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Hsp40 family members assist in clearance of aggregate-initiating polyglutamine peptides

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Several neurodegenerative disorders, including Huntington’s disease, are caused by expansion of the polyglutamine (polyQ) tract over 40 glutamines in the disease-related protein. Protein fragments containing the expanded polyQ tract are thought to initiate aggregation and represent the toxic species. Although it is not clear how these toxic fragments are generated, *in vitro* data suggest that proteasomes release polyQ peptides upon degradation of polyQ-containing proteins. Mimicking polyQ peptide generation in living cells induces aggregation and toxicity when polyQ peptides of disease-related lengths are expressed. Here, we show that DnaJB6 and DnaJB8, two members of the DnaJ (Hsp40) chaperone family that prevent aggregation and toxicity of polyQ-containing proteins, are able to suppress aggregation of expanded polyQ peptides as well. In contrast, the classical chaperones HspA1A (Hsp70) and DnaJB1 (Hsp40) are less effective. DnaJB6 and DnaJB8 require its C-terminal serine-rich region to suppress aggregation, whereas a functional J-domain does not seem to be essential. This suggests that these chaperones do not depend on their interaction with the HspA family to suppress aggregation of polyQ peptides. Furthermore, DnaJB6 but not DnaJB8 reduces soluble levels of polyQ peptides, whereas the levels of polyQ-expanded huntingtin protein remain constant. This indicates that DnaJB6 intervenes at an earlier stage of aggregation than DnaJB8. Taken together, DnaJB6 and DnaJB8 appear to act at the level of polyQ peptides to prevent aggregation and are the first examples of heat shock proteins that can reduce aggregation of toxic peptide species by improving their clearance.
Polyglutamine (PolyQ) disorders are a group of dominantly inherited, progressive neurodegenerative disorders. These disorders are caused by expansion of the polyQ tract over 40 glutamines within coding regions of unrelated proteins. At least 9 different polyQ disorders are known, including Huntington’s disease (HD), various spinocerebellar ataxias, spinobulbar muscular atrophy, and dentatorubral-pallidoluysian atrophy (Orr and Zoghbi, 2007). These disorders are characterized by atrophy of certain regions in the brain and the presence of intracellular aggregates. Several studies showed that these aggregates contained shortened fragments of the disease-related protein including the expanded polyQ tract (DiFiglia et al., 1997; Goti et al., 2004; Lunkes et al., 2002). These fragments are generated by cleavage by caspases, calpains and aspartyl endopeptidases (Gafni et al., 2004; Graham et al., 2006; Lunkes et al., 2002; Wellington et al., 1998). Furthermore, it was shown that truncated forms of huntingtin (Htt), ataxin-3, ataxin-7, atrophin-1 and androgen receptor containing the expanded polyQ tract are more aggregation-prone and enhance toxicity (Cooper et al., 1998; Ellerby et al., 1999; Graham et al., 2006; Haacke et al., 2006; Ikeda et al., 1996; Merry et al., 1998; Young et al., 2007). This was supported by the finding that transgenic mice expressing polyQ-expanded Htt, the protein causing HD, maintained normal neuronal function and did not develop any behavioral deficits or striatal neurodegeneration when the caspase-6 cleavage site was mutated thereby preventing the generation of toxic fragments (Graham et al., 2006). Proteasomes are able to degrade polyQ-containing proteins (Bailey et al., 2002; Iwata et al., 2009; Jana et al., 2005; Rousseau et al., 2009), but they are not able to cleave within polyQ tracts in vitro (Venkatraman et al., 2004). As a consequence, polyQ peptides may be released upon proteasomal degradation of expanded polyQ proteins.

When mimicking this process in living cells, we observed that expression of expanded polyQ peptides is sufficient to induce aggregation and toxicity in cells (Raspe et al., 2009). Aggregates or inclusion bodies (IBs) initiated by expanded polyQ peptides as well as expanded polyQ-containing proteins are decorated with various proteins, such as components of the ubiquitin-proteasome system (UPS), chaperones, and polyQ-containing transcription factors such as TATA-binding protein (TBP), and the CREB-binding protein (CBP) (Chai et al., 1999; Cummings et al., 1998; Holmberg et al., 2004; Kazantsev et al., 1999; Kim et al., 2002; Matsumoto et al., 2006; Raspe et al., 2009; Suhr et al., 2001).

Molecular chaperones transiently bind and stabilize non-native protein conformations. Many fundamental biological processes are thus assisted by chaperones such as protein folding, assembly of proteins, prevention of misfolding and aggregation of damaged or unstable proteins and guidance of misfolded proteins to degradation machineries. Heat shock proteins (Hsps) function as molecular chaperones and are upregulated under stress conditions, such as heat stress (Esser et al., 2004; Hartl and Hayer-Hartl, 2002). Hsps have been classified into a number of families on the basis of their approximate molecular mass and presence of conserved domains (Fink, 1999; Vos et al., 2008). The HspA (Hsp70) family consists of 11 members, whereas the DnaJ (Hsp40) family consists of over 40 members in humans (Vos et al., 2008). Overexpression of several chaperones was shown to reduce aggregation of various disease-related expanded polyQ proteins (Chai et al., 1999; Cummings et al., 1998; Jana et al., 2000;
Kobayashi et al., 2000; Stenoien et al., 1999). In a recent comparative screen of all members of the HspA, HspB (small Hsps), HspH (Hsp110), DnaJA and DnaJB (sub)families, DnaJB6 and DnaJB8 were identified as the two most potent suppressors of aggregation and related toxicity of expanded polyQ proteins (Hageman et al., submitted; Vos et al., in preparation).

