mice were incubated with ABP1. Subsequently, IBs containing mHtt(Q200) and neuronal nuclei were identified using immunohistochemistry using the N18 and NeuN antibodies, respectively. Colocalization of N18, NeuN and probe labeling confirms the presence of active proteasomes in neuronal IBs in the cerebral cortex (upper panel). Lower panel shows zoom-in of two randomly chosen neurons. Scale bar = 20 μm.
MATERIALS AND METHODS

DNA Constructs. mHtt(Q97) exon1 was generated by replacing the C-terminal GFP sequence of polyQ-expanded mHtt exon1-GFP (kindly provided by Prof. RR. Kopito, Stanford University, USA) for a tetracycline C4-tag (F1NCPCGCCMPE) and a stop codon [14]. Ub-Q103 was generated as described previously [7]. Ub-Q99-C4 was generated by introducing a C4-tag after GFP-Ub-Q99 using two annealed C4-primers, forward 5'-GATCTGTTCTAATGTTGTCGTTGGTGTGATAGAACCTTAAT-3′ and reverse 5′-CTAGATAGGCTTCACAACAGCAGTAATCAAACAAAC-3′. PSMB4-mRFP was kindly provided by Prof. NP. Dantuma (Karolinska Institute, Stockholm, Sweden), and the PSMB4-GFP and PSMB4-C4 constructs were generated by replacing the mRFP for an eGFP or C4-tag.

Cell culture and transfection. HeLa, Mel JuSo (melanoma) and HEK293 cells were cultured in DMEM supplemented with 10 % fetal calf serum at 37°C in a 5 % CO₂ atmosphere. HEK293 cells were transfected with jetPEI (Polyplus transfection), HeLa and Mel JuSo cells were transiently transfected with HD Fugene as described by the manufacturer (Roche Applied Sciences, Mannheim, Germany). For confocal microscopy imaging cells were grown on 2 cm coverslips (Menzel Glaser, Braunschweig, Germany) in 6-well plates.

Biosensical labeling and confocal imaging. At 48 h after transfection, HeLa and Mel JuSo cells were stained as described by Martin et al. [14]. Briefly, 1 mM ReAsH which was pre-incubated in 10 mM 1,2- ethanedithiol (EDT, Sigma-Aldrich, Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide (DMSO) for 10 min. Subsequently, cells were washed using PBS (GIBCO/Invitrogen, Breda, The Netherlands) and incubated for 45 min at 37°C with 1 μM ReAsH in Optimem (GIBCO/Invitrogen, Breda, The Netherlands), followed by 4 washes at RT in wash medium (complete DMEM medium with 1 mM EDT). Mel JuSo cells were fixed with 2 % paraformaldehyde (EMS, Hatfield, PA, USA) in 1x PBS (GIBCO/Invitrogen, Breda, The Netherlands) prior to labeling and HeLa cells were fixed after labeling. Samples were examined using a Leica TCS SP2 confocal microscope equipped with Ar/Kr laser and 40x or 63x objective (Leica Microsystems, Mannheim, Germany).

Native gel analysis. HEK293 cells were harvested in TSDG buffer (10 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA and 8 % glycerol) and lysed by 3 freeze/thaw cycles in liquid nitrogen. After centrifugation (15 min, 20,817 x g) the concentration of the supernatant was determined by a Bradford protein assay (Serva, Heidelberg, Germany). Proteasomes were labeled in the lysate using 0.5 μM ABP2 (bodipy-epoxyoximin) [22] for 1 h at 37°C. 4x Native sample buffer (20 mM Tris pH 8.0, 50 % glycerol, 0.1 % bromophenol blue) was added to 30 μg lysate. The samples were loaded on a 4-12 % Criterion XT Precast Bis-Tris gel (Biorad, Hercules, CA, USA) and ran for 4 h at 180V. Fluorescence imaging was performed on a Trio Thypoon (GE Healthcare) using the 580 BP 30 filter to detect the bodipy-epoxyoximin probe and mRFP, the 610 BP 30 filter to detect ReAsH and the 520 BP 40 filter for GFP detection. For western blotting the gels were incubated 0.5 h in transfer buffer with SDS added (25 mM Tris pH 7.5, 192 mM Glycine, 20 % MeOH and 0.1 % SDS) and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). α2 proteins were detected by the MCP236 antibody (1:1000) (kindly provided by Rasmus Hartmann-Petersen, Biologisk Institut, University of Copenhagen), and detection was done by the Odyssey detection system (LICOR Biosciences, Lincoln, NE, USA).

Confocal FLIM. We used EGFP and the red biarsenical fluorophore ReASh as a FRET pair. Data was acquired using an Olympus IX81 inverted microscope with a FV1000- confocal detection head, controlled by FV2.3 software. Donor fluorophores were excited using a 440 nm 20 MHz pulsed laser diode (Picoquant, Berlin, Germany), as controlled by a SepiaII laser driver unit (Picoquant, Berlin, Germany) and attenuated 10 times by a neutral density filter. The light was guided via a D405/480/560/635 primary dichroic mirror (Chroma, Brattleboro, VT, USA) through a water immersed 60x UPlanS-Apo objective (NA 1.2) into the sample. Cellular samples were grown on 24 mm round coverslips (Menzel Glaser, Braunschweig, Germany) fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA) and stored in PBS (GIBCO/Invitrogen, Breda, The Netherlands). The emission light was guided via a size-adjustable pinhole, set at 120 μm, through the Olympus detection box to the fibre output channel. The optical fibre was coupled to a custom-made detection box (Picoquant, Berlin, Germany) containing MPD avalanche photodiodes. The light was guided into one of the MPDs and filtered by a 525/45 emission filter (Chroma, Brattleboro, VT, USA). The photon arrival times were recorded by a Picoharp 300 time-correlated single-photon counting system (Picoquant, Berlin, Germany) and analysed in SymPhoTime 5.13 software (Picoquant, Berlin, Germany). For each ROI the fluorescence decay curve was fitted using a mono-exponential decay fit, including the estimated instrument response function (IRF) and a background term. For data analysis only fluorescence decays having more than 10,000 registered photons in the peak were considered and the quality of the fit was judged by visual inspection of the fitsresiduals and the normalized Chi² value.

Soluble and insoluble fraction analysis. HEK293 cells were lysed in 1.5 % SDS buffer (70 mM Tris pH 6.8, 1.5 % SDS, 20 % glycerol) and sonificated by the Soniprep150 (Sanyo, Leicester, UK), 50 mM EDT was added and samples were boiled for 10 min prior to 1 h centrifugation at 20,817 x g at RT. The pelleted fraction, representing the insoluble fraction, was incubated with 10 μl 100 % formic acid, incubated at 37°C for 30 min and lyophilized for 0/n in a speedvac (Eppendorf, Hamburg, Germany). 1.5 % SDS buffer was added to the pellet, following with 10 μl 100 % formic acid, incubated at 37°C for 30 min and lyophilized for 0/n in a speedvac (Eppendorf, Hamburg, Germany). 1.5 % SDS buffer was added to the pellet, following with 10 μl 100 % formic acid, incubated at 37°C for 30 min and lyophilized for 0/n in a speedvac (Eppendorf, Hamburg, Germany). 1.5 % SDS buffer was added to the pellet, following with 10 μl 100 % formic acid, incubated at 37°C for 30 min and lyophilized for 0/n in a speedvac (Eppendorf, Hamburg, Germany).

Pulse-Chase experiments. HeLa cells were labeled with ReASh as describes above to stain the pre-existing pool of C4-tagged proteins. After washing the cells were incubated at 37°C for 8 or 20 h in 20 % fetal calf serum supplemented DMEM. After the chase, newly synthesized proteins were labeled with...
FLAsH by the same procedure. Subsequently, cells were fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA), embedded in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA) and analyzed by confocal microscopy, using Huygens essentials software (BioVision technologies, Exton, PA, USA) to determine fluorescence intensities of the cross sections.

Proteasome activity labeling in living cells. Mel Ju50 cells were incubated for 1 h at 37°C in serum free medium supplemented with 0.25 μM ABP1 (BodypiFL-Ahx3L3VS) [16]. Cells were subsequently washed 3 times in PBS (GIBCO/Invitrogen, Breda, The Netherlands) and fixed in 2 % paraformaldehyde (EMS, Hatfield, PA, USA) prior to ReAsH staining.

Activity labeling in gel. HEK293 cells were harvested in TSDG buffer(10 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA and 8 % glycerol), lysed by three freeze/thaw cycles in liquid nitrogen and protein levels were determined by a Bradford assay (Serva, Heidelberg, Germany). 40 μg of cell lysates were incubated with 0.5 μM ABP1, 0.5 μM Epoxomicin or similar amounts of DMSO for 1 h at 37°C. Samples were loaded on a 4-12 % Criterion XT Precast Bis-Tris gel (Biorad, Hercules, CA, USA) and subsequently scanned for fluorescence on a Typhoon imager (GE Healthsciences) using the 520 BP 40 filter. The wet gel slab was incubated for 20 min in 20 ml Overlay buffer (20 mM Tris pH 7.5, 5 mM MgCl2, 1 mM ATP and 0.25 μM ABP1 and washed for 3 h in Destain buffer (5 % acetic acid, 20 % MeOH). the wet gel slabs were transferred to PVDF membrane (Millipore, Bedford, MA, USA) and western blotting was performed as described above.

Activity labeling in vivo. Freshly frozen brains of 15-week-old R6/2 mice (mHtt(Q200)) and littermate controls were used (Kindly provided by prof. G. Bates, London). 10 μm thick brain slices were mounted on 1 mm thick PEN membrane slides (Zeiss, Oberkochen, Germany), dried for 10 minutes at room temperature and stored at -20°C until further use. Sections were incubated for 10 minutes with 1 µM ABP2 and similarly treated as described in Supplementary Figure S2.

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**Supplementary Fig. 1.**

PSMB4 is efficiently incorporated in 20S proteasomes. HEK293 cells were transiently transfected with PSMB4-RFP (lane 2), PSMB4-GFP (lane 3) and PSMB4-C4 (labeled with Bodipy-epoxomicin) (lane 4), subjected to electrophoresis on a native gel and scanned for fluorescence. Activity based probe 2 (ABP2) labeled proteasomes were used as a marker for proteasome complexes (lane 1). The fluorescently-labeled subunits can be identified at similar levels of active proteasome labeling, confirming the incorporation into proteasomal complexes (lane 2-4). Right panel, presence of the 20S complex was confirmed by Western blotting against α2.

