The role of the proteasome in Huntington's disease

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

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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 cleavage 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 lacking flanking 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 expanding degraded 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 Epoxomicin 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-exon1-25Q 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 Epoxomicin and 3-MA on the proteasomal catalytic activity in treated Neuro-2a cells, endogenous proteasomes were labeled with a proteasome activity-based probe where the catalytical activities were
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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 proteasomal reporter Ub-G76V-GFP in Neuro-2a cells was analysed. Epoxomicin but not 3-MA increased the protein level of the proteasomal 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 bisarnesial 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

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-disolved and non-disolved (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 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
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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).

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 N-terminal degron on mHtt-exon1 degradation and protein aggregation, Neuro-2a cells were transfected with the constructs Htt-exon1-97Q or Ub-R-KK-Htt-exon1-97Q. Upon expression for 24 hours, cells were treated with the inhibitors Epoxomicin and 3-MA. In contrast to Htt-exon1-97Q, the "N-end rule" Htt protein Ub-R-KK-Htt-exon1-97Q has a low abundance of soluble protein level on western blot and no aggregated mHtt-exon1 detected on filtertrap. Due to the N-degron signal the soluble monomeric Htt-exon1-97Q protein is efficiently targeted to the UPS, as treatment with the proteasomal inhibitor Epoxomicin but not the autophagy inhibitor 3-MA resulted in an accumulation of Ub-R-KK-Htt-exon1-97Q in the soluble and SDS-insoluble fraction (Fig. 3D). This indicates that the specific N-degron signal transforms mHtt-exon1 into a short-lived protein which is targeted via the proteasome before it can accumulate and aggregate within the cell. Smaller partial digest products of the Ub-R-KK-Htt-exon1-97Q protein by the proteasome consisting of putative intact polyQ tracts missing the flanking sequences were not detected on western blot with the specific polyQ antibody 385H10, suggesting that Ub-R-KK-Htt-exon1-97Q is fully degraded by the proteasome.

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.

Fig. 2. Proteasomal degradation of mHtt-exon1 in Atg5-deficient cells.
(A) Quantification of Htt aggregates after expression of Htt-exon1-97Q-C4 in Atg5+/+ and Atg5−/− MEF cells. Six hours after electroporation cells were treated for 16 hours with DMSO or Epoxomicin and fixed on cover slips for staining and aggregate scoring. *p<0.05 (n=3). (B) Western blot analysis for detection of endogenous LC3-I and LC3-II levels in electroporated Atg5+/+ and Atg5−/− MEF cells. (C) The proteasomal catalytic sites (β1, β2 and β5) were labeled in the Neuro-2a cell lysate with the LC3 antibody. β-actin was used as a loading control; *p<0.05 (n=3).

**p<0.01 (n=3).
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 ReAsF 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 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 and Htt-exon1-25Q-GFP or the empty vector as control. Six hours after transfection cells were fixed and aggregates of Q112-peptides co-sequestering the reporter protein Htt-exon1-97Q-C4 for 48 hours. Htt-exon1-97Q-C4 aggregates stained with ReAsH 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-exon1-35Q-GFP. The nucleus is stained with Dapi. Scale bar: 10 µm. (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-exon1-35Q-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 aggregation reporter Ub-Q112-25Q-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 fixed and aggregates of Q112-peptides co-sequestering the reporter protein Htt-exon1-35Q-GFP. The nucleus is stained with Dapi. Scale bar: 10 µm. (F) 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 peptidase II (TPPII), which may 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 in vitro

The previous results with the efficient cellular mHtt degradation, independent of peptidases like TPPII 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 wHtt-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 wHtt-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 3B5H10 against the polyQ tract and HA antibody against the C-terminus. Within 24 hours both mHtt-exon1 (Fig. 5A) and wHtt-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 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 3B5H10, marginal smaller proteasomal cleavage fragments appear within the first 3 hours of digest as putative proteolytic intermediates. After 16 hours of digest only ~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 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 in vitro digest, resulting in unintended protein degradation by the caspase-like activity of the proteasome, purified mHtt-exon1 was incubated with protease K, an enzyme that is capable of cleaving after glutamate but not glutamine. Incubation of mHtt-exon1 with protease K generates a proteolytic product within 2 hours of incubation with a size of ~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 protease K (Fig. 5E). Since glutamine deamidation in the mHtt-exon1 protein would result in various smaller fragments of different sizes after protease K digest, the appearance of a specific monomeric protein band (arrow) and a formic acid-soluble oligomeric species (asterisk) detectable by the polyQ antibody 3B5H10 upon protease K digest but not proteasomal degradation, suggests that the mHtt-exon1 protein is completely degraded by the 20S proteasome.