Here, we show that DnaJB6 and DnaJB8 can suppress aggregation of polyQ peptides. When aggregates are formed, these chaperones are irreversibly trapped in the core of aggregates. The more classical chaperones, HspA1A (Hsp70) and DnaJB1 (Hsp40) are less effective in preventing aggregation of polyQ peptides and are absent from the core of aggregates. Reducing aggregation does not increase the amount of soluble polyQ peptides. This suggests that the ability of DnaJB6 and DnaJB8 to reduce aggregation of expanded polyQ peptides is due to enhanced clearance of polyQ peptides, thereby preventing them to act as inducers of aggregation.

**DNAJ6B AND DNAJB8 REDUCE AGGREGATION OF POLYQ PEPTIDES**

To determine the effect of chaperones on polyQ peptide aggregation in living cells, we used GFP-ubiquitin-polyQ (GFP-Ub-polyQ) constructs that are immediately cleaved upon expression into GFP-Ub and polyQ peptides by Ub C-terminal hydrolases (Figure 1A) (Johnson et al., 1995). Only expanded polyQ peptides induced aggregation, as quantified by the sequestration of GFP-Ub into aggregates (Raspe et al., 2009). Here, we show that approximately 60% of cells expressing Q104 peptides contained aggregates after 72 hours. Co-expression of HspA1A had no significant effect on polyQ peptide aggregation, whereas DnaJB1 slightly reduced aggregation (Figure 1B). Similar results were obtained for Httexon1-Q103-GFP when HspA1A or DnaJB1 were co-expressed (Figure 1C), although DnaJB1 was more effective in reducing aggregation of Httexon1 when compared to polyQ peptides. In contrast, co-expression of DnaJB6b or DnaJB8 with GFP-Ub-Q104 resulted in a dramatic decrease in polyQ peptide aggregation (Figure 1B). DnaJB6 and DnaJB8 also reduced the aggregation of Httexon1-Q103 (Figure 1C), which is in agreement with data of Chuang et al. (2002) and Hageman et al. (submitted).

To examine whether this effect was directly on polyQ peptide aggregation or indirectly by reducing GFP-Ub recruitment, polyQ peptides were tagged with a tetracysteine (C4) motif for direct visualization. The membrane-permeable biarsenical dye ReAsH can bind this small tag and only then becomes fluorescent (Martin et al., 2005). C4-tagging of Q99 peptides did not affect aggregate formation, as untagged and C4-tagged polyQ peptides induced similar amounts of aggregate-positive cells after 72 hours (data not shown). Upon co-expression of GFP-Ub-Q99-C4 and DnaJB8 or DnaJB6b, the number of ReAsH-labeled aggregates was reduced as compared to expression of Q99-C4 alone (data not shown). The aggregates that were present, were positive for both GFP-Ub and ReAsH (Figure 1D), indicating that DnaJB6b and DnaJB8 directly reduced aggregate formation of expanded polyQ peptides and not indirectly by affecting sequestration of GFP-Ub to aggregates.
DnaJb6 and DnaJb8 reduce polyQ peptide aggregation

Figure 1. DnaJb6 and DnaJb8 reduce aggregation of expanded polyQ peptides. (A) Schematic representation of cleavage of GFP-Ub-polyQ constructs by Ub C-terminal hydrolases directly after Ub, thereby separating GFP-Ub and polyQ. Percentage of transfected HEK cells that contained fluorescent aggregates at 72 hours after transfection with GFP-Ub-Q104 (B) and Httexon1-Q103-GFP (Htt-Q103) (C) in combination with DnaJb6a, DnaJb6b, DnaJb8, HspA1A or DnaJ, respectively (data are mean ± SEM of three independent experiments). DnaJb6a, DnaJb6b, DnaJb8 and DnaJb1 reduced aggregation of both Q104 and Httexon1-Q103-GFP (two-tailed unpaired t-test; *** p<0.001; ** p<0.01; * p<0.05). (D) Confocal images of HeLa cells expressing GFP-Ub-Q99-C4 in combination with DnaJb6b or DnaJb8, respectively, labeled with ReAsH to visualize Q99-C4 peptides at 72 hours after transfection. Arrows indicate aggregates. All ReAsH-positive aggregates were also GFP-Ub positive. Scalebar: 25 µm. (E, F) SDS-soluble and SDS-insoluble fractions prepared at 72 hours after transfection with GFP-Ub-Q16 or GFP-Ub-Q104 (E) and Httexon1-Q103-GFP (F) in combination with DnaJb6b and DnaJb8 and analyzed by Western blotting. Blots were stained for polyQ using 1C2 antibody (upper panel), anti-GFP antibody (middle panel) and anti-actin antibody (lower panel). Asterisks indicate endogenous polyQ-containing proteins, arrow and arrowhead indicate specific Q104 peptide bands. Star indicates an aspecific band.
To examine whether expression of DnaJB6b and DnaJB8 also reduced the amount of SDS-insoluble polyQ aggregates, cells expressing GFP-Ub-polyQ in combination with DnaJB6 or DnaJB8 were separated into SDS-soluble and SDS-insoluble fractions. Samples were loaded on SDS-PAGE and subsequently stained for polyQ. As the antibody 1C2 detects polyQ tracts in general, several bands were detected on Western blot that represent endogenously-expressed polyQ-containing proteins (asterisks; Figure 1E). Expression of Q16 peptides did not lead to accumulation of Q16 peptides in the soluble fraction or of SDS-insoluble aggregates (Figure 1E), indicating efficient degradation of short polyQ peptides as shown before (Raspe et al., 2009). Expression of Q104 peptides resulted in specific polyQ-positive bands in both soluble and insoluble fractions (arrow and arrowhead; Figure 1E). Co-expression of DnaJB6b and DnaJB8 remarkably reduced the amount of polyQ peptides present in the SDS-insoluble fraction (Figure 1E), which is in agreement with the reduced number of aggregates visualized in cells. Although GFP-Ub was also sequestered into polyQ peptides-induced aggregates, GFP-Ub was not detected in the insoluble fraction (Figure 1E). This indicates that sequestering of GFP-Ub into aggregates is not resistant to treatment with detergents such as SDS, as was shown before (Raspe et al., 2009). Actin was not sequestered into aggregates and was thus absent in the SDS-insoluble fraction (Figure 1E). Furthermore, expression of DnaJB6b also resulted in a marked decrease in polyQ peptides present in the lower specific Q104 band in the SDS-soluble fraction (arrowhead; Figure 1E). This suggests that DnaJB6b acted at an earlier stage of polyQ peptide aggregation than DnaJB8, where it prevents the formation of small oligomeric complexes. SDS-insoluble levels of Httexon1-Q103 were also decreased by co-expression of DnaJB6b or DnaJB8, as detected by both polyQ and GFP levels in the insoluble fraction. However, SDS-soluble Httexon1-Q103 protein levels were not affected (Figure 1F) (Hageman et al., submitted). Together, these data show that DnaJB6 and DnaJB8 are able to reduce aggregation of expanded polyQ peptides.