**Supplementary Fig. 2.**

Proteasomes are not sequestered in the insoluble fraction of cells with IBs unlike polyQ proteins. HEK293 cells were transiently transfected with mHtt(Q97) and lysed after 72 h in a 1.5 % SDS containing buffer. The insoluble fraction, including aggregated proteins, was separated from the soluble fraction and solubilized using formic acid. Whereas polyQ Htt is present in both the soluble and insoluble fraction, proteasomes were only present in the soluble fraction (as detected by the α2 subunit of the proteasome).

**Supplementary Fig. 3.**

All three catalytic sites remain equally active in cells with IBs as compared to wild type cells. HEK293 cells expressing Htt-exon1(Q107) or Ub-Q99-C4 were incubated with the ABP2 and similarly treated as described in Supplementary Figure S2. While mHtt(Q97) (Arrow) and poly peptides (arrow heads) were detected in the insoluble SDS-fraction, proteasomes were only present in the soluble SDS-fraction. Activity labeling of proteasomes in cells containing polyQ initiated IBs or control cells showed similar efficiencies in labeling of the catalytic subunits.
Dynamic recruitment of active proteasomes into polyglutamine initiated inclusion bodies

REFERENCES


MODULATING PROTEASOME ACTIVITY IN HUNTINGTON’S DISEASE

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Manuscript in preparation
Huntington's disease (HD) is a neurodegenerative disease which is caused by an expansion of the polyglutamine (polyQ) tract in the huntingtin protein. Fragments of the huntingtin protein containing the expanded polyQ tract accumulate into aggregates, thereby recruiting other proteins including components of the ubiquitin proteasome system. The role of proteasomes in HD is controversial. Proteasomes may be unable to degrade the polyQ tracts and become impaired while degrading these polyQ fragments. In addition, induction of immunoproteasomes has been reported in HD and may enable cells to cope with the hazardous protein fragments. In this study, we show that proteasomes are able to cleave within polyQ tracts and that degradation of polyQ peptides can be improved by activation of the proteasome. Increasing proteasomal activity by introduction of the PA28αβ activator improved degradation of expanded polyQ peptides, but these activated proteasomal complexes appeared unable to target the folded huntingtin protein. Alteration of the catalytic activity of proteasomes by introducing immunosubunits in the 20S proteasome increased degradation of mutant huntingtin in vitro but not in cells. Interestingly, modulation proteasomal activity by selective inhibition of individual catalytic sites suggests a specific role for β1 and β5 in mutant huntingtin degradation.

INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disease, resulting in movement disorders and psychological changes [1, 2]. The disease belongs to a group of polyglutamine (polyQ) disorders that are caused by a polyQ expansion in the disease-related protein [3, 4]. When the polyQ repeat, which is located at the N-terminus of the huntingtin (Htt) protein, exceeds the threshold of approximately 37 glutamines, the disease is initiated. This is hallmarkd by the occurrence of nuclear aggregates or inclusion bodies (IB) in neurons of affected brain regions in patients and transgenic mouse models [5-8]. These aggregates are initiated by accumulation of the aggregation-prone N-terminus of mutant huntingtin (mHtt) fragments, which in turn also recruit other cellular proteins [8, 9]. Whether large aggregates, smaller oligomers or monomeric polyQ-expanded Htt fragments represent the most toxic species is still under debate [10-13]. Nowadays, it is even thought that large aggregates (or inclusion bodies) may be protective, and that the intermediate oligomers are particular toxic to cells [14-16]. Therefore, clearance of these fragments in an early stage, prior to oligomerization, would be beneficial in order to prevent aggregation and toxicity. Intracellular protein clearance can be accomplished by two major degradation systems in cells, the ubiquitin proteasome system (UPS) and autophagy. While it has been shown that induction of autophagy leads to amelioration of the HD phenotype, the autophagic machinery is absent in the nucleus and thus will not be able to target nuclear mHtt fragments or aggregates [17, 18]. This emphasizes the importance of studying the role of the UPS in HD, which is present in both the cytoplasm and the nucleus of the cell.

The role of proteasomes in HD disease progression has been controversial. Various studies in cell culture and patient material reported UPS impairment [19-21], with proteasomes being sequestered into aggregates [9, 22, 23]. Meanwhile, other studies showed a temporal UPS impairment prior to IB formation only, which may be due to accumulation of polyQ fragments [24, 25]. We recently observed that proteasomes were not irreversibly trapped in IBs but are dynamically recruited, and remain accessible for substrates (manuscript submitted). Furthermore, proteasomes were shown to be unable to cleave within polyQ sequences, which led to the hypothesis that the polyQ fragments derived from inefficient cleavage can physically impair proteasomes [23, 26]. However, both in vitro and in vivo studies have shown that polyQ peptides and polyQ proteins can actually be degraded by proteasomes [27-29]. Taken together, it is evident that proteasomes play an important role during development of HD and although proteasomes seem to be able to degrade polyQ proteins, it is not sufficient for efficient clearance. This raises the question whether modification of proteasomal activity is beneficial for degrading mHtt fragments more efficiently and to diminish the disease phenotype.

The UPS operates via recognition of proteins which are marked for degradation by poly-ubiquitination [30, 31]. Consequently, poly-ubiquitinated proteins are unfolded by the 19S activator which guides the substrates into the 20S barrel [32]. Together these complexes are called the 26S proteasomes. The 20S proteasome exists of a barrel-shaped core that contains four stacked rings [33-36]. The outer two rings, called the α-rings, are important for opening and closing of the barrel. The inner two β-rings are responsible for protein cleavage since each ring contains 3 catalytic subunits. Each of these subunits have their own specificity, with caspase activity of β1, trypsin-like activity of β2 and chymotrypsin-like activity of β5. One way to modulate the activity of the proteasome is by exchanging these constitutive catalytic subunits by immunosubunits [37-40]. Two of the genes, coding for immunosubunits β1i and β5i, are present on the MHC class II locus and are transcribed after interferon(IFN)γ stimulation [41]. The third immunosubunit β2i is not located on the MHC class II locus but is also induced after IFNγ stimulation [42-44]. After an inflammatory response, these subunits are incorporated into de novo synthesized immunoproteasomes (20S1) that are transiently expressed [45, 46]. Besides immunosubunits, IFNγ also induces subunits of the alternative proteasome activator (PA)28αβ. The 20S core of the proteasome is a latent complex and needs to be activated in order to degrade substrates [47]. The main pool of 20S proteasomes is activated by the 19S activator in an ATP-dependent fashion. Alternatively, the 20S core can also be activated by PA28αβ in order to increase peptidase activity [48-50]. The PA28αβ is formed by PA28α and PA28β subunits which form a hetero heptameric ring structure that binds to the α-rings of the 20S core, thereby opening the normally closed formation of the core and increasing peptidase activity. This type of proteasome activation is ATP-independent, and unlike the 19S complex, PA28 cannot unfold proteins.

In the present study, we explored the effects of altered proteasome activity in mHtt degradation. Proteasomal activation by PA28 increased degradation of polyQ peptides but degradation of mHtt(Q97) did not appear to be improved. However, manipulation of the catalytic activities of the proteasome affected degradation of mHtt, as replacement of the constitutive subunits for immunosubunits showed improved degradation of mHtt in vitro but not in vivo. Inhibition of individual proteasome catalytic sites resulted in different cleavage patterns of mHtt, and especially inhibition of β5 resulted in decreased levels of mHtt in vitro and in cells.

ABSTRACT

Huntington's disease (HD) is a neurodegenerative disease which is caused by an expansion of the polyglutamine (polyQ) tract in the huntingtin protein. Fragments of the huntingtin protein containing the expanded polyQ tract accumulate into aggregates, thereby recruiting other proteins including components of the ubiquitin proteasome system. The role of proteasomes in HD is controversial. Proteasomes may be unable to degrade the polyQ tracts and become impaired while degrading these polyQ fragments. In addition, induction of immunoproteasomes has been reported in HD and may enable cells to cope with the hazardous protein fragments. In this study, we show that proteasomes are able to cleave within polyQ tracts and that degradation of polyQ peptides can be improved by activation of the proteasome. Increasing proteasomal activity by introduction of the PA28αβ activator improved degradation of expanded polyQ peptides, but these activated proteasomal complexes appeared unable to target the folded huntingtin protein. Alteration of the catalytic activity of proteasomes by introducing immunosubunits in the 20S proteasome increased degradation of mutant huntingtin in vitro but not in cells. Interestingly, modulation proteasomal activity by selective inhibition of individual catalytic sites suggests a specific role for β1 and β5 in mutant huntingtin degradation.
RESULTS

PolyQ degradation by IFNγ-modulated proteasomes does not improve mHtt degradation

Conditional mouse models expressing polyQ-expanded Htt show the induction of immunoproteasomes, which may be a cellular response to encounter accumulation mHtt fragments [51, 52]. Since there are discrepancies between the various studies concerning the ability of proteasomes to cleave polyQ peptides, we developed a system where endopeptidase activity against polyQ sequences can be detected via generation of fluorescence. While earlier studies used small polyQ peptides that can be targeted by exopeptidases such as puromycin sensitive aminopeptidase (PSA), we were especially interested in the activation of endopeptidases, as these proteasomes should be able to cleave within larger polyQ sequences [26, 53]. In our approach, we separated proteasomes from cell lysates on native PAGE and incubated the wet gel slab with quenched Q8-peptides that only become fluorescent after cleavage between glutamine residues (Fig. 1A). The flanking sequences of these peptides are synthesized from D-amino acids that makes these peptides inaccessible for exopeptidases, and these peptides can thus only be cleaved within the polyQ sequence via endopeptidase activity. Upon protein complex separation and incubation with the quenched polyQ-peptide reporter, a number of fluorescent bands appeared due to polyQ cleavage by protease activities (Fig. 1B). When the cell lysate was incubated with a proteasome activity-based probe (ABP) [54], which labels active proteasome complexes, a similar cleavage pattern appeared (Fig. 1B) which was absent in lysates that were pre-incubated with proteasome inhibitor (Fig. 1B). Merging both fluorescent signals showed identical patterns confirming that cleavage of the quenched Q8-peptides was achieved by proteasomes (Fig. 1B). Only the band representing the latent 20S complex was lacking after incubation with fluorogenic substrates. This data shows that activated proteasomal complexes are able to cleave between glutaminines. To investigate whether changes in proteasomal composition due to IFNγ stimulation affect activity towards Q8-peptides, we compared peptide cleavage patterns by non-stimulated cell lysates with IFNγ-stimulated cell lysates (Fig. 1C). Increased fluorescence of PA28αβ-capped and so-called hybrid proteasomes (19S-20S-PA28αβ) indicates improved degradation of Q8-peptides due to the induction of PA28αβ. Blotting the native gel with PA28α antibodies confirmed the increased levels of PA28αβ-capped complexes (Fig. 1C). Subsequent western blot analysis after running the cell lysates on SDS-PAGE confirmed that equal amounts of 20S proteasomes were loaded, as indicated by immunostaining of α2 subunits.

