Chapter 3

DNAJB6 is a peptide chaperone that suppresses initiation of aggregation mediated by polyglutamine containing polypeptide fragments

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A DNAJB Chaperone Subfamily with HDAC-dependent Activities Suppresses Toxic Protein Aggregation.


DNAJB6 is a peptide-binding chaperone which can suppress amyloid fibrillation of polyglutamine peptides at substoichiometric molar ratios.

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ABSTRACT

As explained in Chapter 2, misfolding and aggregation are associated with cytotoxicity in several protein folding diseases and large network of molecular chaperones ensures protein quality control. Within the Hsp70, Hsp110, and Hsp40 (DNAJ) chaperone families, members of a subclass of the DNAJ family (particularly DNAJB6b and DNAJB8) were found to be superior suppressors of aggregation and toxicity of disease-associated polyglutamine proteins. This anti-aggregation activity is dependent on a C-terminal serine-rich region and the C-terminal lysine that are crucial to their oligomerization and anti-aggregation activity. Moreover, DNAJB8 is (de)acetylated at two lysine in the C-terminal tail that are not involved in substrate binding, but do play a role in suppressing protein aggregation.

In vitro, purified DNAJB6 protein very efficiently suppressed the fibrillation of purified polyQ peptides, whilst fibrillation of the longer fragment of Huntington Exon-1 (HttEx1), as used in the cellular experiments, was suppressed far less efficiently. The effects were independent of Hsp70s. Together our data demonstrate that suppression of polyQ aggregation is due to a direct protein-protein interaction between DNAJB6 and polyQ-peptides, which are generated during proteolytic degradation of polyQ proteins and that form the seeds that initiate the aggregation unless prevented by the peptide chaperone DNAJB6.
INTRODUCTION

Ensuring and maintaining protein folding is a major challenge faced by all living cells. All cells have an extensive chaperoning network to deal with unfolded proteins. This network is linked to protein degradation systems and maintains cellular protein homeostasis (1). Under proteotoxic stress, the constitutive chaperoning network is augmented by the additional synthesis of chaperones. Critical nodes in the chaperoning network are the HSPA (HSP70) chaperoning complexes. The human genome encodes 13 HSPA and 4 HSPA-related HSPH (HSP110) proteins (2). The activity of the HSPA proteins is regulated by a number of cofactors, such as Hip (3), Bag family members (4), CHIP (5) and especially the DNAJ (HSP40) proteins (6). DNAJs prime the HSPA folding machine: they supply the substrate and thereby determine the specificity of the HSPA chaperone (2); they also stimulate the HSPA ATPase which activates the machine (7). The human DNAJ family has over 40 DNAJ members (2,6) and can be subdivided into 3 subfamilies. DNAJA proteins are most closely related to E. coli DNAJ and contain, besides the N-terminal J domain, a Gly/Phe rich domain, and a Cys rich region. The C-terminal domain is variable. DNAJB proteins lack the Cys rich region. DNAJC proteins lack both the Gly/Phe domain and the Cys rich region and the J domain is not always N-terminal. The J domain stimulates the ATPase activity of the HSPA proteins, while the other domains are thought to bind unfolded protein substrates which are then transferred to HSPA for refolding or to the degradation machinery for disposal. There is evidence that some DNAJA and DNAJB members may have chaperoning activity independently of HSPA (8). For the 13 cytosolic yeast DNAJs both specificity and redundancy have been shown to exist (9). Redundancy and specificity is also likely to hold true for the human DNAJs, but no systematic comparison of the human DNAJ proteins has been reported so far.

Deficiency in coping with proteotoxic stress leads to the accumulation of toxic protein aggregates and cell death. The failure of the heat shock system with age is a likely cause of age-related protein folding diseases (e.g. neurodegenerative diseases) and modulation of the entire heat shock response can affect both the onset of protein folding diseases and the ageing process (1). This further emphasized by the intimate relation between the heat shock response and the IGF signaling pathway (10), the most strongly established pathway related to healthy ageing. Within the context of the Hsp70 machines, especially DNAJ proteins seem to play a crucial role in protein folding diseases (11). However, it is not clear whether there are functional differences within the DNAJ family in this respect.

