The ubiquitin proteasome system in Huntington disease : impairment of the proteolytic machinery aggravates huntingtin aggregation and toxicity

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Chapter 5

Ubiquitin-conjugating enzyme E2-25K / Hip-2 increases aggregate formation and cell death in polyglutamine diseases

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Abstract

Polyglutamine diseases are characterized by neuronal intranuclear inclusions of expanded polyglutamine proteins, which are also ubiquitinated, indicating impairment of the ubiquitin proteasome system. E2-25K (Hip2), an ubiquitin-conjugating enzyme, interacts directly with huntingtin and may mediate ubiquitination of the neuronal intranuclear inclusions in Huntington Disease. E2-25K could thus modulate aggregation and toxicity of expanded huntingtin. Here we show that E2-25K is involved in aggregate formation of expanded polyglutamine proteins and polyglutamine-induced cell death. Both a truncated mutant, lacking the catalytic tail domain, as well as a full antisense sequence, reduce aggregate formation. Strikingly, both E2-25K mutants also reduced polyglutamine-induced cell death. In post-mortem brain material of both Huntington Disease and SCA3, E2-25K staining of polyglutamine aggregates was observed in a sub-set of neurons bearing intranuclear neuronal inclusions. These results demonstrate that targeting by ubiquitination plays an important role in the pathology of polyglutamine diseases.

Introduction

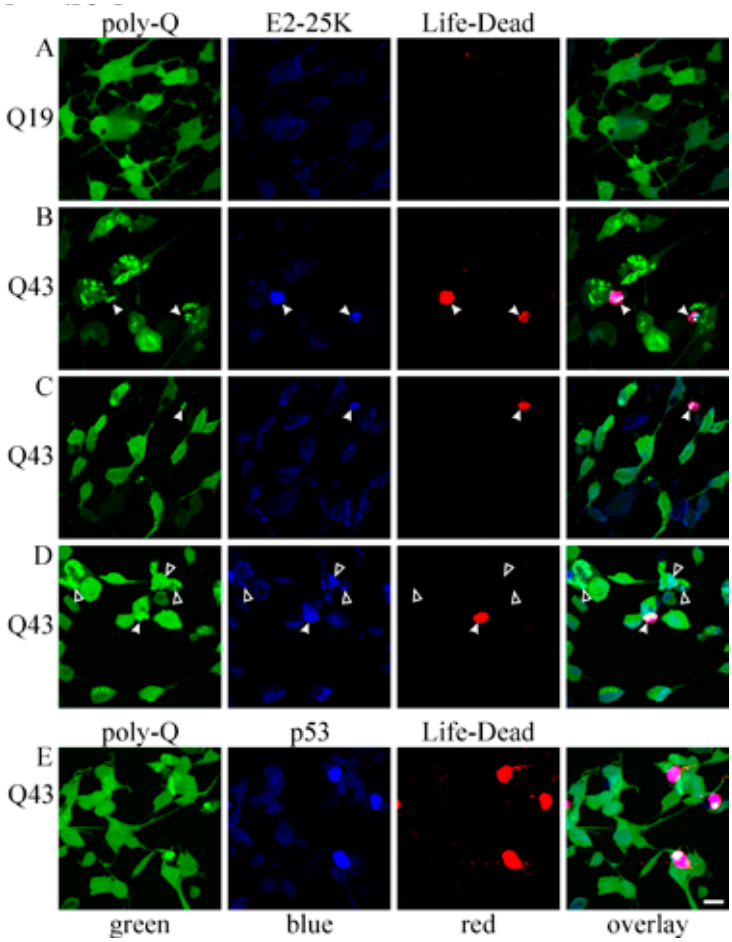
Several neurodegenerative diseases have been shown to be caused by the pathogenic expansion of a polyglutamine repeat, including the Spinocerebellar ataxias (SCAs) and Huntington disease (HD) (Nakamura et al., 2001; Zoghbi and Orr, 2000). Expansion of the repeat above a critical length results in severe neurodegeneration accompanied by the pathological formation of neuronal intranuclear inclusions (NIIs) in the affected areas of the brain (DiFiglia et al., 1997). Although there is still an ongoing debate as to whether these inclusions are detrimental, accumulating evidence points to the favourable formation of large aggregates as a means of sequestering these aberrant proteins (Arrasate et al., 2004; Saudou et al., 1998). However, since the aggregation prone properties of these proteins are the underlying cause of the disease (Perutz et al., 1994; Scherzinger et al., 1997), presumably an early stage of aggregate formation initiates toxicity.

Several studies have implicated the ubiquitin-proteasome system (UPS) in the pathogenesis of polyglutamine diseases and demonstrate an enhancement of neurodegeneration by further impairment of the UPS (reviewed by (Ciechanover and Brundin, 2003)). The fact that NIIs incorporate ubiquitin or ubiquitinated proteins indicates that the aggregating proteins are targeted to, but not efficiently degraded by, the proteasome (DiFiglia et al., 1997; Paulson et al., 1997). *In vitro* studies have furthermore shown that expanded polyglutamines can directly inhibit the proteasome (Bence et al., 2001; Verhoef et al., 2002), resulting in apoptotic cell death (de Pril et al., 2004; Li et al., 2000). In addition, in SCA3 patients, subunits of the 26S proteasome have been shown to be recruited to NIIs (Chai et al., 1999; Schmidt et al., 2002). Finally, in SCA1 transgenic mice, the Purkinje cell pathology was aggravated by mutation of the E6-AP ubiquitin ligase although the number of NIIs was reduced (Cummings et al., 1999).