To examine whether the increased capacity to degrade Q8-peptides could be extrapolated to cells, we compared mHtt degradation in IFNγ-stimulated cells with control cells, both transfected with the N-terminal fragment of mHtt, mHtt(Q97)exon1 (Fig. 1D). Importantly, the induction of immunoproteasomes is a relatively rapid process, while the formation of detectible aggregates takes at least 48 h. To study the effect of IFNγ-modulated proteasomes on soluble levels of mHtt and on aggregate formation of mHtt, we stimulated cells for 24, 48 and 72 h with IFNγ. No significant effects on mHtt(Q97)exon1 protein levels were detected, as quantified by immunoblotting against...
the polyQ tract (Fig. 1D). Moreover, quantification of the aggregated fraction by filter-Trap analysis showed a trend towards increased levels of aggregation when cells were stimulated with IFNγ (Fig. 1D). This data shows that while immunoproteasomes induced by IFNγ are better capable in cleaving Q8-peptides, these modulated proteasomes do not improve mHtt(Q97)exon1 degradation in cells, as IFNγ treatment may even have enhanced aggregation.

Proteasome activation improves mHtt degradation in vitro but not by PA28 capping

IFNγ activates multiple pathways in the cell to induce antiviral responses, and one of the consequences is the production of reactive oxygen species that oxidize and damage the existing protein pool [55, 56]. In addition, IFNγ may induce the synthesis of defective ribosomal intermediate products (DRiPs) [57-59], resulting in an increased pool of oxidized and misfolded proteins that are subsequently targeted for proteasomal degradation [60]. This makes the UPS to become overloaded with substrates, thereby having a limited capacity for mHtt degradation, but these defective proteins are also prone to aggregation and may accelerate mHtt aggregation. To exclude these additional effects by IFNγ on proteasomal dependent degradation and aggregate formation, an in vitro assay was performed to study the effects of proteasome modulation on mHtt degradation. We used H4-tagged mHtt(Q97) exon1 that was isolated from transfected N2a cells as substrate. Once purified, mHtt(Q97)exon1-H4 was incubated with 20S proteasomes that were isolated from human erythrocytes. We recently showed that mHtt(Q97) exon1-H4 is in vitro completely degraded by 20S proteasomes, thereby generating intermediate polyQ containing fragments [61]. To determine how modulation of these proteasomes affect mHtt(Q97)exon1-H4 degradation, we activated purified 20S proteasomes using different activators. One way to activate 20S proteasomes is gate opening by using RPT peptides which represent the C-termini of the 19S subunits RPT2 and RPT5 [62, 63]. Separation of the catalytic subunits on SDS-PAGE after ABP incubation showed increased activation of the catalytic subunits when the RPT peptides were added (Fig. 2A). When mHtt(Q97)exon1-H4 was incubated with RPT-activated proteasomes, RPT2 and RPT5 peptides increased degradation of mHtt(Q97)exon1-H4 as compared to degradation by control 20S proteasomes (Fig.2A). Addition of epoxomicin to the reaction confirmed that mHtt was degraded by proteasomes and not by other enzymes (Fig.2A). An alternative method to obtain open-gated proteasomes, is the use of low concentrations of SDS [64]. Similarly as observed for RPT peptides, activation by 0.01 % SDS increased degradation of mHtt(Q97)exon1-H4 (Fig.1B). In contrast, addition of PA28αβ to the in vitro reaction mixture even prevented proteasomes from degrading mHtt(Q97)exon1-H4, both in the presence and the absence of the 19S cap (Fig.2C). Activity labeling confirmed activation of the proteasomes by PA28αβ, and complex identification on gradient native gel showed the shift to PA28-capped 20S proteasomes that are highly active (Fig.2D). Addition of PA28αβ to 26S proteasomes did not result in hybrid complex formation, as only PA28αβ-capped 20S complexes were present (Fig. 2D, last two lanes).

The inability of PA28αβ-capped proteasomes to degrade mHtt suggests that the PA28 cap forms a physical barrier which prevents entrance into the catalytic cavity, which may be due to the folded structure of the mHtt. The proline-rich region at the C-terminus can form a knick in the secondary structure of mHtt, that may prevent entrance into the PA28αβ-capped core [65]. Another obstacle could be the secondary structure of the expanded polyQ sequence, which is supposed to form multiple β-sheets in its folded state [66]. To examine their contribution to the limited degradation by PA28αβ-capped proteasomes, the proline-rich region was removed from mHtt (mHtt(Q97)exon1-ΔPRO) and incubated with isolated proteasomes, as well as wild-type Htt(Q25)exon1-H4 with a shorter sequence of 25 glutamines (Fig. 2E). Unlike mHtt(Q97-exon1-H4, mHtt(Q97)exon1-ΔPRO-H4 degradation by PA28αβ activated proteasomes was as efficient as by non-capped proteasomes (Fig. 2E). Additionally, wild type Htt(Q25)exon1-H4 was rapidly degraded by PA28-capped proteasomes although at slower rate than when compared to non-capped proteasomes (as shown by the longer exposure time). Together, this indicates that the presence of the proline-rich region obstructs entering...
Modulating proteasome activity in Huntington's disease

Previous results showed that mHtt cannot be degraded by PA28αβ-capped proteasomes in vitro. However PA28β-capped proteasomes in lysates of IFNγ-stimulated cells had increased activity towards quenched Q8-peptides. Moreover, overexpression of PA28αβ in cells increased cleavage of quenched Q8-peptides two-fold, which is considerably more when compared to IFNγ-induced proteasome activation (Fig. 3A). To analyze whether expanded polyQ peptides that exceed the disease-related threshold of 36-40 glutamines are also better degraded by PA28αβ-capped proteasomes in cells, HEK293 cells were transfected with PA28αβ or the individual PA28 subunits together with pure Q54-peptides using a GFP-Ub-Q54 construct. Upon synthesis of GFP-Ub-Q54, the GFP-Ub is cleaved immediately by C-terminal hydrolases and as a consequence the Q54-peptides are released as pure peptides without a starting methionine [67]. As a control, we cotransfected the chaperone DnaJβ which keeps polyQ peptides in a soluble form and reduces aggregation [68]. After 72 hours of expression, cells were harvested and lysates were blotted with antibodies against polyQ sequences and β-actin (Fig.3B). The aggregated fraction was loaded on a filtertrap assay and blotted for polyQ aggregates (Fig.3B). When PA28αβ was co-expressed, a significant reduction of both soluble and aggregated Q54-peptide levels was observed (Fig. 3B), although expression of PA28α alone also seemed to have an effect on polyQ peptide degradation. This can be explained by the fact that homomeric PA28α is also able to activate proteasomes, although with less affinity than heteromeric complexes [49, 69]. Additionally, it was shown in HEK293 cells that overexpression of PA28α stabilizes endogenous PA28β, thus increasing proteasome activation [70]. Proteasome complex identification on native gel indeed showed a shift towards PA28-activated proteasomes when only PA28α was expressed (Supplementary Fig. 1). To confirm whether the effects were proteasome dependent, we incubated the cells the last 16 hours in the presence of the proteasome inhibitor epoxomicin (Fig. 3C). Inhibition of proteasomes reduced the observed PA28αβ effect on soluble polyQ peptides and aggregation levels, indicating that the soluble Q54-peptides were degraded by proteasomes (Fig. 3C). The limited decrease in aggregation after epoxomicin incubation can be explained by the relatively short incubation time with the inhibitor compared to the long expression time of PA28αβ.

While degradation of polyQ peptides is apparently improved by PA28αβ both in vitro and in cells, isolated mHtt was not degraded by PA28-capped proteasomes (Fig. 2E). This may be due to the requirement to unfold mHtt prior to proteasomal digestion which may be facilitated by the proteostasis network in cells. When HEK293 cells were cotransfected with mHtt(Q97) and PA28αβ, PA28α or PA28β, and lysates were blotted to demonstrate polyQ levels, effect of PA28 on both soluble and insoluble levels was not observed (Fig. 3D). Together, this data shows that proteasome activation by PA28αβ improves polyQ peptide degradation in cells but does not improve mHtt turnover. This may well be due to the inability of folded mHtt to enter the proteasome.

**Immunoproteasomes improve mHtt degradation in vitro but not in cells**

In addition to manipulation of the accessibility and activation of 20S proteasomes via protein activators, the proteasomal catalytic activities themselves can also be modulated by exchanging the three catalytic subunits β1, β2 and β5 for the immunosubunits β1i, β2i and β5i, respectively. To evaluate the direct effect of these alterations in catalytic sites on mHtt degradation, we compared
in vitro degradation of mHtt(Q97)exon1-H4 by constitutive proteasomes and immunoproteasomes that were isolated from human spleen (Fig. 4A). Interestingly, not only was mHtt more rapidly degraded by immunoproteasomes since a faster decrease of input levels was observed, but also the appearance of additional proteolytic fragments suggests different rates of cleavage activities (Fig. 4A, fragments are indicated by asterisks). This effect was solely due to altered catalytic activities since equal levels of 20S proteasomes were determined by α2 antibodies and the absence of PA28α excluded a potential effect of PA28αβ on degradation velocity, as shown with the use of PA28α antibodies (Fig. 4A). The differences in degradation patterns were reproduced with different badges of proteasomes isolated from different donors (data not shown), indicating that replacement of constitutive subunits for immunosubunits improves degradation of mHtt(Q97)exon1-H4.

To investigate whether degradation of mHtt(Q97)exon1 is also improved in cells, HeLa cells that stably expressed the immunosubunits β1i, β2i and β5i were used to optimize incorporation in the 20S core [71]. To determine the rate of incorporation, 2D electrophoresis was used to separate all constitutive subunits and immunosubunits. Lysates of both control HeLa cells and HeLa cells that stably expressed all three immunosubunits were incubated with ABP for labeling of the active catalytic subunits. Subsequently, proteins were separated in two dimensions to identify all 6 catalytically-active subunits. Fluorescence analysis showed almost complete replacement of the constitutive subunits by immunosubunits (Fig. 4B). Immunoblotting for LMP2 and α2 confirmed the presence of the immunosubunit in the stable cell line only, whereas α2 was present at a similar levels (Fig. 4B).

To analyze whether the exchange by cellular immunoproteasomes improves quenched Q8-peptide degradation, cell lysates were subjected to zymography (Fig. 4C). Quantification of the fluorescent signal emitted by the quenched peptide, however, did not reveal a significant difference between control and immuno HeLa cell line (Fig. 4C). To further examine whether mHtt degradation is affected by immunoproteasomes in cells, mHtt(Q97)exon1 and GFP were expressed for 24 hours in HEK293 cells. GFP was cotransfected as an internal control, to normalize for transfection differences between the cell lines. Cell lysates were subjected to western blotting and filtertrap assays, and mHtt levels were normalized to GFP levels (Fig. 4D). After 24 hours expression, aggregates were not yet detected and analysis of the soluble fraction did not reveal differences between both cell lines. Taken together, this shows that immunoproteasomes better deal with mHtt in vitro, but that polyQ peptide degradation and mHtt degradation in cells is not affected.