NUCLEAR DNAJB6A REDUCES AGGREGATION IN BOTH CYTOPLASM AND NUCLEUS

The effect of DnaJB6b on SDS-soluble levels of polyQ peptides suggests that it counteracts early oligomerization steps of these aggregation-prone peptides. To examine this phenomenon in more detail, we reasoned that only monomeric or small oligomers can shuttle freely through the nuclear pore by diffusion. If correct, the presence of DnaJB6 in a restricted compartment such as the nucleus should lead to a reduction in aggregation in both compartments, as DnaJB6 keeps polyQ peptides soluble. To test this hypothesis, we used two isoforms of DnaJB6: DnaJB6b which is present in both cytoplasm and nucleus, and DnaJB6a which contains a putative nuclear localization signal and is therefore localized in the nucleus only (Figure 2A) (Hageman et al., submitted). Expression of fluorescently-tagged DnaJB6a confirmed that its localization was indeed restricted to nuclei. Fluorescence was not observed in the cytoplasm (Figure 2B). Fluorescently-tagged DnaJB6b and DnaJB8 were present in both compartments, with DnaJB6b slightly enriched in the nucleus and DnaJB8 mainly present in the cytoplasm (Figure 2B). Despite the exclusive localization of DnaJB6a in the nucleus, expression of DnaJB6a was as effective as DnaJB6b and DnaJB8 in reducing polyQ-peptide aggregation (Figure 1B).
To examine whether DnaJB6a only reduced aggregation in the nucleus, polyQ peptides were expressed together with the various chaperones. Subsequently, specific localization of aggregates in nucleus, cytoplasm, or both compartments was determined at 48 and 72 hours after transfection. Co-expression of the various chaperones reduced the amounts of aggregates as shown before (Figure 1A), but reduction in aggregate formation was not limited to the compartment where these chaperones were residing. Co-expression of DnaJB8 did not alter the ratio of nuclear and cytoplasmic aggregates after 48 hours, whereas co-expression of DnaJB6b resulted in a slight change in this ratio in favor of nuclear aggregates (Figure 2C). Expression of DnaJB6b and DnaJB8 resulted in even a higher percentage of aggregates present in nuclei after 72 hours (Figure 2C). The nuclear localization of DnaJB6a did not lead to the exclusive presence of aggregates in the cytoplasm, because the ratio of nuclear and cytoplasmic aggregates remained unaltered after expression of DnaJB6a (Figure 2C). It is concluded that although DnaJB6a is present in the nucleus only, its effect on aggregation is not limited to the nuclear compartment.

Using fluorescence loss in photobleaching (FLIP) (Lippincott-Schwartz et al., 2001), we showed that upon repeatedly bleaching of a part of the cytoplasm, fluorescence loss was measured in the nucleus (Figure 2D). Expression of DnaJB6a, DnaJB6b or DnaJB8 did not affect the mobility of expanded polyQ peptides, as a similar loss in fluorescence was detected upon FLIP analysis (Figure 2D). This suggests that expanded polyQ peptides indeed easily diffuse from cytoplasm to nucleus and back.

**DNAJB6A, DNAJB6B, AND DNAJB8 ARE IRREVERSIBLY SEQUESTERED IN POLYQ PEPTIDE INITIATED AGGREGATES**

HspA1A and DnaJB1 are known to be recruited to aggregates of polyQ-expanded disease-related proteins (Chai et al., 1999; Kobayashi et al., 2000; Stenoien et al., 1999; Suhr et al., 2001). DnaJB6b is present in Lewy bodies (Durrenberger et al., 2009) and DnaJB6b and DnaJB8 colocalize to Htt aggregates as well (Hageman et al., submitted) (Chuang et al., 2002). HspA1A is usually found in a ring around the core of aggregates (Figure 3A) (Kim et al., 2002; Matsumoto et al., 2006; Raspe et al., 2009). Here, we find that DnaJB1 is also associated with aggregates, but only weakly (Figure 3A). However, in the few cells that contained aggregates upon DnaJB6a, DnaJB6b or DnaJB8 co-expression, these three chaperones were present in the core of aggregates (Figure 3A). This suggests that DnaJB6 and DnaJB8 are recruited in an early stage into aggregates induced by polyQ peptides.