Recently, we reported that an aberrant form of ubiquitin (UBB^{+1}) accumulates in the NIIs and the cytoplasm of neurons within the affected areas of HD and SCA3 (de Pril et al., 2004). The accumulation of UBB^{+1} in post-mortem brain material of different neuropathological disorders acts as a marker for proteasomal impairment (de Pril et al., 2004; Fischer et al., 2003). Moreover, we demonstrated that UBB^{+1}, being a substrate and an inhibitor of the proteasome, enhances aggregation of expanded polyglutamine proteins and synergistically aggravates polyglutamine-induced cell death. These findings demonstrate the importance of an efficient UPS in neurodegenerative diseases such as the polyglutamine disorders.

Ubiquitin-conjugating enzyme E2-25K (or Hip2; Huntingtin interacting protein 2) is highly expressed in the brain and was found in a yeast two-hybrid screen to interact with huntingtin in a repeat-independent matter (Kalchman et al., 1996). Recently, E2-25K has been implicated in the mediation of amyloid-β neurotoxicity and proteasome inhibition *in vitro* (Song et al., 2003). Despite the interaction with huntingtin and the

Figure 1: E2-25K colocalizes with polyglutamine aggregates in dead cells.



Differentiated SH-SY5Y neuroblastoma cells were lentivirally transduced with truncated huntingtin constructs containing 19 or 43 glutamine repeats (Qxx-HTT-GFP). A cell death assay was performed 4 days post transduction. Cells were subsequently fixed and stained for endogenous E2-25K. Cells that are transduced with the Q19-HTT-GFP construct show a diffuse cytoplasmic staining for E2-25K (A; blue; panel 2) and cytoplasmic localization of Q19-HTT-GFP (green; left panel). Upon transduction with Q43-HTT-GFP we once more find cytoplasmic staining for E2-25K (B-D; blue panel 2) and the formation of aggregates of expanded polyglutamines (green; left panel). Cells that are subject to expanded polyglutamine induced cell death (red; panel 3; cell death reporter) show upregulation or nuclear translocation of E2-25K and coaggregation of the enzyme with the expanded polyglutamine proteins (closed arrowheads). Normally, even cells with a high aggregate load (C) do not show coaggregation of E2-25K. Occasionally we do find aggregates that are positive for E2-25K and are not stained by the cell death fluorescent reagent (D; open arrowheads) suggesting that upregulation or nuclear translocation and consecutive coaggregation is one of the events preceding apoptosis. Staining for p53 shows diffuse staining in most cells and a clear colocalization with the cell death reporter (E).The right column shows the overlay of E2-25K (or p53), cell death and poly-Q. Magnification bar is 50 µm.

importance of E2-25K as part of the UPS machinery, there have been no reports on the influence of E2-25K on HD or even its sub-cellular localization in this disease.

In the present paper we studied the role of E2-25K in aggregate formation and cell death in polyglutamine disease.

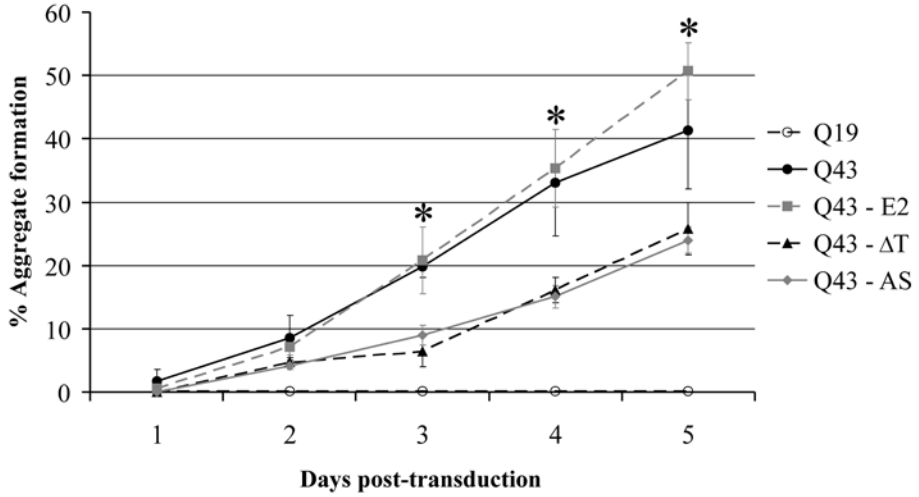
Results

We used neuronally differentiated SH-SY5Y cell lines, which were transduced with a lentiviral vector expressing a truncated huntingtin fragment containing 19 or 43 glutamine repeats. Staining of these cells for endogenous E2-25K showed a diffuse cytoplasmic staining in all cells (Figure 1A-D; panel 2). However, we noticed that, although E2-25K did colocalize in some of the aggregates, this was not the case for all aggregate-forming cells. The morphology of these cells suggested that this might reflect the viability of the cells and lead us to perform a cell death assay. Surprisingly, it appeared that the ubiquitin-conjugating enzyme E2-25K was found in the aggregates primarily in the cells that were in the process of dying (Figure 1B-D; closed arrowheads). Occasional colocalization of E2-25K with the polyglutamine aggregates in living cells, suggests that the upregulation or nuclear translocation of E2-25K and its subsequent colocalization with polyglutamine aggregates, precedes cell death (Figure 1D; open arrowheads). However, using Western blot analysis we were unable to demonstrate an obvious upregulation of the enzyme over the entire population of cells (data not shown). Staining for the tumour suppressor protein p53 demonstrates a clear upregulation and colocalization with the cell death reporter (Figure 1E). Indeed, p53 has been shown to play an important role in determining whether a cell will go into apoptosis and has been shown to coaggregate with polyglutamine inclusions (Levine, 1997; Steffan et al., 2000; Suhr et al., 2001).