Contribution of individual proteasome subunits in mHtt degradation

Since mHtt fragments and also polyQ peptides are degraded by proteasomes, we next aimed to determine the importance of each individual catalytic subunit in mHtt degradation, using inhibitors that are directed against a specific catalytic site [72-74]. To examine whether a particular site is mainly responsible for cleaving between glutamines, the quenched polyQ peptides were added to...
isolated proteasomes that were pretreated with the selective inhibitors (Fig. 5A). Prior to loading on a native gel, constitutive proteasomes were incubated with PA28αβ and the inhibitors. Activation by PA28αβ was necessary to increase the degradation signal to levels that were detectable by fluorescence scanning. After electrophoresis, the gel was incubated with the quenched Q8-peptides and fluorescence was imaged (Fig. 5A). Largest decrease in fluorescence signal was observed when β5 activity was inhibited, indicating that the chymotryptic activity is mainly responsible for polyQ cleavage, as has been suggested before [27].

To explore the effects of specific inhibition on mHtt degradation, the inhibitors were used in the in vitro degradation of mHtt(Q97)exon1-H4 (Fig. 5B). ABP labeling of proteasomes demonstrates complete abrogated β1 activity whereas little activity of β2 and β5 remained after addition of the corresponding inhibitors. Due to cross reactivity between the β5 inhibitor and β2, we were not able to increase inhibitor concentrations. Nevertheless, proteasomes were still capable to degrade mHtt(Q97)exon1-H4 after inhibition of β2 or β5, as degradation products were generated (Fig. 4B). When compared to non-inhibited proteasomes, β5-inhibited proteasomes showed faster loss of mHtt input levels (Fig. 5C). In contrast, inhibition of β1 activity prevented the generation of the smaller cleavage product (Fig. 5B, indicated by arrow heads). Additionally, immunostaining of the C-terminal His-sequence showed that the lower fragment was not stained, suggesting that the β1 cleavage site is in the C-terminus of the mHtt protein. Next, we examined for potential alterations in the mHtt cleavage pattern and degradation velocity after β1 or β5 inhibition in cells, respectively. HEK293 cells were transfected with mHtt(Q97) exon1 for 24 hours and inhibitors were added 16 hours before harvesting. Compared to the DMSO control, β2 inhibition did not affect mHtt levels, while β1 inhibition slightly increased the soluble levels (Fig. 5E). When β5 was inhibited with two different concentrations (at 5 μM β2 activity was affected as well), decreased soluble mHtt(Q97) exon1 levels were detected (Fig. 5E). To determine whether this decrease was due to more efficient degradation or a shift towards the insoluble fraction, we immunostained mHtt aggregates with 1C2 antibodies in a filtertrap assay (Fig. 5F). Since only at high concentrations of β5 inhibition (which also affected β1 and β2 activity) caused a significant increase in aggregate formation, our results suggest improved mHtt(Q97)exon1 degradation only when the β5 activity is impaired. Together, these results suggest important but opposing functions for β1 and β5 in mHtt degradation. While β5 seems to be the main activity able to cleave within polyQ sequences, inhibition of this activity also seemed to accelerate mHtt degradation.

**DISCUSSION**

The role of proteasomes in HD has been an issue of a long-time discussion. Most studies examining proteasome activity in HD-related models have shown impairment of proteasomal functions, which is explained by the observed sequestration of proteasomes into aggregates and the incompetence of proteasomes to properly degrade mHtt proteins [19, 22, 23, 26]. The inability of proteasomes to cleave within polyQ sequences is considered to cause release of polyQ fragments or peptides by the proteasomes or even clogging of proteasomes by nondegradable polyQ fragments. More
recent in vivo studies indicated that proteasomal impairment is only a temporal effect prior to the formation of large inclusion bodies, which rescues the cell by sequestering the hazardous, degradation-resistant proteins [24, 25]. Yet, we recently showed that the proteasome is able to degrade mHtt [61], that the activity of proteasomes is not impaired and that proteasomes are not irreversibly sequestered into aggregates (manuscript submitted). These findings raised the question whether the proteasome is not only a contributor to the disease due to reduced activity but may actually be a potential target for intervention, especially since reduction of mHtt levels has been shown to be an effective way of restricting the disease phenotype [75-78]. Additionally, since HD is an age-related disease, reduced activity of proteasomes in aging cells may be linked to the onset of the [79, 80]. Independent of the underlying cause of reduced proteasomal activity, amelioration of the proteasomal system to improve mHtt degradation is an interesting therapeutic option.

Activation of the proteasome

When we examined whether proteasomes can degrade polyQ sequences, we showed that the proteasome cleaves within polyQ peptides, mainly by its β5 catalytic site. Thusfar, only the aminopeptidase PSA and to a lesser extent tripeptidyl peptidase II (TPPII) have been identified as peptidases that are able to cleave small polyQ peptides by nibbling off glutamine residues from the N-terminal side. PSA is not able to target expanded polyQ peptides as it can only degrade peptides up to 30 amino acids, and lacks endopeptidase activity to cleave within polyQ sequences [81]. Increased in polyQ peptide degradation was observed when cells were stimulated with IFNγ, which was due to the activation of proteasomes by PA28αβ. However, IFNγ stimulation of cells did not improve degradation of polyQ-expanded proteins or polyQ peptides (data not shown) and even appeared to induce increased aggregation. This could be explained by the broad cascade of anti-inflammatory response events triggered by IFNγ, including increased generation of both oxidized proteins and DRiPs that are aggregation prone and compete with mHtt for degradation by the induced immunoproteasome. Indeed, Sefert et al. have shown a direct effect between upregulation of oxidized protein levels and DRiPs, and their clearance via immunoproteasomes [60]. To study the effects of proteasome modifications independently of other factors, we performed in vitro experiments using isolated proteasomes, immunoproteasomes and isolated PA28 caps to examine direct effects of altered proteasome activity on mHtt(Q97)exon1 degradation. Activation of the proteasome via opening of the 20S barrel by SDS or RPT peptides, to mimic gate opening via the 19S cap, resulted in increased degradation of mHtt(Q97)exon1. However, whereas activation by P28αβ resulted in more activity labeling by ABPs when compared to the other activators, degradation of mHtt(Q97)exon1 did not occur. Removal of the proline-rich region allowed degradation by PA28-capped proteasomes, suggesting that the folded mHtt fragment simply cannot enter PA28-capped proteasomes. Unfolding by the 195 cap may therefore be needed to enable entrance into the proteasomes. Alternatively, degradation of mHtt by PA28αβ-capped proteasomes may be improved by chaperones. Whereas various chaperones have been identified that prevent mHtt aggregation [82-88], no direct link between mHtt solubilization by chaperones and subsequent targeting for degradation by the proteasome has been established.

Yet, the expanded polyQ stretch is considered as the obstacle in mHtt degradation, but we show here that proteasome activation by PA28αβ improves degradation of polyQ peptides with a disease-related length. This is in agreement with earlier studies that showed proteasomal cleavage of short polyQ-peptides in the presence of the PA28γ (K188E) activator [27]. Additionally, a role in the clearance of misfolded proteins during oxidative stress has been assigned to PA28αβ [70]. These findings indicate that PA28αβ improves clearance of misfolded and aggregation-prone polyQ peptides, but is unable to deal with folded mHtt. When mHtt can be unfolded first by the 195 cap, PA28αβ may become more relevant in mHtt processing, either by forming hybrid proteasomes or by acting downstream of the 26S proteasome to target released polyQ products. However, in this scenario it is crucial that mHtt is efficiently targeted for 19S recognition.

Another interesting feature for modulation of proteasomal activity is the proposed interplay between PA28 and HSP90. HSP90 is important for the maintenance of intracellular homeostasis by supporting protein folding as a molecular chaperone [89, 90]. HSP90 is also involved in activation and capping of proteasomes and is, in association with the 20S proteasome, responsible for degradation of partly-unfolded oxidized proteins [91, 92]. Since PA28 has been identified as a crucial mediator in the refolding mechanism of HSP90, PA28-mediated refolding of protein substrates by HSP90 and degradation by the proteasome may be linked [93, 94]. This hypothesis is supported by the molecular-coupling hypothesis that suggests that specific inserts at the exterior of the PA28 cap function as binding domains for chaperones, thus targeting unfolded proteins to the proteasome [95]. In addition, it was recently reported that HSP90 is indeed involved in the solubilization of mHtt [88].

Modulation of proteasomal activity

When isolated mHtt is degraded by proteasomes in vitro, it appeared that immunoproteasomes can degrade mHtt with highest efficiency. Replacing all constitutive catalytic sites by immuno catalytic sites results in an general upregulation of trypsin-like and chymotrypsin-like activity [38, 40]. However, focusing on de activity changes by individual subunits, the largest difference is caused by the replacement of β1 for β1i. Replacement by β1i reduces caspase activity, and increases chymotrypsin-like activity [96]. Experiments with specific inhibitors showed that β5, which has chymotrypsin-like activity, is mainly responsible for cleavage between glutamines, thus supporting the conclusion that mHtt is more efficiently degraded by immunoproteasomes. However, when we extrapolated these in vitro findings to cells, we did not find significant differences, either in polyQ peptide cleavage or mHtt degradation. The inability of immunoproteasomes to accelerate mHtt degradation in living cells may be due to inefficient targeting of mHtt to (immuno)proteasomes, which is in agreement with the findings that cytoplasmic mHtt is to a large extent cleared by autophagy [61]. This, however, does not explain why immunoproteasomes do not degrade Q8-peptides faster in zymography assays.
The results that we obtained with subunit-selective proteasome inhibitors seem contradictory with the data showing that β5 is responsible for quenched Q8-peptide cleavage. Yet, our in vitro data indicates increased clearance of mHtt after β5 inhibition, which may be caused by the (relative) increase of the remaining proteasomal activities, thereby altering protein turnover and generating different products [97]. Indeed, we observed that inhibition of β5 induces increased ABP labeling of the β1 site of isolated proteasomes (Fig. 5B). Additionally, we show by specific inhibition of β1 that this site is important for the generation of a specific cleavage site in the C-terminus and in cells it causes a small increase in soluble mHtt levels. This may suggest that an improved function of β1, maybe in concert with β2, improves mHtt degradation.