While mHtt-exon1 and wHtt-exon1 are efficiently degraded by the proteasome, we analysed whether the expanded polyQ stretch affect 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 wHtt-exon1.
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Anti-Htt antibody polycl. 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 polycl 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 polycl. (E) In vitro degradation of the mutant Htt protein with proteasome K. Purified mHtt was incubated with proteasome K at 37°C for the time points indicated. After digest aggregates were separated 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 polycl. The pure polyQ fragment (arrow) and a soluble oligomeric intermediate (asterix) generated by the proteasome K digest were detected by the polycl 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).

wHtt-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.

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 wHtt-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.

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

<table>
<thead>
<tr>
<th>Sequence</th>
<th>PEP</th>
<th>Score</th>
<th># detection events</th>
<th>Intensity</th>
<th># detection events</th>
<th>Intensity</th>
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<tr>
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<td>2.6-11</td>
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<td>1</td>
<td>4E+05</td>
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<tr>
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<tr>
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<tr>
<td>MGMGGGVVPDEYVYD</td>
<td>4.0-09</td>
<td>69</td>
<td>1</td>
<td>8E+05</td>
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</tr>
<tr>
<td>PVDVDPYVNNH</td>
<td>1.6-35</td>
<td>147</td>
<td>2</td>
<td>7E+05</td>
<td>1</td>
<td>7E+04</td>
</tr>
<tr>
<td>PVDVDPYVNWNNH</td>
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<td>2E+05</td>
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DISCUSSION

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-signals 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

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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 proteasomalt 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 non-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 degrad 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 proteinase 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), HSPA3 (HSC70), HSPA9 (mHSP70), HSPA9 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 Spino cerebellar 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 2114A 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 tetracystine 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 Xhol/Ncol fragment followed by klenow fill from the plasmid R15Q (kindly provided by M.D. Kaytor, Emory University, USA) [76] and cloned into a 5’Xhol 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 tetracystrine C4-tag sequence (FLNCPP-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’Xhol and 3’ BamHI site into a vector encoding a C-terminal H4-tag (HA-His-His-HA, kindly provided by J. Steffen, 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), Tppl-/- 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-ethylhenyleneimine) according to the manufacturer’s instructions. Atg5+/+ MEF, Atg5-/- MEF and Tppl-/- 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 TSGD 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 Thphoon imager (GE Healthcare) with the S808P30 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 laemmli 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
Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes

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 20S 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 µM Epoxomicin and DMSO as control were added to living Neuro-2a cells, respectively, incubated for 16 hours and subsequently 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-LLVY-AMC, 150 µM Ac-RLR-AMC and 150 µM Ac-GPDL-ACM (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 20S proteasome in vitro chymotrypsin-like activity of the proteasome was monitored by Suc-LLVY-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 20S 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 20S 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

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, MA81574), anti-polyQ (1:1000, Sigma-Aldrich 3B5H10), 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-[ρ]-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). Filtertrap 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.

ReAASH 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 ReAASH (kindly provided by H. Overkleeft, University Leiden, The Netherlands) and 10 µM 1,2-ethanediithiol (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 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 DM2). 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 immunoprecipitated for 2 hours using EzView Red Anti-HA Affinity Gel (Sigma-Aldrich) to purify Htt-exon1-25/97Q-H4. Proteins were eluated 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. Dahlmann, Charité, Germany) were purified from human erythrocytes as described previously [79].

Expanded polyglutamine-containing N-terminal huntingtin fragments are entirely degraded by mammalian proteasomes
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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, 82] using the Andromeda search algorithm against a manually generated fasta-file containing the sequence of Htt-exon1-25/9Q-H4. Search parameters: Enzyme: any cleavage allowed; max. miscleavages: 40. To determine Htt-exon1-97Q:H4 and 205 proteasome co-precipitated proteins, the 3 kDa filter supernatant (≥3kDa fraction) was diluted in 100 µL 50 mM NH₄HCO₃ (ABC) buffer and trypsin digested at 37 C overnight. Digests were stopped by adjusting the pH with 10 % TFA 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.

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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