HspA1A is only transiently associated and not irreversibly trapped within aggregates (Kim et al., 2002; Raspe et al., 2009). To examine whether DnaJB6a, DnaJB6b and DnaJB8 were able to dissociate from the core of aggregates, we studied the mobility of the sequestered chaperones, using fluorescent recovery after photobleaching (FRAP) (Lippincott-Schwartz et al., 2001; Reits and Neefjes, 2001). Fluorescent recovery was observed of HspA1A, but not of DnaJB6a, DnaJB6 and DnaJB8 (Figure 3B). Since most DnaJB6a, DnaJB6b, and DnaJB8 was sequestered into aggregates (Figure 3A), we also measured FLIP. Here, the entire cell was repeatedly bleached with exception of one fluorescent aggregate. The rate of fluorescence loss of that aggregate represents the on/off rate of chaperones. Similar to FRAP analysis, fluorescence loss was measured of HspA1A, whereas DnaJB6a, DnaJB6b and DnaJB8 present in the core of aggregates.
Figure 2. DnaJb6 and DnaJb8 prevent aggregation of Q104 peptides by keeping them soluble. (A) Schematic representation of domains present in DnaJb6a, DnaJb6b and DnaJb8. (B) Confocal images of HeLa cells transfected with DnaJb6a, DnaJb6b or DnaJb8, respectively that was stained with Hoechst and fixed at 72 hours after transfection. Scalebar: 20 µm. (C) Ratio of aggregates present in cytoplasm, nucleus or both compartments at 72 hours after transfection with GFP-Ub-Q104 in combination with DnaJb6a, DnaJb6b or DnaJb8, respectively, in Mel JuSo cells (data are mean ± SEM of three independent experiments). Chaperones did not specifically reduce aggregation in the compartment where they reside. (D) A representative graph showed that repetitive bleaching of the cytoplasm resulted in fluorescence loss of Q99-C4-tagged peptides in the nucleus irrespective of co-expression with DnaJb6b or DnaJb8.

were hardly affected by bleaching (Figure 3C). These data show that DnaJb6a, DnaJb6b and DnaJb8 behave differently than HspA1A and DnaJb1, with HspA1A transiently associated to the periphery of aggregates and DnaJb1 only weakly associated to aggregates. In contrast, DnaJb6a, DnaJb6b and DnaJb8 were irreversibly sequestered in the core of polyQ-induced aggregates. This indicates that once the activity of these chaperones has failed to prevent aggregation, they are irreversibly trapped in the inert core of aggregates.

SERINE-RICH REGION IN DNAJB6 AND DNAJB8 IS ESSENTIAL FOR REDUCTION OF AGGREGATION

The DnaJ family is defined by the presence of a J-domain that can regulate chaperone activity of the HspA family by stimulating ATP hydrolysis (Figure 2A) (Liberek et al., 1991; Qiu et al., 2006). The sequence HPD in the J-domain is essential for accelerating ATPase activity of HspA (Tsai and Douglas, 1996). The C-terminus of DnaJ family members is involved in recognition
DnaJB6 and DnaJB8 reduce polyQ peptide aggregation

DnaJB6 and DnaJB8 reduce polyQ peptide aggregation (Qiu et al., 2006). To verify which domain(s) in DnaJB6 and DnaJB8 were required to reduce polyQ peptide aggregation, we used several mutations within these chaperones. Mutating His to Glu (H31Q) in the HPD motif resulted in an inactivate J-domain (Tsai and Douglas, 1996), but only slightly impaired the ability of DnaJB6 and DnaJB8 to reduce polyQ peptide aggregation (Figure 4A). This was also found when using expanded polyQ-containing Htt fragments (Hageman et al., submitted). In agreement, a similar reduction of polyQ peptides was detected in the SDS-insoluble fraction on Western blot when compared to expression with wild-type chaperones (Figure 4B).

Figure 4. DnaJB6 and DnaJB8 reduce polyQ peptide aggregation. (A) HeLa cells were transfected with GFP-Ub-Q104 in combination with tagRFP-tagged DnaJB6a, DnaJB6b, DnaJB8, or DnaJB1 or with RFP-Ub-Q104 in combination with HspA1A-GFP, respectively. Confocal images were obtained after 72 hours. DnaJB6a, DnaJB6b and DnaJB8 were recruited into the core of aggregates. HspA1A was present in an additional ring around the RFP-Ub ring, whereas DnaJB1 was hardly attracted to polyQ-induced aggregates. Scalebar: 20 µm. (B) FRAP analysis of chaperones recruited into polyQ-induced aggregates at 72 hours after transfection. Fluorescent aggregates in the highlighted area were subjected to FRAP analysis. Images were taken before (pre-bleach) and at indicated time points after photobleaching. DnaJB6a, DnaJB6b and DnaJB8 did not recover after photobleaching, whereas HspA1A fluorescent levels recovered. Scalebar: 20 µm. (C) Fluorescence loss of aggregates was measured using FLIP analysis by repetitive photobleaching of another part of the cytoplasm. Fluorescence loss of HspA1A was observed, whereas fluorescent levels of DnaJB6a, DnaJB6b and DnaJB8 were not affected.