On the basis of this data we conclude that modification of the catalytic activity in the core of the 20S proteasome is effective with respect to mHtt degradation. Remarkably, it seems that not polyQ degradation is improved but that cleavage in the flanking sequences is improving mHtt turnover. This assumption is supported by the observations that immunoproteasomes do not improve Q8-peptide degradation in zymography in gels and that inhibition of β5, which is the major site for polyQ degradation, is not a limiting factor in mHtt processing. Exploring the relevance of cleavage sites in the flanking sequences and identification of the catalytic subunit responsible for this cleavage site may improve mHtt clearance.

Conclusion

In the present study, we show that both isolated proteasomes and proteasomes in cell lysates can cleave within polyQ sequences. Proteasomal degradation of short polyQ peptides and polyQ peptides of disease related length can be increased by the PA28αβ cap. However, the folded structure of the mHtt protein seems to prevent entering into PA28αβ-capped proteasomes, both in vitro and in cells. Modulation of the activity by replacement of the catalytic subunits for immunosubunits or by remodeling activity by using inhibitors did not improve polyQ degradation but enhanced mHtt degradation in vitro, suggesting that the cleavage sites in the flanking sequences of N-terminal mHtt(Q97)exon1 are also relevant for mHtt degradation by the proteasome. However, induction of immunoproteasomes in cells by constant expression of immunosubunits did not improve mHtt degradation. Insufficient targeting towards proteasomal degradation may be the underlying cause of this observation. Inhibition of the β5 activity did show a decrease in mHtt levels in cells, however activation of the autophagy machinery due to proteasomal inhibition has to be excluded.

Finally, while we focussed on proteasomal degradation of mHtt, recent reports also suggest a role for immunoproteasomes in maintenance of cellular homeostasis [60, 70, 98, 99]. Prior to inclusion body formation, proteasomes may become temporarily impaired due to an overload of the proteostasis network, leading to misfolding and accumulation of other proteasomal substrates that may start to aggregate [24, 25]. It will be interesting to study the indirect effects of proteasome modulation in HD, as the burden of aggregation-prone substrates in the cell could be counter-acted by immunoproteasomes which are presumably more efficient in degrading ubiquinated conjugates than constitutive proteasomes. Induction of immunoproteasomes in HD patients has already been established, though as a secondary response to inflammation [51, 52]. Earlier activation of the UPS could decrease general protein accumulations and thereby reducing the disease phenotype.

MATERIALS AND METHODS

Constructs. The constructs mHtt(Q97)exon1-H4 and mHtt(Q97)exon1-ΔPRO-H4 were generated by cloning the mHtt(97Q)exon1 (kindly provided by Prof. RR Kopito, Stanford University, USA) and mHtt(Q97)exon1-ΔPRO (kindly provided by J. Steffan, University of California, USA) sequence with a 5’ Xhol and 3’ BamHI site into a vector encoding a C-terminal 4-His tag (His-HA-His, kindly provided by J. Steffan, University of California, USA). GFP-Ub and GFP-Ub-Q54 were generated as described previously [67]. PA28α and PA28β (kindly provided by Prof. PM Kloetzel, Charité Universitätsmedizin Berlin, Germany) were cloned into an pcDNA3 vector using EcoRI. DNAJB6 was generated after cloning the mHtt(97Q)exon1 (kindly provided by Prof. RR Kopito, Stanford University, USA) by cloning the mHtt(97Q)exon1 (kindly provided by Prof. RR Kopito, Stanford University, USA) into a vector encoding a C-terminal 4-His tag (His-HA-His, kindly provided by J. Steffan, University of California, USA). DNAJB6 was kindly provided by Prof. HS Overkleeft, Leiden Institute of Chemistry, The Netherlands).

Cell culture and transfection. HEK293 and the stable cell line HeLa A2(clone 33) and clone 33/2 (HeLa A2 +β1, β2 and β5i) (kindly provided by Prof. PM Kloetzel, Charité Universitätsmedizin Berlin, Germany) were cultured in DMEM supplemented with 10 % fetal calf serum at 37°C in a 5 % CO₂ atmosphere [71]. HEK293 cells were transfected with jetPEI as described by the manufacturer (Polyplus transfection). The stable cell lines were transfected with X-treme GENE HP DNA transfection reagent as described by the manufacturer (Roche diagnostics, Mannheim, Germany). HeLa A2(clone 33) cells were kept on selection in 2 μg/ml puromycin and 300 μg/ml hygromycin. Cells were stimulated with 100 U/ml IFNy for 72 h unless indicated otherwise. Proteasomes were inhibited with 250 nM epoxomicin, 5 μM β1 inhibitor (MVBl111) [72], 10 μM β2 inhibitor (PR671A) [74] and 2.5 or 5 μM β5 inhibitor (PR523A) (73) for 16 h (Selective inhibitors are kindly provided by Prof. HS Overkleeft, Leiden Institute of Chemistry, The Netherlands).

Visualizing peptide degradation in gel. HEK293 cells were harvested in TSDG buffer (10 mM Tris pH7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA and 8 % glycerol) and lysed by 3 freeze/thaw cycles in liquid nitrogen. After centrifugation (15 min, 20,817 x g) the protein concentration of the supernatant was determined by Bradford protein assay (Serva, Heidelberg, Germany). For proteasome labeling 0.5 μM ABP was added to 40μg lysate [54]. For proteasome inhibition 0.5 μM Epoxomicin was added or similar amounts of DMSO in control lysates and incubated for 1 h at 37°C. After incubation 4x Native sample buffer (20 mM Tris pH 8.0, 50 % glycerol, 0.1 % bromophenol blue) was added. The samples were loaded on a 3-12 % NativePAGE Novex Bis-Tris Gels (Innogen, Life Technologies Europe BV, Bleiswijk, Netherlands) and separated by electrophoresis (3 h at 150V). When isolated proteasomes were used, 4 μg of 205 proteasomes were pre-incubated for 1 h with proteasome inhibitors; 10 μM epoxomicin, 2 μM β2 inhibitor (PR671A) and 1 μM β1 (MVBl111) and β5 inhibitor (PR523A). 6 μg of purified PA28αβ caps (kindly provided by Prof. M. Rechsteiner, University of Utah School of Medicine, USA), were added 30 min prior to loading. To detect peptide cleavage in gel, the wet gel slab was incubated directly after electrophoresis for 20 minutes in 20 ml
Modulating proteasome activity in Huntington's disease

Overlay buffer (20 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM ATP) with 400 µM of the quenched peptides. Fluorescent intensities were measured on a Typhoon imager (GE Healthsciences) using the 580 BP 30 filter. For Western blotting, native gels were transferred to a PVDF membrane (Millipore, Bedford, MA, USA) in transfer buffer (25 mM Tris pH 7.5, 192 mM Glycine, 20 % MeOH) using the Cretener blotter (Biorad, Hercules, CA, USA). α2 proteins were detected by the MCP236 antibody (1:1000, kindly provided by Prof. Rasmus Hartmann-Petersen, Biologisk Institut, University of Copenhagen, Copenhagen), PA28α were directed against RVQPEAQAKVDVFRED (1:3000, kindly provided by Prof. M. Groettrup, University of Konstanz, Germany) [100] and antibody detection was done by the Odyssey detection system (LI-COR Biosciences, Lincoln, NE, USA).

Western blot analysis and filtertrap assay. Cells were harvested in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton-X100, supplemented with complete mini protease inhibitor cocktail (Roche)). After centrifugation (15 min, 20.817 x g) the concentration of the supernatant was determined by Bradford protein assay (Serva, Heidelberg, Germany). 30 µl supernatant was boiled for 10 min at 99 C with 6x sample loading buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % glycerol, 6 % 1-mercaptoethanol, bromophenol blue) added. Proteins were fractionated on 12,5 % SDS-PAGE gels and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Western blot membranes were blocked with 5 % milk, incubated with primary antibodies anti-polyQ 1C2 (1:1000, Millipore, MAB1574), anti-polyQ (1:1000, Sigma-Aldrich 385H10), polyclonal rabbit anti-GFP (1:1000, kindly provided by Prof. J. Neefjes, NKI, The Netherlands), anti-β-actin (1:1000, Santa Cruz, SC-130656), anti-α2 (1:1000, MCP236, kindly provided by Prof. Rasmus Hartmann-Petersen, Biologisk Institut, University of Copenhagen, Copenhagen), anti-PA28α (1:3000, kindly provided by Prof. M. Groettrup, University of Konstanz, Germany) [100], anti-β (1:1000, Abcam, ab3328), anti-2 (1:2000, kindly provided by Prof. M. Groettrup, University of Konstanz, Germany) [81] and subsequently incubated with secondary antibodies IRDye 680 or IRDye 800 (1:10,000; LI-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (Licor). Filtertrap assay was performed with the pellet obtained after centrifugation of the cell lysate. Pellet with aggregates was resuspended in benzonase buffer (1 mM MgCl₂, 50 mM Tris/HCl pH 8.0 and Benzonase freshly added (Merck, Darmstadt, Germany) and incubated for one hour at 37°C with. Reaction was stopped by adding 2x termination buffer (40 mM EDTA, 4 % SDS, 100 mM DTT fresh). Samples with 50 µg protein extract, as determined from the supernatant, were diluted in 2 % SDS wash buffer (2 % SDS, 150 mM NaCl, 10 mM Tris/HCl pH 8.0). Cellulose acetate membrane (Schleicher & Schuell) with 0.2 µm pore size was pre-equilibrated in 2 % IPG buffer (pH 3-10 NL, GE Healthcare) and incubated o/n at room temperature. IEF was performed on a Protean IEF Cell (Biorad, Hercules, CA, USA) using the following program; 0.1min 50 V, 30 min 200 V; 30 min 200 V, 30 min 400 V, 30 min 600 V, 60 min 3500 V, 240 min 3500 V, 10 min 200 V. After focusing, the strips were incubated for 0.5 h in equilibration buffer (50 mM Tris pH 8.8, 6 M Urea, 30 % (v/v) Glycerol, 20 % (w/v) SDS, BPB) with 10 mg/ml fresh DTT. Subsequently, the strips were directly transferred in equilibrium buffer with 25 mg/ml IAA and incubated for 0.5 h. The strips were recovered, loaded on top of a 12 % SDS-PAGE gel and fixed in agarose sealing solution (15 % w/v glycerol, 1 % agarose, 1x Lammmi electrophoresis buffer, BBP). Electrophoresis was performed at 30 mA per gel. Fluorescent detection was done on a Trio Typhoon (GE Healthcare) 580 BP 30 filter to detect the ABP.

Supplementary fig. 1. 20S proteasomes are activated when only PA28α is overexpressed.