Amongst various neurodegenerative diseases, Polyglutamine diseases are a set of diseases caused by a CAG triplet expansion in the affected gene, leading to a prolonged polyglutamine (polyQ) stretch in the encoded protein (12). PolyQ stretches are a normal feature of many human proteins (13,14), suggesting an important cellular function. The normal form of the
human Huntingtin (Htt) protein contains a polyQ stretch of 11 to 34 glutamines in Htt Exon-1 (HttEx1). An expansion of the number of glutamines to above 35 leads to Huntington’s disease (HD), a severe disease hallmarked by protein fibrillation and neuronal inclusion body formation, linked to a progressive neurodegenerative process with uncontrolled movements, personality changes, and cognitive impairment. The length of the PolyQ stretch is statistically related to the age of onset of HD: the longer, the earlier the onset of symptoms (15). Fibrillation propensity is also dependent on polyQ length as shown in several cell and animal models as well as \textit{in vitro} data with purified proteins (16,17). This all suggests that aggregation initiates disease, although the nature of the toxic entities (fibrils or precursor oligomeric forms) that cause neurodegeneration is subject to debate.

Several reports suggest that aggregation initiation is associated with processing of polyQ-containing proteins by proteases, since the full length polyQ-containing proteins are less prone to aggregation (18,19). The proteasome is unable to cleave expanded polyQ stretches (20,21). Proteasomal processing of polyQ-containing proteins may therefore yield highly aggregation-prone polyQ peptides (22), not readily digested by peptidases (23). These polyQ peptides, by seeding the aggregation of polyQ-containing proteins, could thus be a major initiator of aggregation and disease.

In the current study, we compared the diverse members of the HSP70 machine for their capacity to prevent protein aggregation and related toxicity in cellular models of polyglutamine mediated diseases. DNAJB6 and DNAJB8 were the most potent suppressors of protein aggregation and its associated toxicity. They form polydispersed oligomeric complexes that bind to and suppress aggregation of non-foldable proteins in an HSPA-independent manner, and maintain them competent for degradation, which is a HSPA dependent process. Further, mutation of the two acetylated C-terminal lysine results in reduced aggregation suppressive activity. Using mass spectrometry, we unexpectedly found many peptides to be associated with DNAJB6. \textit{In vitro} data with purified DNAJB6 next revealed that also here it forms heterogeneous complexes, as found in the cells, and suppresses the fibrillation of initiated by polyQ peptides under conditions where the DNAJB1 and the HSPA machinery had no effect.

**RESULTS**

\textit{Among the members of the HSP70 machine DNAJB6 and DNAJB8 are the most potent suppressors of polyglutamine aggregation}

We tested the members of the HSP70 (HSPA), HSP110 (HSPH) and HSP40 (DNAJ) chaperone families for efficacy in suppression of the aggregation using HDQ119-EYFP (eYFP-tagged fragment of the exon-1 of the huntingtin gene containing 119 glutamines) as challenge.
None of the (V5-tagged) HSPH, HSPA or DNAJA proteins inhibited poly-Q aggregation as assayed by the formation of High Molecular Weight (HMW) material (Fig. 1A), SDS-insoluble aggregates retained in cellulose-acetate filters (Fig. 1B) and large cytosolic inclusions (Fig. 1C). However, expression of 2 members of the DNAJB subfamily, DNAJB6b and DNAJB8,
did do so (Fig. 1A,B). Cells expressing these proteins showed a diffuse staining pattern of HDQ119-EYFP (Fig. 1C), with less than 1% of cells containing inclusions compared to 25-30% in cells without chaperone co-expression (data not shown). Comparable results were obtained with non-tagged constructs (Fig. S1A, B). DNAJB6b and DNAJB8 also were effective in suppressing poly-Q aggregation in the brain derived cell lines N2A and SHSY-5Y (Fig. S1C), demonstrating that their effect is not cell type specific. The HDQ119 data thus revealed the greater potency of DNAJB6b and DNAJB8 as suppressors of protein aggregation.

Figure 2. The J-domain of DNAJB6-like proteins facilitates the degradation of polyglutamine expanded proteins. (A) Western blot and (B) Filter trap assay on extracts of cells coexpressing EGFP-HDQ74 and DNAJB J-domain mutants 48 hours after transfection (details as in fig. 1) (C) Schematic representation of DNAJB6b (upper panel). Western blot and filter trap assay on extracts of cells coexpressing HDQ119-EYFP and J-domain mutants of DNAJB6b (lower panel). Details as in fig 1. (D) Same as panel C for DNAJB8

The J-domain of DNAJB6b and DNAJB8 is not absolutely required for anti-aggregation activity. To investigate whether DNAJB6b and DNAJB8 depend on collaboration with HSPA proteins, we co-expressed lower levels of these proteins (Fig. S2 A, B) with HSPA family members. None
of the HSPA chaperones significantly increased the anti-aggregation effect of DNAJB6b (Fig. S2 B,C). DNAJB6b or DNAJB8 also completely inhibited poly-Q aggregation in O23 hamster lung fibroblasts (data not shown) that lack HSPA1A and HSPA1B under normal conditions (24). Thus, HSPA1A/B is not required for the activity of DNAJB6b or DNAJB8. However, other constitutively expressed members like HSPA8 (HSC70) might be the interaction partners of DNAJB6b or DNAJB8. Down-regulation of HSPA8 was toxic to the cells and its effect could thus not be tested.