Figure 3. DnaJB6a, DnaJB6b and DnaJB8 are irreversibly trapped in the core of polyQ-induced aggregates. (A) HeLa cells were transfected with GFP-Ub-Q104 in combination with tagRFP-tagged DnaJB6a, DnaJB6b, DnaJB8, or DnaJB1 or with RFP-Ub-Q104 in combination with HspA1A-GFP, respectively. Confocal images were obtained after 72 hours. DnaJB6a, DnaJB6b and DnaJB8 were recruited into the core of aggregates. HspA1A was present in an additional ring around the RFP-Ub ring, whereas DnaJB1 was hardly attracted to polyQ-induced aggregates. Scalebar: 20 µm. (B) FRAP analysis of chaperones recruited into polyQ-induced aggregates at 72 hours after transfection. Fluorescent aggregates in the highlighted area were subjected to FRAP analysis. Images were taken before (pre-bleach) and at indicated time points after photobleaching. DnaJB6a, DnaJB6b and DnaJB8 did not recover after photobleaching, whereas HspA1A fluorescent levels recovered. Scalebar: 20 µm. (C) Fluorescence loss of aggregates was measured using FLIP analysis by repetitive photobleaching of another part of the cytoplasm. Fluorescence loss of HspA1A was observed, whereas fluorescent levels of DnaJB6a, DnaJB6b and DnaJB8 were not affected.
In the C-terminal region of DnaJB6 and DnaJB8 a conserved serine-rich region (SSF-TST and SSF-SST, respectively) is present, which is absent in DnaJB1 or its related subfamily members (Figure 2A) (Hageman et al., submitted). Deletion of this serine-rich region was found to be crucial for interaction of these chaperones with histone deacetylases (HDACs) that regulate their function as suppressors of expanded polyQ protein aggregation (Hageman et al., submitted). Similar to what was observed for polyQ-expanded Htt fragments (Hageman et al., submitted), deletion of this serine-rich region severely affected the ability of these chaperones to reduce polyQ peptide aggregation (Figure 4A), resulting in more SDS-insoluble material on Western blot (Figure 4B). This suggests that the serine-rich region within DnaJB6 and DnaJB8 is more important for their anti-aggregation properties than their J-domain.

Figure 4. Serine-rich region within DnaJB6 and DnaJB8 is essential for reduction of polyQ peptide-induced aggregation. (A) Percentage of transfected HEK cells that contained fluorescent aggregates at 72 hours after transfection with GFP-Ub-Q104 in combination with DnaJB6b or DnaJB8 and their J-domain (H31Q) and serine-rich region mutants (ΔSSF/TST and ΔSSF/SST, respectively; data are mean ± SEM of three independent experiments). An inactive J-domain induced a small increase in aggregation, whereas deletion of the serine-rich region severely increased aggregation of Q104 peptides (two-tailed unpaired t-test; *** p<0.001; ** p<0.01; * p<0.05). (B) Western blot analysis of the SDS-insoluble fraction of HEK cells expressing GFP-Ub-Q104 in combination with DnaJB6b or DnaJB8 and their mutants at 72 hours. Western blots were stained for polyQ with 1C2 antibody. Arrows indicate specific Q104 peptide bands. More polyQ peptides were present in SDS-insoluble fraction when mutant chaperones were expressed.

In the C-terminal region of DnaJB6 and DnaJB8 a conserved serine-rich region (SSF-TST and SSF-SST, respectively) is present, which is absent in DnaJB1 or its related subfamily members (Figure 2A) (Hageman et al., submitted). Deletion of this serine-rich region was found to be crucial for interaction of these chaperones with histone deacetylases (HDACs) that regulate their function as suppressors of expanded polyQ protein aggregation (Hageman et al., submitted). Similar to what was observed for polyQ-expanded Htt fragments (Hageman et al., submitted), deletion of this serine-rich region severely affected the ability of these chaperones to reduce polyQ peptide aggregation (Figure 4A), resulting in more SDS-insoluble material on Western blot (Figure 4B). This suggests that the serine-rich region within DnaJB6 and DnaJB8 is more important for their anti-aggregation properties than their J-domain.

VARIOUS PROTEASE INHIBITORS DO NOT AFFECT ANTI-AGGREGATION PROPERTIES OF DNAJB6B AND DNAJB8

We showed that the ability of DnaJB6a, DnaJB6b and DnaJB8 to reduce aggregation of expanded polyQ peptides was not restricted to the compartment where they resided. Furthermore, DnaJB6b reduced polyQ peptide levels not only in the SDS-insoluble fraction, but also in the SDS-soluble fraction. This suggests that these chaperones may assist in the disposal of polyQ peptides by keeping them in a monomeric and/or small oligomeric stadium that can be degraded. These monomeric or small oligomeric polyQ peptides can be degraded by peptidases that degrade monomeric peptides or via macroautophagy that can degrade oligomeric stages of aggregated polyQ peptides. To elucidate the degradation pathway of these polyQ peptides, cells co-expressing expanded polyQ peptides and DnaJB6b or DnaJB8 were
DnaJb6 and DnaJb8 reduce polyQ peptide aggregation incubated with various inhibitors. Macroautophagy was inhibited by 3-methyladenine (3-MA) (Seglen and Gordon, 1982). Various families of peptidases were inhibited: cysteine proteases by E64, aspartyl proteases by pepstatin A, aminoproteases by bestatin, serine proteases by phenylmethylsulfonyl (PMSF) and metalloproteases by phenanthroline. To exclude redundancy between these peptidases, also a cocktail containing all peptidase inhibitors was used. Tripeptidyl peptidase II, a cytoplasmic peptidase with endopeptidase activity (REF), was inhibited by Ala-Ala-Phe-chloromethylketone (AAF-CMK). Addition of these inhibitors to cells expressing DnaJb6b and DnaJb8 did not affect the ability of these chaperones to suppress aggregation, as a similar percentage of cells containing aggregates was detected after 72 hours (Figure 5A). The reduction in aggregation of Q65 peptides upon expression of DnaJb6 or DnaJb8 was not affected after incubation with any of the inhibitors or the cocktail of all inhibitors when analyzed by filtertrap analysis (Figure 5B). The fact that these protease inhibitors did not alter reduced aggregation by DnaJb6 and DnaJb8 suggests either that the included peptidases or macroautophagy are not involved in polyQ peptide clearance, or that a reduced rate of polyQ peptide breakdown is not detectable by filter retardation assays or Western blotting.