HEK293 cells were transfected with PA28α or PA28β for 72 h. After harvesting cells were incubated with ABP and loaded in a native gel. Scanning for fluorescence revealed a shift towards PA28-capped proteasomes compared to the non transfected control cells when PA28α or PA28β alone were expressed. Immunoblotting for α2 determined the presence of 20S complexes.
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Modulating proteasome activity in Huntington's disease


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SUMMARY AND CONCLUSION
In this thesis, we examined the role of the proteasome in Huntington’s disease by addressing the following questions. Are intracellular polyQ fragments peptidase resistant and aggregation prone? Can we prevent their aggregation by chaperones? Are proteasomes sequestered and inactivated in HD? Can proteasomes degrade polyQ-expanded mHtt fragments, and if so, can we modify proteasome activity to improve mHtt degradation? These questions should resolve whether proteasomes are the good or the bad guys in HD, and whether manipulating proteasomal function can be used as a therapeutic strategy. To date, the proteasome has been considered as the bad guy in HD due to either the release of toxic polyQ fragments when degrading mHtt or due to its impairment.

Summary

In chapter 1 we give an short introduction to HD and proteasomes and their connection with each other in the cell, and we introduce the topics discussed in this thesis. In chapter 2 we review studies related to the role of proteasomes in HD to get a better insight in the current views and perspectives. The general idea is that proteasomes are impaired in HD and are unable to cleave polyQ sequences, although some studies show contradictory results. This may partly be due to the comparison of in vitro data to animal studies. Performing additional studies in living cells could give important answers that cannot be found in in vitro or in animal studies. In addition, we discuss the composition of the proteasome and how the activity of the proteasome can be modified by immunosubunits and proteasome activators.

The claim that proteasomes are unable to cleave inside polyQ sequences led to two possible scenarios. First, proteasomes could become clogged by the expanded polyQ sequences as they cannot be released by the proteasome. Second, polypeptide fragments are released into the cellular environment where they rapidly initiate aggregation. In chapter 3 we show that when polyQ peptide release by the proteasome is mimicked, aggregation is initiated when the peptides exceed the disease-related threshold of approximately 37 glutamines. As has been observed in HD, other proteins with short polyQ tracts are recruited into polyQ peptide-initiated aggregates, as well as components of the UPS and chaperones, albeit during different stages of aggregation. Taken together, this indicates that when proteasomes release expanded polyQ peptides, it represents a model for aggregate formation that is common in all polyQ disorders.

In chapter 4, we examined whether the chaperones DNAJB6 and DNAJB8, which suppress aggregation and toxicity of polyQ-expanded proteins, also suppress aggregation induced by polyQ peptides. We show that both chaperones efficiently reduce aggregation of polyQ peptides, whereas the soluble levels of these peptides increase. Therefore, we conclude that these chaperones keep polyQ peptides in a soluble form, which allows degradation of these hazardous fragments before aggregation can occur. Furthermore, we also observed that these chaperones are recruited into polyQ peptide aggregates, which is a contra-productive process as the sequestered chaperones cannot affect peptide aggregation anymore. This data is in agreement with the findings of Månsson and colleagues, who showed that DNAJB6 efficiently prevents aggregation of polyQ peptides in vitro, but not of polyQ-expanded proteins.

While polyQ peptides were proven to be aggregation prone and initiate similar aggregation features as observed in HD, we next examined whether proteasomes are restricted in degrading polyQ-expanded mHtt fragments. In chapter 5, we studied whether proteasomes are capable of degrading mHtt entirely or whether expanded polyQ peptides are released. In order to circumvent degradation by the autophagic pathway, we added a degradation signal to mHtt for targeting towards proteasomal degradation. In a ubiquitin-dependent fashion and in agreement with the Nend rule, mHtt became a short-lived protein that was rapidly recognized and degraded by the proteasome. Surprisingly, no aggregation was observed when mHtt was targeted to the proteasome, and fragments containing the polyQ sequence were only temporarily observed in vitro. This indicates that proteasomes can efficiently degrade mHtt both in cells and in vitro, but we cannot exclude that the proteasome generates polyQ peptides while degrading the mHtt protein and that these polyQ peptides are subsequently degraded via additional rounds of 20S degradation in vitro or by other proteasomes or peptidases in vivo.

To further explore the role of the proteasome in HD, we next focused on proteasomal functioning in cells expressing mHtt. The questions that we wanted to address were, if proteasomes are indeed irreversibly sequestered into aggregates, is the activity affected and can we manipulate proteasome activity to improve mHtt degradation? To answer these questions, we developed various methods to examine proteasome subunit incorporation, intracellular proteasome distribution and dynamics, proteasome activity and interaction of proteasomes with proteasomal activators. In chapter 6, we describe these methods and show that the efficiency of incorporation dramatically differs between various fluorescently-tagged proteasomal subunits, with β5i being the best candidate to be used for proteasome studies. We further explored and compared various methods to determine proteasome activity in cells and cell lysates, and after separation on SDS and native gels. Finally, we discuss how interactions between the 20S core and proteasome activators, such as the PA28αβ activating cap, can be measured by FRET-FLIM in living cells and which steps are needed to optimize interactions for FRET-FLIM measurements.

These techniques, we used to challenge the generally accepted concept of proteasome impairment in HD, which is mainly based on immunostainings of fixed cells and short-term FRAP experiments. As a consequence, it is assumed that proteasomes may become impaired due to an irreversible withdrawal from the cell when recruited into aggregates, or proteasomes could become impaired due to clogging by the aggregating polyQ fragments. In chapter 7 we show by fluorescence pulse-chase experiments that proteasomes are not trapped in aggregates, but are dynamically recruited albeit with slow kinetics. When proteasomes that are recruited into aggregates, were visualized in cells with fluorescent activity based probes, we observed that these proteasomes are active and accessible for substrates, indicating that the UPS is not as severely affected by aggregate formation as assumed and that proteasomes remain functional in HD.
Since we show that proteasomes are able to degrade mHtt completely and its functioning is not as severely affected as initially suggested, we examined in chapter 8 whether modulation of the proteasomal activity improves mHtt clearance. *In vitro* degradation experiments revealed that activation of the 20S proteasome via opening of the α-ring accelerates mHtt degradation, although activation by the PA28αβ cap prevented folded mHtt from entering the catalytic cavity. When we modulated proteasome activity by exchanging constitutive catalytic subunits by immunosubunits, or by specific inhibition of the β5 activity, we observed improved mHtt degradation in *vitro*. Inhibition of β5 also resulted in reduced mHtt levels in cells, whereas exchanging the active sites in cells did not result in improved degradation. We conclude that modifying proteasomal activity does affect mHtt cleavage and improves its degradation but these results cannot simply be translated into a cellular system, most likely due to inefficient targeting of mHtt to the proteasome and competition by the autophagic pathway.

**Conclusion and future perspective**

In conclusion, we show in the present thesis that mHtt can be cleared by proteasomes *in vitro* and in cells, but apparently not efficiently enough to prevent protein accumulation in cells. By using microscopy techniques in living cells, we show that proteasomes remain functional when recruited into polyQ initiated IBs. In addition, modulation of proteasomal activity has proven to be an effective strategy for improving mHtt degradation, although targeting of mHtt for proteasomal degradation appears to be insufficient in cells. Our study contributes to a better understanding of proteasomal functioning in HD. Especially, since we give new perspectives on mHtt processing by proteasomes, which contradict the general assumptions. We propose that proteasomes may become a target for therapeutic intervention, particularly for clearance of nuclear mHtt fragments that cannot be cleared by autophagy. However, targeting of mHtt towards proteasomal degradation should be optimized to increase the effects of proteasomal modulation. Interestingly, we recently observed that nuclear mHtt fragments are ubiquitinated in a different way when compared to cytoplasmic mHtt, which may explain the low efficiency in mHtt turnover by proteasomes in cells. Whereas mHtt may be poly-ubiquitinated in the cytoplasm in order to facilitate degradation via the autophagic machinery, the limited levels of nuclear ubiquitinated mHtt may be due to the absence of a dedicated E3 ligase, thereby preventing mHtt degradation by the 26S proteasome.

*Månsson C, Kakkar V, Monsellier E, Härmark J, Kampinga HH, Melki R, Emanuelsson C, DNAJB6 is a peptide-binding chaperone which can suppress amyloid fibrillation of polyglutamine peptides at substoichiometric molar ratios, Cell Stress Chaperones, 2013*

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**Final figure. Illustration of proteostasis components involved in mHtt degradation and their intracellular connections.**

The research questions that we addressed in this thesis are indicated by the chapter numbers where they are discussed.
ADDENDUM

Nederlandse samenvatting en conclusie
Curriculum Vitae
Portfolio
Publications
Abbreviations
Dankwoord
**Korte introductie**


In de ziekte van Huntington is het huntingtin eiwit gemuteerd, waardoor een reeds aanwezige reeks van glutamine amino zuren (polyQ) in het eiwit verder expanderen tot een reeks van meer dan 37 glutamines achter elkaar. Hierdoor kan het eiwit niet goed meer functioneren en gaat het aggregeren. Dit resulteert uiteindelijk in celdood gevolgd door neurodegeneratie, hetgeen een symptoom van de ziekte van Huntington is. Dit suggereert dat het proteostasis netwerk niet goed genoeg functioneert om het geneesmiddel huntingtin (mHtt) te kunnen handhaven. De rol van het proteasoom in dit proces kan echter niet beantwoord in vitro in levende cellen kan daarom antwoorden genereren die men in diermodellen niet kan beantwoorden

De veronderstelling dat proteasomen niet kunnen knippen in een glutamine sequentie leidt tot twee mogelijke scearios. Het eerste scenario is dat proteasomen letterlijk verstopt raken door de lange polyQ sequenties welke in het proteasoom blijven steken. In het tweede scenario worden de polyQ peptiden niet door het proteasoom afgebroken uitgescheiden in het cytoplasma of de celkern om vervolgens te aggregeren. In hoofdstuk 3 laten we zien dat wanneer we het tweede scenario nablootstellen, de polyQ peptiden met een ziekte gerelateerde lengte van meer dan 37 glutamines niet worden afgebroken door peptidasen en vervolgens aggregatie initiëren. Verder laten we zien dat, net als in de ziekte van Huntington, andere eiwitten met korte glutamine reeksen, maar ook componenten van het ubiquitine proteasoom systeem en chaperonnes, in de polyQ peptide aggregaten accumuleren gedurende verschillende stadia van het aggregatieproces. Hieruit kunnen we concluderen dat wanneer polyQ peptiden worden uitgescheiden door het proteasoom, dit een model is voor aggregaat vorming in alle polyQ ziekten.