As an alternative approach to test for functional interaction with the HSPA machine, we generated DNAJB6b and DNAJB8 mutants. Mutating the highly conserved HPD motif in the J domain is known to impair the functional cooperation between DNAJs and HSPAs (8). However, DNAJB6b and DNAJB8 mutants in which the histidine residue in the HPD motif was substituted by a glutamine (H/Q) (Fig. 2A,B) still were nearly fully capable of inhibiting EGFP-HDQ74 aggregation unlike the DNAJB1 H/Q mutant (Fig. 2A,B) (25). When a longer poly-Q tract was used (HDQ119-EYFP), the H/Q mutants as well as mutants lacking the complete J domain were significantly less active (Fig. 2C, D). These data show that the J domain is not absolutely required for the anti-aggregation activity of DNAJB6b or DNAJB8, although it does contribute to full activity.

**The C-terminus of DNAJB6b and DNAJB8 is crucial for the anti-aggregation activity**

The J-domain is not strictly required for the anti-aggregation activity of DNAJB6b and DNAJB8. In contrast, the C-terminus is crucial for its anti-aggregation activity. In search for functional regions within the C-terminus, we generated deletion mutants from both the C-terminal and N-terminal end of DNAJB8ΔJ (Fig. 3A). Activity was largely retained even upon deletion of the J domain and the first 80 AA of the C-terminal region (Fig. 3B). Thus the main activity resides within the C-terminal region (AA 152-232). Within this region a short sequence (AA 195-217: TTK-LKS) is rather conserved in the DNAJB6/DNAJB8 subfamily but not in the DNAJB1-like proteins. However, mutants lacking this sequence (ΔTTK-LKS), were still capable of preventing HDQ119-EYFP aggregation, although slightly less efficiently than the wild type protein (Fig. 3C). In contrast, deletion of a serine-rich region (AA 149-186: SSF-SST) characteristic for the DNAJB6/DNAJB8 subfamily but absent in members from the DNAJB1-subfamily resulted in a substantial loss of anti-aggregation capacity (Fig 3C: ΔSSF-SST). Combining this mutant with a deletion of the entire J-domain (ΔJ ΔSSF-SST) led to complete loss of function (Fig. 3C). Strikingly, also a minimal 24 amino acid C-terminal deletion combined with the J-domain deletion led to an almost complete loss in activity (Fig. 3B), indicating that also the C-terminal end is critical for DNAJB6/8 function.
Figure 3. The importance of the C-terminal domain of DnaJB8 for activity, substrate binding and oligomerization.

(A) Schematic representation of systematic DNAJB8 deletion mutants and (B,C) their effect on HDQ119-EYFP aggregation measured as in fig. 1.

(D) Cells transfected with YFP-HDQ119 and CFP-tagged DNAJB8 wildtype and mutant variants as indicated were analyzed for fluorescence resonance energy transfer (FRET) using fluorescence lifetime (FLIM) analysis; data are mean ± SD (N>9 individual cells).

(E) Sucrose density gradient fractionation of cells expressing DNAJB8 wildtype and mutant variants as indicated. DNAJB8 and its mutants were detected using anti-V5 antibody whereas endogenous DNAJB1 and HSPA1A/B with DNAJB1 or HSPA1A/B specific antibodies.
The SSF-SST domain in DNAJB6b and DNAJB8 is essential for substrate binding and formation of high molecular weight complexes

Time lapse microscopy on the dynamics of aggregate formation of HDQ119-EYFP in the presence or absence of mRFP-DNAJB6b or mRFP-DNAJB8 revealed that, in the few cells (< 1%) that did contain aggregates, DNAJB6b and DNAJB8 clearly co-localized with the inclusion at a very early stage (Fig. S3A), suggesting that they interact directly with HDQ119-EYFP. To conclusively address this, we used fluorescence life time microscopy (FLIM) to measure fluorescence resonance energy transfer (FRET) between DNAJB8 and polyQ. FRET efficiency indeed increased for YFP-HDQ119 and CFP-DNAJB8 to levels close to that of co-aggregating YFP-HDQ119 and CFP-HDQ74 (Fig. 3D), indeed implying direct interaction between the chaperones and the poly-Q substrates. Interestingly, the ΔSSF-SST mutant showed even lower FRET efficiency than CFP only (Fig. 3D), indicating its complete loss of interaction with the poly-Q substrate.