To test the influence of E2-25K on aggregate formation and cell-viability we constructed lentiviral vectors containing either the full length E2-25K enzyme, the enzyme lacking its C-terminal catalytic domain or the complete anti-sense sequence. These constructs were cotransduced with the truncated huntingtin fragments. The E2-25K protein contains a tail region that is necessary for the generation of K48 linked polyubiquitin chains (Haldeman et al., 1997). The E2-25K protein lacking its tail region still interacts with ubiquitin activator E1. Expression of the truncated enzyme will thus compete for activated ubiquitin without tagging target proteins for degradation. Furthermore, the full anti-sense sequence of the E2-25K mRNA has been shown to be sufficient to knock down the expression of E2-25K in cell lines (Song et al., 2003).

We quantified the aggregate formation after lentiviral transduction of SH-SY5Y neuroblastoma cells with polyglutamine vectors in combination with one of the E2-25K vectors. The truncated huntingtin fragment with 43 glutamines fused to green fluorescent protein (Q43-HTT-GFP) shows a marked time-dependent increase

Figure 2: E2-25K mediates aggregate formation of expanded polyglutamines.



*Differentiated SH-SY5Y neuroblastoma cells were lentivirally transduced with constructs with truncated huntingtin fragments containing 19 or 43 glutamine repeats (Qxx-HTT-GFP) in combination with either E2-25K, a truncated Δtail mutant, or the full antisense sequence. Cells were quantified for aggregate formation every 24 hours after transduction. Upon addition of E2-25K there is no significant increase in aggregate formation of Q43-HTT-GFP, probably due to sufficient levels of endogenous E2-25K in the cells. Addition of either the Δtail mutant or the antisense sequence of E2-25K results in a highly significant decrease in aggregate formation. E2-25K clearly mediates the ubiquitination and subsequent aggregate formation of the expanded polyglutamines. Statistical analysis by three-way ANOVA confirmed that the combination of E2-25K with constructs of 43 glutamines has a pronounced, highly significant effect on the aggregate formation in time *P<0.001.*

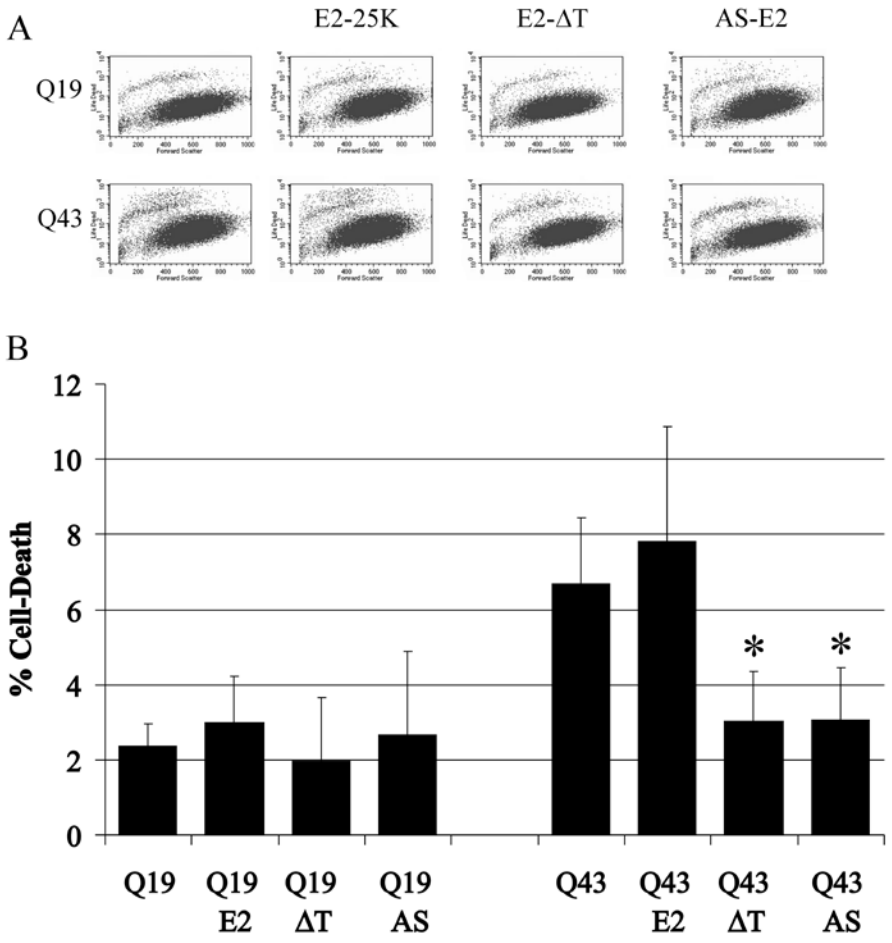
in aggregate formation compared to the Q19-HTT-GFP construct (Figure 2). Co-transduction with the E2-25KΔtail mutant or with the antisense sequence reduces the aggregate formation of Q43-HTT-GFP, compared to a mock-treated condition or with addition of E2-25K ($P<0.001$). Co-transduction of Q43-HTT-GFP with E2-25K did not enhance aggregate formation; apparently the levels of endogenous E2-25K are not rate-limiting for aggregate formation.