In hoofdstuk 4 hebben we onderzocht of de chaperonnes DNAJB6 en DNAJB8, waarvan is aangetoond dat ze aggregatie en toxiciteit van polyQ eiwitten kunnen verminderen, ook aggregatie van polyQ peptiden kunnen reduceren. We laten zien dat terwijl deze chaperonnes efficiënt aggregatie verminderen, de diffuse fractie van de peptiden juist toeneemt. Hieruit concluderen we dat de chaperonnes polyQ peptiden in een opgeloste staat houden zodat ze beter gepresenteerd kunnen worden aan afbraaksystemen in de cel, waardoor ze de kans krijgen te aggregeren. Daarnaast konden we aantonen dat wanneer de chaperonnes aanwezig zijn in aggregaten, deze niet meer functioneel zijn en geen effect hebben op de aggregatie van polyQ peptiden. Deze bevindingen zijn in overeenstemming met data van Månsso et al.. In hun in vitro studie laten zij zien dat DNAJB6 aggregatie van polyQ peptiden kan voorkomen, maar niet de aggregatie van polyQ eiwitten.
Omdat we hebben aangetoond dat de polyQ peptiden die mogelijk door het proteasoom gegeneereerd worden aggregeren en ook hetzelfde accumulatiepatroon vertonen als mHtt eiwitten, was de volgende vraag of proteasomen daadwerkelijk niet in staat zijn om mHtt, met een expansie in de polyQ reeks, volledig af te breken. In hoofdstuk 5 hebben we onderzocht of proteasomen het mHtt eiwit in zijn geheel afbreekt of dat inderdaad de polyQ reeksen als peptiden worden uitgescheiden, zoals gepubliceerde in vitro data suggerereert. Om effecten van autofagie te omzeilen hebben we een afbraaksgaans aandacht voor het proteasoom we gekoppeld om deze direct naar het proteasoom te sturen. Hiertoor werd het eiwit een kortlevend eiwit dat snel en ubiquitine-afhankelijk door het proteasoom werd herkend en afgebroken. Verassend genoeg werd er geen aggregatie meer waargenomen wanneer mHtt direct naar het proteasoom werd gestuurd, en polyQ fragmenten werden alleen tijdelijk waargenomen in in vitro experimenten. Dit laat zien dat proteasomen beter in staat zijn om mHtt volledig af te breken, zowel in cellen als in vitro. We kunnen echter niet uitsluiten dat polyQ peptiden wel gegeneereerd worden, maar dat ze vervolgens in meerdere afbraakrondes verder worden afgebroken door het 20S proteasoom in vitro of door andere proteasen of peptidases in cellen.

Om de rol van het proteasoom in de ziekte van Huntington nog uitgebreider te onderzoeken, hebben we ons vervolgens gefocust op het functioneren van proteasomen in cellen die mHtt tot expressie brengen. De vragen die we wilden beantwoorden waren, of proteasomen onomkeerbaar accumuleren in de polyQ aggregaten, of de proteasoom activiteit verminderd in cellen met aggregaten en of de activiteit van proteasomen kunnen moduleren zodat ze beter in staat zijn om mHtt eiwitten af te breken. Om deze vragen te beantwoorden hebben we verschillende methoden ontwikkeld. Met deze methoden kunnen we de incorporatie van proteasoomsubunits, diens intracellulaire distributie en dynamiek, proteasoomactiviteit, en interactie tussen proteasoom complexen en proteasoomactivatoren bestuderen. In hoofdstuk 6 beschrijven we deze methoden en laten we vervolgens zien dat de incorporatie-efficiëntie van verschillende proteasoomsubunits erg uiteenloopt, waarbij β5i het meest representatief is voor proteasoomstudies. Verder hebben we verschillende methoden om proteasoomactiviteit te bepalen onderzocht en vergeleken. Hierbij konden we proteasoom activiteit in cellen, in cel lysaten, na scheiding op SDS-PAGE en in native gels bepalen. Als laatst bespreken we ook hoe interacties tussen het 20S complex en proteasoomactivatoren, zoals de PA28β ring, gemeten kunnen worden door middel van FRET-FLIM in levende cellen en welke stappen genomen moeten worden om deze interactiemesingen te optimaliseren.

Met behulp van deze technieken betwisten we het algemeen geaccepteerde concept dat proteasomen niet goed functioneren in de ziekte van Huntington, wat voornamelijk gebaseerd is op immunokleuringen van gefixeerd cellen en kortdurende FRAP experimenten. Op basis van deze bevindingen wordt aangenomen dat proteasomen minder functioneel zijn doordat ze ontstoken zijn aan cellulair metabolisme wanneer ze in aggregaten accumuleren of doordat ze geblokkeerd raken door de polyQ fragmenten. In hoofdstuk 7 laten we door middel van fluorescerende pulse-chase experimenten zien dat proteasomen niet onomkeerbaar accumuleren in aggregaten, maar dat ze dynamisch blijven hoewel met lage kinetiek. Wanneer we de activiteit van proteasomen in aggregaten visualiseren door middel van fluorescente probes zien we dat deze proteasomen actief zijn en toegankelijk voor substraten. Dit toont aan dat het ubiquitine-proteasoom systeem niet geïnactiveerd wordt door aggregaat vorming zoals wordt aangenomen, maar de ze functioneel blijven in de ziekte van Huntington.

Omdat we hebben aangetoond dat proteasomen in staat zijn om het mHtt eiwit volledig af te breken en dat proteasomen functioneel blijven, onderzoeken we in hoofdstuk 8 of modificatie van de activiteit van het proteasoom, het opruimen van mHtt kan bevorderen. In vitro afbraakexperimenten laten zien dat mHtt versneld wordt afgebroken na activatie van het 20S proteasoom door opening van diens α-ring. Activatie door de β5 ring verhinderde echter dat gevouwen mHtt het 20S complex kon binnen gaan. Wanneer de activiteit van het proteasoom werd veranderd door de normale actieve subunits te vervangen door immunosubunits of door specifiek het actieve subunit β5 te remmen, werd mHtt beter afgebroken in vitro. Remming van β5 resulteerde ook in een afname van mHtt eiwitten in cellen, terwijl de uitwisseling van actieve subunits geen effect had op mHtt afbraak in de cel. Hieruit kunnen we concluderen dat wanneer de activiteit van het proteasoom gemoduleerd wordt, dit wel degelijk invloed heeft op de afbraak van mHtt waardoor deze ook verbetert. De bevindingen zijn echter moeilijk te vertalen naar celsystemen, waarschijnlijk omdat mHtt niet goed door het proteasoom herkend wordt voor afbraak, en door competitie met autofagie.

Conclusie en perspectieven

Concluderend kunnen we stellen dat we in dit proefschrift laten zien dat mHtt door proteasomen kan worden afgebroken, zowel in vitro als in cellen. Dit gebeurt klaarblijkelijk niet efficiënt genoeg aangezien aggregaat vorming niet kan worden voorkomen. Door gebruik te maken van levende cel microscopic, laten we zien dat proteasomen functioneel blijven wanneer ze aanwezig zijn in aggregaten. Daarnaast hebben we aangetoond dat modulatie van het proteasoom en daarmee ook de activiteit, een effectieve strategie is om afbraak van mHtt door het proteasoom te bevorderen. Het lijkt er echter op dat de signalering voor afbraak, door middel van ubiquitinering, niet toereikend is in de cel. Onze bevindingen dragen bij aan een beter begrip van de functie van proteasomen in de ziekte van Huntington, met name van de nieuwe inzichten die we leveren in het afbraakproces van mHtt, welke in tegenspraak zijn met algemene opvattingen. Wij stellen voor dat het proteasoom een mogelijk doel is voor therapeutische interventie, zeker voor het afbreken van mHtt in de kern van de cel omdat autofagie daar afwezig is. We zullen in de toekomst de signalering voor herkenning door het proteasoom moeten optimaliseren zodat proteasoommodulatie een groter effect heeft in de cel. Een fascinerende observatie is dat mHtt in de kern anders geubiquitineerd is dan mHtt in het cytoplasma, wat mogelijk de inefficiënte herkenning door het proteasoom in de cellkern verklaart. Mogelijk wordt mHtt in het cytoplasma geubiquitineerd om vervolgens naar autofagie te worden gestuurd. Terwijl de geringe poly-ubiquitnatie in de kern wijst op een mogelijke afwezigheid van de juiste E3-lijgase, waardoor mHtt niet kan worden afgebroken door het proteasoom.
CURRICULUM VITAE

Sabine Schipper-Krom werd geboren op 6 juli 1983 te Beverwijk. In 2001 behaalde zij haar HAVO diploma aan het Jac P. Thijssse College te Castricum. Het daaropvolgende jaar is zij gaan werken en reizen in Australië en Thailand om vervolgens in 2003 aan haar studie Biologie & Medisch Laboratoriumonderzoek aan de Hogeschool Inholland te Alkmaar te beginnen. Tijdens deze studie volbracht zij 2 stages. De eerste stage was bij de afdeling Immunohaematologie Experimenteel bij Sanquin Bloedvoorziening te Amsterdam (onder supervisie van Dr. M. de Haas), de tweede stage was bij de afdeling Celbiologie en Histologie aan het AMC te Amsterdam (onder supervisie van Dr. E.A.J. Reits). Gedurende haar HLO opleiding is zij lid geweest van de opleidingscommissie ter ontwikkeling en verbetering van de opleiding. In 2007 rondde zij haar opleiding cum laude af om vervolgens door te stromen in het masterprogramma Biomolecular Sciences aan de VU. Gedurende dit masterprogramma heeft zij twee stages volbracht. De eerste stage was op de afdeling Celbiologie aan het Nederlands Kanker Instituut te Amsterdam (onder supervisie van Prof. dr. J. Neefjes). Gedurende deze stage deed zij onderzoek naar het transport en de fusie van late endosomen. De tweede stage was wederom op de afdeling Celbiologie en Histologie aan het AMC te Amsterdam (onder supervisie van Dr. E.A.J. Reits). Tijdens deze stage deed zij onderzoek naar de rol van proteasomen in de ziekte van Huntington. De scriptie die zij heeft geschreven aangaande dit onderwerp is beloond met een Anna Wichersprijs. Haar masterdiploma in de studierichting Molecular Cell Biology behaalde zij in 2009. In mei 2009 zette zij haar stage onderzoek naar de rol van proteasomen in de ziekte van Huntington voort in een promotieonderzoek, resulterend in dit proefschrift.