Figure 5. Protease inhibitors do not affect ability of DnaJb6 or DnaJb8 to prevent polyQ peptide aggregation. (A) Percentage of transfected HEK cells that contained fluorescent aggregates at 72 hours after transfection with GFP-Ub-Q104 in combination with DnaJb6b (left panel) or DnaJb8 (right panel). Various protease inhibitors were added after 48 hours and cells were incubated for a subsequent 24 hours (data are mean ± SEM of three independent experiments). Similar amounts of polyQ-induced aggregates were measured when expressing chaperones with or without incubation with protease inhibitors (two-tailed unpaired t-test; *** p<0.001; ** p<0.01; * p<0.05). (B) Filter retardation assay of HEK cells expressing GFP-Ub-Q65 in combination with DnaJb6b (left panel) or DnaJb8 (right panel) at 48 hours after transfection. Cells were incubated during 24 hours with the autophagy inhibitor 3-MA and various peptidase inhibitors. Filtertrap was stained for polyQ. DnaJb6b and DnaJb8 reduced the amount of trapped polyQ-positive aggregates and addition of various inhibitors to DnaJb6b- or DnaJb8-expressing cells did not affect the amount of trapped aggregates.
PolyQ disorders are hallmarked by aggregation and toxicity of polyQ protein fragments (Cooper et al., 1998; DiFiglia et al., 1997; Ellerby et al., 1999; Goti et al., 2004; Graham et al., 2006; Haacke et al., 2006; Ikeda et al., 1996; Lunkes et al., 2002; Merry et al., 1998; Young et al., 2007). Improving the clearance of these toxic fragments should delay the age of onset and severity of the disorders. Clearance may be facilitated by chaperones that prevent aggregation, allowing proteases to remove hazardous fragments. In this study, we examined mechanisms to prevent aggregation of polyQ fragments, focusing on expanded polyQ peptides that can initiate aggregation and toxicity.

We found that the DnaJ family members DnaJB6 and DnaJB8 were efficient suppressors of polyQ peptide aggregation. The classical chaperones HspA1A and DnaJB1 were less able to prevent aggregation of expanded polyQ peptides. At first sight, this is similar to their effects on aggregation of expanded polyQ proteins (Hageman et al., submitted). However, these chaperones are the first examples of Hsps that reduce aggregation of toxic peptides.

A reduction in aggregates did not lead to an increase in soluble polyQ levels. This suggests that DnaJB6 and DnaJB8 inhibit oligomerization of polyQ peptides leading to improved clearance of polyQ peptides. Expression of DnaJB6b, but not of DnaJB8, even resulted in a reduction in the level of soluble polyQ peptides. This indicates that DnaJB6 prevented aggregation of polyQ peptides at an earlier stage than DnaJB8 (Figure 6). It remains to be established whether DnaJB6 keeps polyQ peptides in a degradation-competitive state, or also control their degradation by targeting them to proteases. Although inhibition of various degradation pathways showed little effect, it may be difficult to detect small differences in polyQ-peptide half-life by Western blotting experiments. In contrast, DnaJB6b did not affect detergent-soluble levels of Httexon1, suggesting that DnaJB6 acts on polyQ-containing fragments smaller than Httexon1.

In contrast to polyQ peptides, fluorescently-tagged expanded polyQ proteins such as Httexon1-GFP, were not present in the core of aggregates, but sequestered in a ring around the aggregate (Chai et al., 2002; Matsumoto et al., 2006; Raspe et al., 2009; Stenoien et al., 1999; Wyttenbach et al., 2000). This suggests that smaller fragments containing the polyQ tract initiate aggregation and that the original expanded polyQ proteins are sequestered in a later stage. Different chaperones may therefore act at different levels, either by preventing sequestration of polyQ proteins into aggregates, or by preventing aggregate formation of the aggregate-initiating polyQ fragments. DnaJB6 and DnaJB8 reduced aggregation of expanded polyQ peptides, thereby preventing that these polyQ peptides can act as nucleators of aggregation and subsequent sequestration of larger polyQ-containing fragments, such as Httexon1. Whereas DnaJB6 and DnaJB8 were trapped in the core of aggregates together with polyQ peptides, expanded polyQ proteins were absent from the core. This supports our model where these chaperones act on the level of aggregate-initiating polyQ peptides. In contrast, the recruitment of HspA1A to the outer ring of aggregates may be due to its recognition of sequestered, possibly unfolded proteins and its attempt to solubilize these proteins. Therefore, HspA1A may be more involved in prevention of recruitment of other proteins into aggregates, than in prevention of the initiation of aggregation.