Since it is still under debate whether aggregate formation is either beneficial or detrimental we measured the viability of the cells. As we showed previously there is a low level of cell death due to viral transduction and culturing that is represented by the levels of Q19-HTT-GFP (de Pril et al., 2004). Here we found that co-expression of E2-25K and Q19-HTT-GFP does not result in an effect on cell death (Figure 3B). Co-expression of either the Δtail mutant or the antisense sequence with Q19-HTT-GFP neither had an effect on these levels.

As we have shown previously, expansion of the polyglutamine repeat results in a marked increase in cell death (de Pril et al., 2004). Overexpression of E2-25K in combination with Q43-HTT-GFP does not increase cell death, as was also seen for aggregate formation. Co-expression of either the Δtail mutant or the antisense sequence with Q43-HTT-GFP however resulted in a significant decrease in the levels of cell death (Figure 3B; $P<0.05$). Inhibition of E2-25K thus results in a reduction of expanded polyglutamine toxicity to background levels.

We found that E2-25K has a role in both aggregate formation and cell death by expanded polyglutamine proteins and colocalizes in aggregates in a cell-culture model. To reveal whether these findings are relevant for Huntington Disease, and for other polyglutamine diseases, we stained post mortem brain material of both HD and Spinocerebellar Ataxia type 3 (SCA3) for E2-25K reactivity. All patient material was characterized previously and found to be immunopositive for ubiquitin, the UBB^{+1} protein, and for the respective expanded polyglutamine proteins (see Table 1 of (de Pril et al., 2004)). We found that a fraction of NIIs in all cases of both HD (Figure 4A-C; closed arrowheads) and SCA3 (Figure 4D) were immunopositive for E2-25K. To our knowledge this is the first evidence that E2-25K is indeed localized in the inclusions in polyglutamine diseases. Double staining of the polyglutamine antibody 1C2 with E2-25K shows that, in contrast to many cellular proteins that reportedly coaggregate in all NIIs (Mitsui et al., 2002), E2-25K is not found in all the inclusions (Figure 4C; open arrowhead). The presence of E2-25K in part of the NIIs indicates a differential mechanism that might reflect the disease state of the respective aggregate bearing neurons. This finding corresponds to our *in vitro* observations, that co-aggregation of E2-25K with polyglutamine aggregates marks cell death and coincides with p53 upregulation. Indeed, staining for the tumour suppressor protein p53 showed an upregulation and coaggregation in the E2-25K-positive neurons suggesting that these neurons are likely to proceed into apoptosis (Levine, 1997). The finding of E2-25K localization to polyglutamine aggregates in disease, underlines the importance of E2-

Figure 3: E2-25K mediates polyglutamine-induced cell death.



*Cell death of differentiated SH-SY5Y neuroblastoma cells, as assessed by a cell survival assay 6 days after transduction. Constructs with 19 or 43 glutamine repeats (Qxx-HTT-GFP) were cotransduced with either E2-25K, a truncated Δtail mutant or the full antisense sequence of E2-25K. Polyglutamine-GFP positive cells were analyzed by flow cytometry for conversion of red-fluorescent cell death reporter (A; representative FACS analysis). Scatter plots clearly show the differential distribution of living cells (lower population) and dead cells (higher population; bright red-fluorescent). The bars (B) show the percentage of cell death that was observed under different conditions. Q19-HTT-GFP shows the background percentage of cell death due to transduction or culturing. Expansion of the polyglutamine repeat to Q43-HTT-GFP gives a marked increase in cell death that is not changed by addition of E2-25K. Both Δtail as well as the antisense sequence give a significant reduction in the levels of cell death. Statistical analysis by two-way ANOVA confirmed that cotransduction of both the Δtail and the antisense E2-25K constructs with Q43-HTT-GFP caused a pronounced, significant decrease of the cell death compared with Q43-HTT-GFP with wt E2-25K or mock treated *P<0.05.*

25K for HD and also SCA3 and demonstrates that it is not restricted to our *in vitro* model.