PORTFOLIO

PhD period: May 2009-december 2013
Name PhD promotor: Prof. dr. C.J.F. van Noorden
Name PhD co-promotor: Dr. E.A.J. Reits

Courses
- AMC World of science
- Advanced Microscopy course
- Two weeks training traject in the proteasome lab of Prof. P.M. Kloetzel

Lectures and Masterclasses
- Weekly department seminars
- Masterclass by Prof. D.C. Rubenzstein

Year ECTS

2010 0.70
2011 3.10
2011 0.20
2012 2.00
2013 2.00
2013 1.75
2013 1.50
2013 0.75
2013 0.75
2012 2.50
2012 2.50
2012 2.50
2013 2.50
2012 2.00
2011 2.50
2011 1.75
2011 1.50
2011 0.75
2013 0.75
2011 0.75
2011 0.20
2013 2.25
2013 0.75
2013 0.75
2011 0.20
2010 0.70

Masterclass by Prof. J. Neefjes
Masterclass Prof. A. Ciechanover

Conferences and presentations
- GRC, CAG Triplet Repeat Disorders, Waterville Valley, USA 2013 2.25
- Poster presentation
- The 3th Dutch HD Symposium, Leiden 2013 0.75
- Presentation
- 3th Dutch Chaperone Meeting, Utrecht 2013 0.75
- Poster presentation
- FEBST, 7th Inproteolys meeting, Kusadasi, Turkey 2013 1.50
- Poster presentation
- The 2th Dutch HD Symposium, Maastricht 2013 0.75
- Presentation
- GRC, CAG Triplet Repeat Disorders, Lucca, Italy 2011 1.75
- Poster presentation (poster award)
- The 1st Dutch HD Symposium, Groningen 2011 0.75
- Presentation

TEACHING

Lectures
- Cellulaire Oncologie 2011-2012 2.00
- bachelor BioMedische Wetenschappen (UvA)
- Clinical Cell Biology 2011-2012 2.00
- master BioMedische Wetenschappen (UvA)
- College zenuwweefsel 2011-2012 2.00
- bachelor Medische Informatiekunde (UvA)

Supervision
- Master student, Biomedical Sciences, Medical Biology (UvA) 2011 2.50
- Bachelor student, Biomedical Sciences, Medical Biology (UvA) 2012 2.50
- Bachelor student, Biomedical Sciences, Medical Biology (UvA) 2012 2.50
- Master student, Biomedical Sciences, Neurobiology (UvA) 2013 2.50


*these authors contributed equally to this work

ABBREVIATIONS

- 20S: constitutive proteasome
- 20Si: immunoproteasome
- 3-MA: 3-Methyladenine
- ABP: activity based probe
- ATG: autophagy related gene
- ATP: adenosinetrifosfaat
- BaFA1: Bafomyycin A1
- C4: tetracycline
- CBP: CREB-binding protein
- DMSO: Dimethyl sulfoxide
- DRIPs: defective ribosomal intermediate products
- FLIM: fluorescence lifetime imaging
- FLIP: fluorescence loss in photobleaching
- FRET: fluorescence resonance energy transfer
- GFP: green fluorescent protein
- HD: Huntington’s disease
- HDAC: histone deacetylase
- HEK: human Embryonic Kidney
- HeLa: Henrietta Lacks (human cervix epithelial)
- His: histidine
- HPRT: hypoxanthine phosphoribosyltransferase
- HSC: heat shock cognate protein
- HSP: heat shock protein
- Htt: huntingtin
- IB: inclusion body
- IFNγ: interferon gamma
- K: lysine
- LC3: microtubule-associated protein 1 light-chain 3
- LC-MS: Liquid chromatography–mass spectrometry
- mHtt: mutant huntingtin
- MODC: mouse ornithine decarboxylase
- MS: mass spectrometry
- N2A: neuroblastoma 2A (mouse)
- PA: proteasome activator
- PolyQ: polyglutamine
- PSA: Puromycin-sensitive aminopeptidase
- Q: glutamine
- QBP: Q-binding protein
- R: argenine
- RPT: regulatory particle triple-A
- SDS: Sodium dodecyl sulfate
- TBP: TATA- binding protein
- TPII: tripeptidyl peptidase II
- TRIC: TCP-1 Ring Complex
- Ub: Ubiquitin
- UPS: ubiquitin proteasome system
- WT: Wild type
- τₚ: phase lifetime
- UFD: Ub-fusion degradation
- ALIS: aggresome-like induced structures
DANKWOORD

Nou, het is dan zover. Ik heb net de laatste teksten geschreven, mijn proefschrift is klaar!

Ik heb mijn promotieonderzoek altijd met veel plezier en enthousiasme uitgevoerd en hoop dat ik ook een klein stentje heb kunnen bijdragen aan het onderzoek naar de ziekte van Huntington. Ik had mijn promotieonderzoek natuurlijk niet op deze manier kunnen volbrengen zonder de hulp van de mensen om mij heen. Ik wil dan ook de laatste pagina’s van mijn boekje gebruiken om deze mensen te bedanken. Als eerste wil ik graag mijn familie bedanken omdat zij altijd voor mij klaar staan en mij altijd een warm thuis bieden. Mijn moeder wil ik bedanken omdat zij mij altijd mijn eigen keuzes heeft laten maken en mij geleerd heeft veel waarde aan te hechten aan de leuke dingen in het leven. Kees wil ik bedanken voor zijn goede zorgen en vooral voor al zijn gezelligheid. Mijn zus Judith, die ook mijn aller liefste vriendin is en altijd voor me klaar staat, en Wouter wil ik bedanken voor alle gezelligheid, leuke avondjes, weekendjes en vakanties en voor alles wat jullie altijd voor ons doen. Dat ik altijd even heerlijk mijn hart kan lichten en dat we altijd elk moment van de dag welkom zijn. En lieve Isabelle, Bo en Bibi, het is jullie altijd weer gelukkig om met jullie drukte en gekkigheid mijn gedachten weer even te verzetten als ik weer veel met mijn werk in mijn hoofd zat. En natuurlijk wil ik mijn lieve Robbie bedanken, maar dat bewaar ik nog even tot het einde. Verder wil ik ook nog mijn dank betuigen aan Rien Walrave, aangezien diegene is geweest die mijn interesse voor biologie heeft gewekt en mij kennis heeft laten maken met wetenschap. Eric Reits en Ron van Noorden wil ik bedanken voor de mogelijkheid om op de afdeling Celbiologie en Histologie mijn promotie onderzoek te kunnen doen. En dat ik hier de kans heb gekregen om ook mijn deel bij te dragen aan het onderzoek ter voorkoming van de ziekte van Huntington waar ook helaas mijn familie door is getroffen. Ron, bedankt voor al je positieve woorden en voor jouw geloof in mij. Maar natuurlijk ook voor het corrigeren van al die lappen tekst die ik bij je inleverde en waar nogal eens aan gespierd moest worden. Eric, ook bedankt voor al je positiviteit. Als ik dacht dat het weer allemaal nergens toe leidde, wist ik je altijd weer te enthousiasmeren om weer een opening te vinden naar een vervolgonderzoek. Voornamelijk op het moment dat ik alles op moest gaan schrijven en ik dacht dat alles in het water was gevallen, heb je me zo weten te motiveren dat ik uiteindelijk alles toch heb kunnen samenbrengen in dit proefschrift. Joachim Goedhart en Mark Hink wil ik bedanken voor al hun hulp bij het FLIMen en voor het feit dat ik altijd langs kon komen om hun microcoop faciliteiten te gebruiken en dat zij ook altijd klaar stonden om te helpen. Dave Spijer wil ik bedanken voor zijn hulp bij het uitleggen van de TD techniek en alle biochemische weetjes er omheen die ik vol enthousiasme heb proberen in te absorberen, want voor al die kennis heb ik heel veel bewondering. Professor Kloetzel wil ik bedanken voor de vruchtelijke werkbesprekingen en ook grote dank voor de mogelijkheid om 2 weken mee te mogen kijken en verschillende technieken voor het bestuderen van het proteasome te leren. Dit heeft mijn erg geholpen bij het uitvoeren van het verdere onderzoek. Frédéric Ebstein en Andreas Lehmann wil ik bedanken voor hun hulp en uitleg tijdens het leren van deze technieken, en voor het feit dat zij daarna altijd nog bereid waren om mijn vragen te beantwoorden. Ook wil ik de studenten bedanken die bijgedragen hebben aan dit onderzoek, Anouk Zaal en Emma van Bodegraven. Meiden, ik heb het als erg leuk ervaren om jullie te begeleiden en heb ook daar een hoop van geleerd. Jullie waren beiden super gemotiveerd en hebben dus ook erg geholpen. Ik hoop dat jullie allebei over een aantal jaar ook een mooi proefschrift af kunnen leveren, succes bij alles wat nog komen gaat. De ‘microscopiemensen’, Jan, Ron en Henk, en de ‘Oogjes’ wil ik bedanken voor hun hulp bij de dagelijkse belastingen en voor de sociale ‘touch’ op het lab. Ise wil ik extra bedanken omdat zij het lab altijd in het gareel weet te houden en ervoor zorgt dat het almaar blijft lopen. Joanna wil ik bedanken voor de gezellige onderonsjes waarbij we altijd even lekker over alles en iedereen konden klagen, om vervolgens te beseffen dat het almaar zo erg niet is. Ik hoop ook dat jij je promotie straks succesvol afrond en een leuke vervolg baan kunt vinden waar je je helemaal goed bij voelt. En natuurlijk wil ik de meiden uit mijn eigen groep bedanken. Katrin en Anne, jullie hebben beide zeer veel kennis in jullie koppies en bedankt dat ik daar zo af en toe wat van heb mogen meekrijgen en kunnen leren. Anne (Jannie), je bent nu een jaarje bezig en hebt de groep al aardig weten op te fleuren. 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En nu is het klaar. Op naar de volgende uitdaging!
Huntington’s disease is a heritable neurodegenerative disease with a slow but fatal progression. Affected patients suffer from involuntary movements, changes in behavior and cognitive impairment. The disease is initiated by an expansion of the polyglutamine sequence in the huntingtin protein. Due to this expansion, the protein becomes misfolded and forms intracellular aggregates. Normally, the proteostasis network is responsible for maintaining proteins in optimal condition and protects them from all kinds of damage. However, in Huntington’s disease this protective network does not function sufficiently to cope with the increasing burden of misfolded huntingtin proteins. A major player in this network is the ubiquitin proteasome system that degrades proteins. Short-lived or damaged proteins are marked for degradation by a ubiquitin chain and subsequently recognized by the proteasome. After recognition, the substrate protein enters the cylindrical-shaped core of the proteasome and is degraded into smaller fragments. It has been suggested that the ubiquitin proteasome system does not function properly in Huntington’s disease and cannot efficiently clear misfolded huntingtin proteins. In this thesis, the role of the proteasome in Huntington’s disease is studied. Questions such as ‘is the proteasome capable of degrading glutamine sequences?’, ‘is the proteasome still functional in Huntington’s disease?’ and ‘can we improve proteasomal degradation of mutant huntingtin?’ were the basis of an exploration whether proteasomes are contributors to Huntington’s disease or potential therapeutic targets.