Deletion of the serine-rich region within DnaJB6 and DnaJB8 severely reduced the anti-aggregation properties of these chaperones. This suggests that this region is essential to
recognize polyQ peptides and to reduce their aggregation. An active J-domain was less important for DnaJB6 and DnaJB8 anti-aggregation properties. Aggregation of Httexon1 was similarly affected by these DnaJB6 and DnaJB8 mutants (Chuang et al., 2002) (Hageman et al., submitted). Several other DnaJ family members with a deleted J-domain were still partially effective in the reduction of aggregation of polyQ-containing proteins (Chai et al., 1999; Jana et al., 2000). Since the J-domain is known to interact with HspA1A and stimulates its ATPase activity (Liberek et al., 1991; Qiu et al., 2006), DnaJB6 and DnaJB8 do not seem to absolutely require HspA1A to reduce polyQ peptide aggregation. Little is known about the function of the serine-rich region in DnaJB6 and DnaJB8, although this region is suggested to be involved in histone deacetylase (HDAC) binding (Hageman et al., submitted). Since the microtubule-associated deacetylase HDAC6 interacts with polyubiquitinated proteins and is required for degradation of misfolded proteins via macroautophagy (Iwata et al., 2005; Pandey et al., 2007), DnaJB6 and DnaJB8 may be involved in stimulating autophagic degradation of expanded polyQ peptides. Since we did not observe an effect of the autophagy inhibitor 3-MA, the chaperonic effect of the serine-rich region may be exerted via other mechanisms.

We show that the ability of nuclear DnaJB6a to reduce polyQ peptide aggregation was not restricted to the nuclear compartment, as aggregation within the cytoplasm was reduced similarly. This suggests that DnaJB6a keeps polyQ peptides in a soluble intermediate, which allows these peptides to freely translocate between cytoplasm and nucleus. This is of importance as most clearance mechanisms such as peptidases and the autophagic machinery are present in the cytoplasmic compartment only (Reits et al., 2003; Rubinsztein, 2006). This may also explain our observation that the ratio of cytoplasmic versus nuclear aggregates decreased in time, as cytoplasmic aggregates may be more efficiently cleared as compared to aggregates in the nucleus. The presence of the microtubule network in the cytoplasm may contribute to the relative decrease in cytoplasmic aggregates in time as well, as concentration of aggregation-prone proteins via microtubules in perinuclear inclusions facilitate their degradation (Taylor et al., 2003).

Previous studies showed that DnaJB6 is highly enriched in the central nervous system (Chuang et al., 2002; Hageman and Kampinga, 2009) and colocalizes to Lewy bodies and aggregates induced by Httexon1 (Chuang et al., 2002; Durrenberger et al., 2009). However, expression of DnaJB8 is restricted to the testis (Hageman and Kampinga, 2009). Therefore, DnaJB6 may be the best candidate for potential therapeutic approaches in the fight against polyQ disorders, for example by inducing endogenous DnaJB6 expression. A combination of solubilizing polyQ peptides by expression of DnaJB6 and stimulating their subsequent degradation seems to be the most attractive alternative.
DNA constructs. GFP-Ub-polyQ constructs were obtained in several steps. Generation of GFP-Ub-Q16/65/112 was described before (Raspe et al., 2009), however the initial polyQ peptides started with a Leu residue and a Glu-Thr-Ser-Pro-Arg sequence at the C-terminus. This Leu residue was changed into a Gln using Quikchange II site directed mutagenesis (Stratagene) with forward primer 5'-CTCAGAGGTGGGCAGCAGCAGCAG-3' and reverse primer 5'-CTGCTGCTGCTGCTGCCCACCTCCTGAG-3'. At the C-terminus a stop-codon was introduced directly after the polyQ stretch using site directed mutagenesis with forward primer 5'-AGCAGCAGCAGCAGCAGCAATAAACTAGCCCCAGGTAAGC-3' and reverse primer 5'-GCTTACCTGGGGCTAGGATCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
DnaJb6b ΔSSF-TST was generated by deleting amino acids 155–195 within DnaJb6b via two successive PCRs. First, two fragments were generated containing either amino acids 1-154 fused to 195-199 using forward primer 5’-GA ATTCCCACCATGGTGGATTACTA-3’ (Δ1F) and reverse primer 5’-GCCATTAACCATTTGATTCAC-3’ (Δ1R) or amino acids 150-154 fused to 195-241 using forward primer 5’-CTTTGGAGTGGATTTAAATGTTAATGGCAGAAAATC-3’ (Δ2F) and reverse primer 5’-GCTGCGCTTGATTAAGTGATCC-3’ (Δ2R). These fragments were isolated using a PCR purification kit (Qiagen). Subsequently, a second PCR was performed to generate DnaJb6b ΔSSF-TST using primers Δ1F and Δ2R, thereby fusing the two fragments generated by the first PCR. Httexon1-Q103-GFP was kindly provided by Ron Kopito (Stanford University, Stanford USA), HspA1A-GFP by Harm Kampinga (University Medical Center Groningen, Groningen, the Netherlands).

**Cell culture and transfection.** Human Embryonic Kidney (HEK293T) cells and the human melanoma cell line Mel Juso were cultured in Iscove’s Modified Eagle Medium (IMDM; Gibco) supplemented with 10% FCS and 25 mM Hepes, penicillin/streptomycin/glutamine (Gibco) and maintained at 37°C in 5% CO₂. HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented as described above, and maintained at 37°C in an atmosphere of 10% CO₂. Cells were dissociated using trypsinization. HeLa and Mel Juso cells (0.15x10⁶) were plated onto glass coverslips (24 mm; Fisher scientific) in a 6-well and were transiently transfected using Lipofectamine 2000 (Invitrogen) or FuGeneHD (Roche), respectively at 24 hours after plating. HEK293T cells (0.1x10⁶) were seeded in 6-well plates and transiently transfected with either FuGene6 (Roche) or polyethyleneimine (PEI; Polysciences) at 24 hours after plating.