Discussion

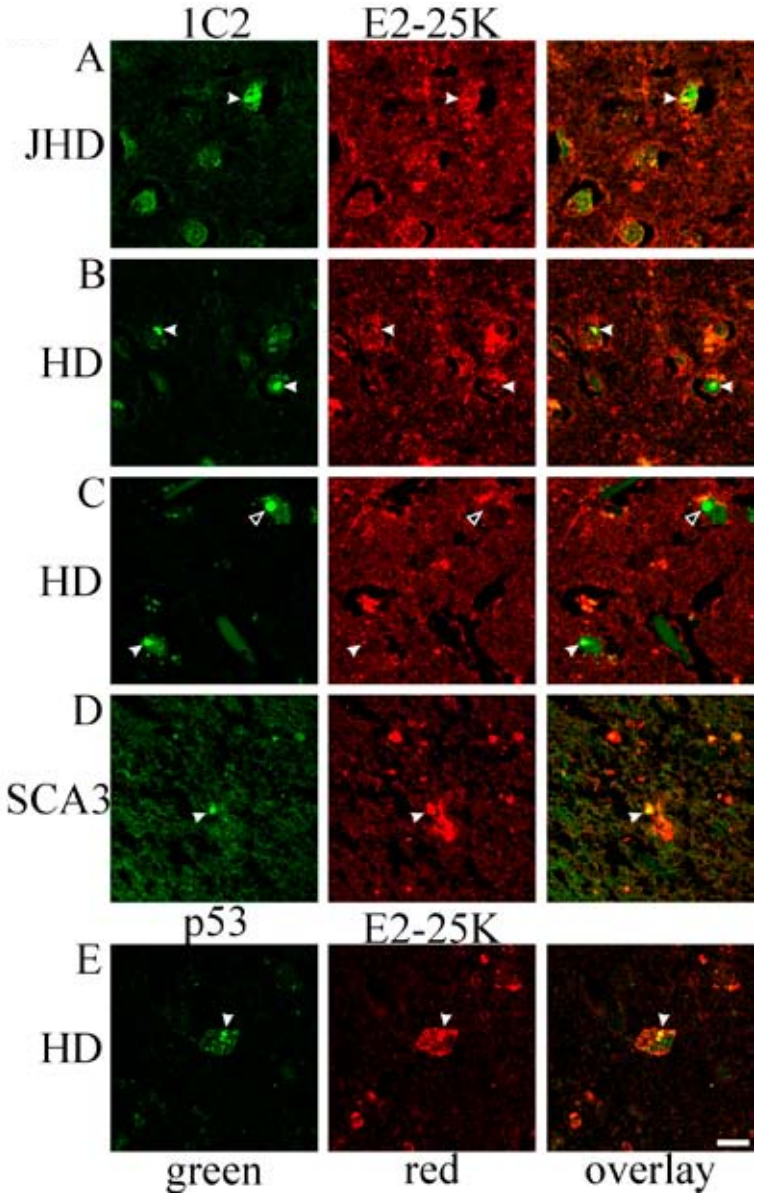
We previously showed that NIIs in HD and SCA3 were immunopositive for the respective expanded polyglutamine proteins (huntingtin and ataxin 3) as well as for ubiquitin and UBB^{+1} (de Pril et al., 2004). The aberrant UBB^{+1} protein appeared to be present in all inclusions and in addition showed a diffuse cytoplasmic staining indicating impaired proteasomal function in the affected neurons (Fischer et al., 2003). The ubiquitination machinery is an obvious candidate to promote the degradation of aberrant proteins such as expanded polyglutamines or UBB^{+1}. Ubiquitination is performed by a cascade of enzymes that activate (E1), conjugate (E2) and ligate (E3) ubiquitin to target proteins (Glickman and Ciechanover, 2002; Pickart, 2001). E2 proteins play an indispensable role in the ubiquitination of proteins that are to be degraded and interact specifically with a subset of E3 and target proteins.

The ubiquitin-conjugating enzyme E2-25K was reported to be expressed in all areas of the brain with higher levels in the areas that are affected in HD, i.e. the striatum and frontal cortex (Kalchman et al., 1996). Interaction of E2-25K with huntingtin, as found in a yeast two hybrid screen, was however not affected by repeat length. So far there have not been any reports on the influence of this interaction on disease and the localization in HD. Furthermore, E2-25K was shown to be involved in Aβ-mediated neurodegeneration and could play a role in Alzheimer pathogenesis (Song et al., 2003).

Staining of neuronally differentiated cell-lines for E2-25K shows a diffuse cytoplasmic localization in all cells. Interestingly, the E2-25K protein coaggregates only in part of the polyglutamine inclusions and this appeared to coincide with cell death. Aggregate forming cells that go into apoptosis might upregulate E2-25K in an attempt to rid the cells of the overload of aberrant proteins resulting in coaggregation in these cells. This indicates that E2-25K is an important factor in polyglutamine-induced neurodegeneration. It is remarkable that Kalchman et al found no obvious upregulation of E2-25K on Western blot of the frontal cortex of HD patients compared to controls, despite the relatively high expression in the frontal cortex and striatum of controls (Kalchman et al., 1996). However, an upregulation in the remaining neurons might be masked by neuronal loss in these areas.

Here we demonstrate that ubiquitination by E2-25K enhances the aggregate formation of expanded polyglutamine proteins. Although we do not find an increase in aggregate formation upon addition of E2-25K, both the Δtail mutant as well as antisense E2-25K give a large decrease in the aggregate load. The lack of increase upon addition of E2-25K can be explained by sufficient endogenous protein that is present in these

Figure 4: E2-25K colocalizes with the NIIs in HD and SCA3.



Double stainings were performed on paraffin sections of HD patients and SCA3 patients for E2-25K (red; middle panel) and 1C2 (green; left panel). Besides cytoplasmic staining, E2-25K staining was found in part of the inclusions positive for 1C2 in frontal cortex tissue of juvenile HD patients (JHD; A), and HD (B-C; closed arrowheads) as well as the pons of SCA3 (D). However, E2-25K does not localize to all the NIIs (C; open arrowhead; HD) and appears to be more prone to colocalize with the cytoplasmic aggregates that are found (A-D). p53 staining of HD frontal cortex shows colocalization with E2-25K (E) suggesting that there is in fact a link between cell death and E2-25K coaggregation in disease. Magnification bar is 50μm.

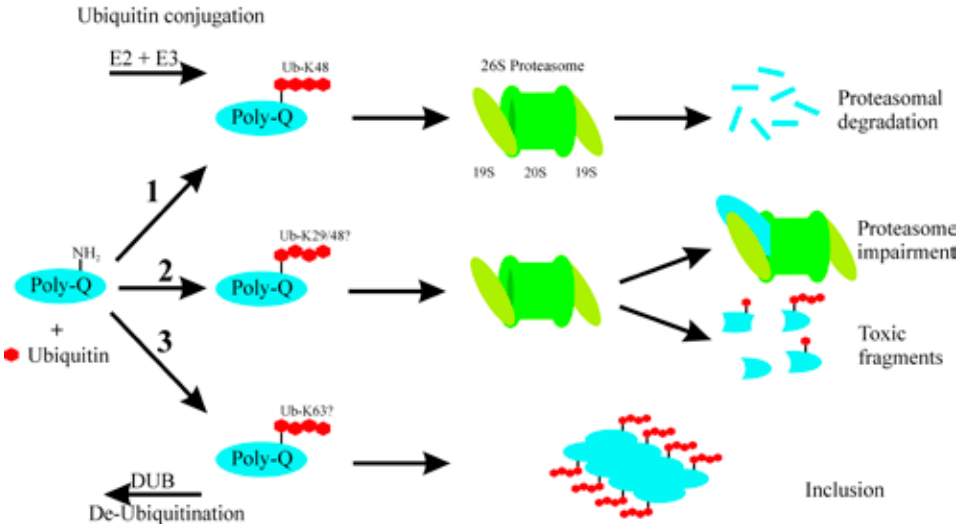
cells, as we did not use a knock-out cell line. It appears that ubiquitination by E2-25K is a stimulatory mechanism for aggregate formation in polyglutamine disorders. This suggests that ubiquitination is either needed for the translocation to the inclusions or indirectly, for the formation of more aggregation prone fragments.

Analysis of cell survival showed that E2-25K mediates polyglutamine-induced cell death. For a normal repeat length (Q19) no change in the levels of cell death was found upon addition of either E2-25K, the Δtail mutant or the antisense sequence. Expansion of the glutamine repeat to Q43 results in an increase in cell death, and addition of E2-25K has no effect, which demonstrates that endogenous E2-25K is not a limiting factor in either the toxicity or the degradation of polyglutamine fragments in the cell. However, addition of either the Δtail mutant or the antisense sequence results in a drop of toxicity to background levels. The Δtail mutant that lacks its functional domain for polyubiquitination will still interact with E1 and accept the activated ubiquitin but will no longer transfer the ubiquitin molecule to target proteins like huntingtin (Haldeman et al., 1997). Apparently this mutant has a similar dominant negative effect on ubiquitination, as does the knock-down of E2-25K by use of the full antisense sequence. These results show that ubiquitination of E2-25K targets does not only influence the aggregate formation of expanded polyglutamine proteins but more importantly triggers polyglutamine-induced cell death. Ubiquitination supposedly results in the targeting of the expanded polyglutamines to the proteasome, resulting in either proteasome impairment or the formation of toxic fragments.

Staining of post mortem brain material of both HD and SCA3 for E2-25K showed that here as well, a differential staining is found between the NIIs. Although we do find staining of aggregates in both disorders and all patients examined, not all aggregates are immunopositive for E2-25K. These results are in agreement with our finding in neuronally differentiated cell lines where E2-25K preferentially coaggregates with expanded polyglutamine proteins in apoptotic cells (Figure 1). E2-25K thus appears to indicate the disease state of the respective neurons. Colocalization of E2-25K seems to coincide with polyglutamine-induced neuronal death, which occurs both in HD as well as in SCA3 (Munoz et al., 2002; Vonsattel et al., 1985).

The relevance of ubiquitination is furthermore demonstrated by the general finding of ubiquitin immunoreactivity in the neuropathological hallmarks of numerous neurodegenerative diseases including HD and SCA3 (DiFiglia et al., 1997; Paulson et al., 1997). In addition, we have shown for aberrant ubiquitin (UBB^{+1}) that it is necessary to ubiquitinate this protein to be translocated to the inclusions, since a mutant that lacks the lysine moieties at positions 29 and 48 of the ubiquitin molecule does not coaggregate and is no longer toxic (de Pril et al., 2004). Mutating the lysine residues at positions 6, 9 and 15 in a truncated fragment of huntingtin similarly reduces neuropathology (Steffan et al., 2004).

Figure 5: Model for targeting of expanded polyglutamines by differential ubiquitination.



Ubiquitination of proteins can play different roles in polyglutamine diseases directly or indirectly affecting polyglutamine toxicity. The ubiquitin conjugation by a certain set of E2 and E3 is proposed to regulate the position of ubiquitin attachment to the target protein and the ubiquitin lysine linkage. Their role in one of these pathways can explain the differences that are found between all ubiquitination proteins that have been described for involvement in polyglutamine pathology (Table 1). Role 1: Ubiquitination results in targeting of the aberrant proteins to the proteasome where they are properly degraded. Role 2: Ubiquitination results in proteasomal targeting but inefficient degradation of aberrant proteins, causing either impairment of the UPS or the formation of more toxic fragments. Role 3: Ubiquitination is followed by transport of the expanded polyglutamine proteins to inclusions as a non-toxic storage for aberrant proteins.