HEK cells expressing GFP-Ub-Q104 and DnaJb6 or DnaJb8 for 48 hours or GFP-Ub-Q65 and DnaJb6 or DnaJb8 for 24 hours were treated with various inhibitors for a subsequent 24 hours: 500 µM 1,10-phenanthroline (Sigma), 1 mM 3-MA (Sigma), 1 µM AAF-CMK (Enzo lifesciences AG), 5 µM bestatin (Enzo lifesciences AG), 10 µM E64 (Sigma), 1 µM pepstatin A (Sigma) or 100 µM PMSF (Sigma).

**Biarsenical labeling.** HeLa cells were transfected with GFP-Ub-Q99C4 and DnaJb6 or DnaJb8 and biarsenical labeling was performed at 72 hours after transfection, as described before (Martin et al., 2005). Briefly, 1 mM ReAsH was pre-incubated in 10 mM 1,2-ethanedithiol (EDT; Sigma) in DMSO for 10 minutes. Cells were labeled with 1 μM ReAsH in 10 μM EDT in OptiMEM for 45 minutes at 37°C, 10% CO₂, in the dark, and subsequently washed several times with 1 mM EDT in OptiMEM containing 10% FCS during 30 minutes at room temperature to remove unbound dyes.

**Fluorescence and confocal microscopy.** HEK293T cells were co-transfected with GFP-Ub-polyQ and various chaperones as indicated in figure legends and the percentage of aggregate-positive cells was determined after 72 hours on a Leica IRB inverted fluorescence microscope. For determining the amount of aggregates present either in cytoplasm or nucleus, Mel Juso cells were transfected with GFP-Ub-Q104 and indicated chaperones. 1 µg/mL Hoechst 33342 (Sigma) was added prior to scoring to better discriminate between nuclei and cytoplasm. The number of cells containing aggregates in nucleus, cytoplasm or both compartments was determined at 48 and 72 hours after transfection on a Leica DM RA HC fluorescence microscope. Confocal images were obtained at 48 hours after transfection using a Leica TCS SP2 confocal system equipped with an Ar/Kr laser with a 63x objective. For FLIP analysis, either a part of the cytoplasm or the whole cell except for the aggregate was photo-bleached at full laser power repeatedly. Fluorescence loss was measured either within the nucleus or within the non-bleached region containing the aggregate, respectively. For FRAP analysis, fluorescently-tagged chaperones trapped in polyQ peptide-induced aggregates were bleached at full laser power and fluorescence recovery was measured in time.

**Immunoblotting.** Preparation of SDS-soluble and SDS–insoluble protein fractions was performed as described before (Carra et al., 2008). Briefly, cells were trypsinized, homogenized, and heated for 10 min at 99°C in sample buffer (SB; 70 mM Tris pH 6.8, 1.5% SDS, 20% glycerol) supplemented with 50 mM DTT at 72 hours after transfection. Cell lysates were centrifuged for at least 30 minutes at 14,000 rpm at room temperature. Supernatants were used as SDS-soluble fraction to which 0.05% bromophenol blue was added. Pellets representing the SDS-insoluble fraction were dissolved in 100% formic acid, incubated 30 minutes at 37°C, lyophilized overnight in a speed vac (Eppendorf) and resuspended in a quarter of the volume of SB (containing 0.05% bromophenol blue) added to soluble fraction. Samples were separated on
either 18% SDS-PAGE (anti-polyQ), or 12.5% SDS-PAGE (anti-GFP/anti-actin). After electrophoresis, proteins were transferred onto a 0.2 µm pore size nitrocellulose membrane filter (Schleicher & Schuell) and blocked in 5% dry milk in Tris-buffered saline (TBS). Western blots were incubated with primary antibodies against polyQ (1C2; 1:1.000; Millipore), GFP (1:1.000; Invitrogen), or β-actin (1:10,000; Sigma) in TBS containing 0.1% Tween-20, and subsequently with secondary antibodies IRDye 680 or IRDye 800 (1:10,000; LI-COR Biosciences). Signal was detected using the Odyssey imaging system (Westburg).

**Filter retardation assay.** Filter retardation assay was performed as described before (Wanker et al., 1999). Briefly, HEK293T cells expressing GFP-Ub-Q65 together with DnaJ6b or DnaJ8 for 72 hours incubated with the various inhibitors, were lysed for 30 minutes on ice in Nondinet P-40 (NP-40) buffer (100 mM TrisHCl, pH 7.5, 300 mM NaCl, 2% NP-40, 10 mM EDTA, pH 8.0, supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). After centrifugation 15 minutes at 14,000 rpm at 4°C, cell pellets were resuspended in benzonase buffer (1 mM MgCl$_2$, 50 mM Tris-HCl; pH 8.0) and incubated for 1 hour at 37°C with 250U benzonase (Merck). Reactions were stopped by adding 2x termination buffer (40 mM EDTA, 4% SDS, 100 mM DTT). Aliquots of 30 µg protein extract were diluted into 2% SDS buffer (2% SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and filtered through a 0.2 µm cellulose acetate membrane (Schleicher and Schuell) pre-equilibrated in 2% SDS buffer. Filters were washed twice with 0.1% SDS buffer (0.1% SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and subsequently blocked in 5% dry milk in TBS. Captured aggregates were detected by incubation with 1C2 antibody and further treated like Western blots.

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DnaJb6 and DnaJb8 reduce polyQ peptide aggregation