We propose here that ubiquitination of proteins plays different roles in polyglutamine diseases directly or indirectly affecting polyglutamine toxicity (Figure 5). The best known function for ubiquitination is targeting of the aberrant proteins to the proteasome where they are properly degraded. The second possibility is proteasomal targeting but inefficient degradation of aberrant proteins, causing impairment of the UPS or the formation of more toxic fragments. The third option is a role in transporting the expanded polyglutamine proteins to inclusions as a non-toxic storage for aberrant proteins. Indeed, ubiquitination has different effects on polyglutamine-induced pathology and toxicity depending on the ubiquitination enzyme that is targeted (Table 1). For example, E4B, a mammalian chain assembly factor (E3), is required to degrade the expanded form of ataxin 3 and is able to prevent aggregate formation of polyglutamines and even neurodegeneration in a Drosophila model of SCA3 (Matsumoto et al., 2004). In contrast, mutation of E6-AP (Ube3a), an ubiquitin ligase (E3), was shown to aggravate the Purkinje cell pathology in SCA1 transgenic mice but to reduce the number of NIIs (Cummings et al., 1999).

This synopsis recapitulates many different mechanisms that are involved in the cellular digestion of polyglutamine proteins (Table 1). The formation of different ubiquitin trees by linkage to another ubiquitin lysine (K6, 29, 48 or 63) will result in a different cascade, i.e. transport to the proteasome or to inclusions (Pickart and Fushman, 2004). The specificity of ubiquitin binding proteins for a certain ubiquitin chain linkage will eventually define the translocation of the target proteins (Verma et al., 2004). The location of ubiquitin attachment on expanded polyglutamines or other target proteins itself might influence the recognition and binding to the proteasome and thereby the subsequent degradation. The specific transport of aberrant proteins to non-toxic inclusions making use of the cytoskeleton also implies specific targeting to these inclusions and is likely to be regulated by ubiquitination (Taylor et al., 2003). Cells are accordingly protected from higher levels of free polyglutamine proteins that would have a higher probability to interfere with cellular processes eventually resulting in neuronal death (Arrasate et al., 2004). We propose that E2-25K is involved in the second pathway, whereby the unfavourable formation of ubiquitin trees results in an increase in either proteasome impairment or the formation of toxic fragments.

Our results demonstrate that E2-25K is a contributing factor in mediating aggregate formation and cell death. These findings illustrate the importance of ubiquitination for the cellular clearance or storage of toxic proteins that extends beyond the proteasome itself. The reduced toxicity of expanded polyglutamines in the absence of functional E2-25K makes it an attractive target in polyglutamine diseases. A small ubiquitin-like modifier (SUMO; a post-translational modifier) has recently been shown to modify E2-25K (Pichler et al., 2005), impairing ubiquitin chain formation by interfering with E1 interaction, making SUMO an interesting molecule to use for gene therapy. Interference with SUMOylation has already been shown to enhance neurodegeneration in a Drosophila polyglutamine model (Chan et al., 2002), which could be linked to E2-25K. Controversially however, reduction of SUMOylation has

Table 1: Ubiquitination and polyQ toxicity.

Gene	LOF / overexpression	Enzyme	Aggregation	Toxicity	Role	References
UBC	LOF	Ub	+	N.D.	1,2,3	(Nollen et al., 2004)
Uba1	LOF	E1	+	N.D.	1,2,3	(Nollen et al., 2004)
E2-25K	LOF	E2	-	-	2	**this paper**
UbcE2D2	LOF	E2	N.D.	+	1/3	(Fernandez-Funez et al., 2000)
Ubc2EH	LOF	E2	N.D.	+	1/3	(Fernandez-Funez et al., 2000)
Cdc34	LOF	E2	-	+	3	(Saudou et al., 1998)
Ube3a	LOF	E3	-	+	3	(Cummings et al., 1999)
E4B	LOF	E3	+	N.D.	1/2	(Matsumoto et al., 2004)
E4B	overexpression	E3	-	-	1	(Matsumoto et al., 2004)
CHIP	overexpression	E3	-	-	1	(Jana et al., 2005)
Parkin	overexpression	E3	-	-	1	(Tsai et al., 2003)
UCH-L3	LOF	DUB	N.D.	+	2	(Fernandez-Funez et al., 2000)
Ataxin-3	overexpression	DUB	-	-	2	(Warrick et al., 2005)
UCH-L8	LOF	DUB	+	N.D.	3	(Nollen et al., 2004)

Overview of the involvement of ubiquitination enzymes in polyglutamine aggregation and toxicity. Numerous studies have been conducted on the influence of a loss-of-function (LOF) or overexpression of ubiquitination enzymes in polyglutamine models. Indicated is the influence of the respective change in expression on the polyglutamine aggregation and toxicity. Related enzymes appear to result distinctly in an increase (+) or decrease (-) of aggregate formation and toxicity. Their influence on these processes implicates the ubiquitination enzymes in either of three different pathways: (1) Proteasomal targeting followed by proper degradation of polyglutamine fragments. (2) Proteasomal targeting resulting in either UPS impairment or the formation of more toxic fragments. (3) Formation of innocuous inclusions. See also Figure 5 for a schematic representation.

N.D.: not determined
DUB: de-ubiquitination enzyme

been shown to reduce pathology in another Drosophila polyglutamine model, and in cell-lines expression of SUMO accelerates polyglutamine-induced cell death (Steffan et al., 2004; Terashima et al., 2002).

The UPS remains an attractive candidate for therapy in polyglutamine diseases and other neurodegenerative disorders. Shifting the balance from UPS impairment and formation of toxic fragments to harmless inclusions and efficient degradation would be a promising avenue in solving these severe diseases.

Materials and Methods

Transduction

Polyglutamine plasmids containing a truncated *huntingtin* fragment with different polyglutamine repeats (19 and 43) flanked by a HA tag and green fluorescent protein (GFP) reporter sequence were provided by Dr. de Cristofaro (de Cristofaro et al., 1999). E2-25K, E2-25K-Δtail and E2-25K-antisense constructs were identical to those described by Song et al. (Song et al., 2003).

cDNAs for HA-Q19-HTT-GFP, HA-Q43-HTT-GFP, E2-25K, E2-25K-Δtail and E2-25K-antisense, were cloned in the lentiviral transfer plasmid pRRLsin-PPThCMV-GFP-pre (Naldini et al., 1996). VSV-G pseudotyped lentivirus was produced by cotransfection of the transfer plasmid and helper plasmids (pCMVdeltaR8.74 and pMD.G.2) in 293T cells. Medium was harvested 24 h after transfection and concentrated by ultracentrifugation. Virus pellets were resuspended in PBS containing 0.5% bovine serum albumin (Sigma). Stocks were titered with a HIV-1 p24 coat protein ELISA (NEN Research, Boston, USA). Lentiviral vectors were used to infect SH-SY5Y neuroblastoma cells at a multiplicity of infection of 50.

Cell lines

Human SH-SY5Y neuroblastoma cells (ATCC:CRL-2260) were cultured in high-glucose Dulbecco's modified Eagle medium containing 15% fetal calf serum, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (all Invitrogen). Cells were cultured in 6-well plates (Nunc) 1 day before transduction with addition of 4 μM all-trans retinoic acid (Sigma) for differentiation.

Immunocytochemistry

Cells were cultured on collagen-coated glass coverslips and subjected to a Live-Dead® assay (red fluorescence; Molecular Probes, Leiden, The Netherlands) 4 days after transfection. Cells were subsequently fixed and stained for E2-25K (1:400) and visualized with donkey-anti-rabbit-Cy5 (1:200; Jackson laboratories). After staining

for E2-25K, pictures were obtained using a confocal laser scanning microscope (Zeiss 510).

Quantification of cells

For aggregate formation, cells were counted manually at different time intervals after transduction. The number of aggregate forming cells was quantified in three randomly selected fields per experiment. Experiments were performed in triplicate and statistics was performed using three-way ANOVA, testing the interaction between repeat expansion, E2-25K protein expression and time course.

Cell survival assay

We assessed SH-SY5Y cell death using a Live-Dead® kit (red fluorescence; Molecular Probes, Leiden, The Netherlands) and analyzed on a flow cytometer (Becton Dickinson, Palo Alto, USA) according to the manufacturer's protocol. In short, we harvested lentivirally transduced cells (see before) 6 days after transduction, washed them in PBS and incubated them for 30 minutes with the fluorescent reactive dye. Subsequently, we fixed cells with 4% formaldehyde for 15 minutes and resuspended in PBS-BSA (1x PBS pH-7.6, 0.5% BSA, 0.1% NaN_3). We analyzed polyglutamine-GFP expressing cells by flow cytometry for conversion of the Live-Dead® kit reactive dye. At least 10^4 polyglutamine-GFP positive cells were examined per sample. Dead cells showed a marked increase of red-fluorescence over living cells due to conversion of the dye (Figure 3A). Experiments were performed in triplicate and statistics were done using two-way ANOVA, testing the interaction between E2-25K protein expression and repeat expansion.

Human post-mortem tissue

Autopsy material was obtained from the Huntington bank (Leiden University Medical Center, The Netherlands) for HD cases, and the Laboratory of Pathology East Netherlands (Enschede, The Netherlands) and University Hospital Groningen (Groningen, The Netherlands) for SCA cases (for details see Table 1 of (de Pril et al., 2004)). We analyzed striatal tissue from 4 different HD patients and frontal cortex tissue of in total 30 HD patients with polyglutamine expansion of various lengths. Furthermore, we examined the hippocampus, pons, mesencephalon and medulla oblongata of 7 different SCA3 patients. All brain areas were fixed in formaldehyde and subsequently embedded in paraffin.

Immunohistochemistry

For double stainings, we subjected paraffin sections (6 µm) to irradiation with a broad spectrum lamp for at least 24 hours to reduce autofluorescence (Hol et al., 2003; Neumann and Gabel, 2002). We then deparaffinized sections and incubated them with

the first antibodies overnight in a humid chamber at 4°C. We used 1C2 monoclonal antibody against expanded polyglutamine repeats (Trottier et al., 1995) (1:10,000; Chemicon, Temecula, USA) monoclonal p53 (1:400; Chemicon) and E2-25K (1:400; Affinity) antibody. We enhanced the E2-25K signal using the avidin-biotin-complex and tyramine procedure (Kerstens et al., 1995) (Sigma-Aldrich, St.Louis, USA) and stained with streptavidin-conjugated-Cy5 (Jackson Laboratories, West Grove, USA). 1C2 and p53 were directly visualized using donkey-anti-mouse-Cy2. Signal specificity was demonstrated by swapping fluorescent dyes and omission of primary antibodies to exclude aspecific staining or signal enhancement. Images were obtained using a confocal laser scanning microscope (Zeiss 510).

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