The C-terminal domain of DNAJB1 is involved in the formation of homodimers, and homodimerization has been proposed to be essential for function (26). To investigate whether DNAJB8 is also dimeric, we performed sucrose gradient with DNAJB8.

On 10-80% sucrose gradients, DNAJB8 was present in most fractions, indicating polydispersity (Fig. 3E). In contrast, DNAJB1 as well as HSP1A/B were recovered only in the first 4 fractions. The ΔSSF-SST mutant was also recovered only in the first fractions, indicating that loss of anti-aggregation (Fig. 3E) correlates with the loss of the ability to form larger oligomeric species.

The SSF-SST domain interacts with HDACs and HDAC activity is required for DNAJB8 anti-aggregation activity

Interestingly the SSF-SST region that is crucial for DNAJB8 function also partially overlaps with a region in DNAJB6 responsible for interaction with HDAC4 (27). Using pull-down experiments in cells co-expressing his-tagged DNAJB6b and DNAJB8 together with Flag-tagged HDACs representative of the 3 well-known HDAC families, type-I (HDAC1, HDAC3, HDAC8), type II (HDAC4, HDAC5, HDAC6, HDAC7) and type III (SIRT2), we found that HDAC4, HDAC6, and SIRT2 interacted with DNAJB6b and DNAJB8 (data not shown). Co-immunoprecipitation (IP) analyses confirmed that full length DNAJB6b and DNAJB8 indeed bound HDAC4, HDAC6 and SIRT2 whereas DNAJB8 ΔSSF-SST did not (Fig. 4A,B), showing that the SSF-SST motif is responsible for interaction with HDACs. DNAJB1, which lacks the SSF-SST motif, did not show interaction with these HDACs (data not shown).

To test whether interaction with these HDACs is functionally relevant, we treated cells with Trichostatin A (TSA), a general inhibitor of class I and II histone deacetylases. Although TSA
did not affect the complex size of DNAJB8 (data not shown), it did inhibit the anti-aggregation activity of DNAJB6b and DNAJB8 in a dose dependent manner (Fig. 4C, D; S3B,C). We also tested the effect of Tubacin, a specific inhibitor of de alpha-tubulin deacetylase activity of HDAC6 (28)(Haggarty et al., 2003) as HDAC6 has been implicated in quality control in poly-Q disease models (29)(Pandey et al., 2007). Tubacin did not inhibit DNAJB8 activity on HDQ119-EYFP aggregation (Fig. 4C,D) suggesting that it is either HDAC4 or the second, Tubacin insensitive, domain of HDAC6 that regulates the activity of DNAJB6b and DNAJB8.

We next used siRNA to down-regulate HDAC4, HDAC6 or SIRT2 (Fig. 4E; S3D). In cells in which HDAC6 or SIRT2 were functionally down-regulated (as evidenced by increased alpha-tubulin acetylation: Fig. S3E), DNAJB8 could still suppress HDQ119-EYFP aggregation (Fig. 4F). HDAC4 down-regulation was found to be rather toxic to cells expressing HDQ119-EYFP. In the surviving cells DNAJB8 overexpression no longer showed a protective effect (Fig. 4F), supporting a functional relation between HDAC4 and DNAJB8.

To test whether DNAJB8 itself is (de)acetylated, we treated cells with TSA and analyzed DNAJB8 by mass spectrometry. We could resolve 3 peaks in the TSA-treated samples that were absent in the control sample (VSEAYEVLSDSKK* VEVEEDGQLK*SVTVNGK, and SVTVNGK*EQLK (Fig. 4G).

These exactly matched the predicted molecular weight of DNAJB8 fragments with acetylated lysines (marked with asterix). The first lysine (K61) is in the J-domain and is conserved in all DNAJA and DNAJB as well as in most DNAJC proteins. The two other acetylated lysines (K216 and K223, Fig. S4) are in the C-terminal end of both DNAJB6b and DNAJB8. K216 is highly conserved amongst the DNAJB6/DNAJB8-like proteins In fact, only DNAJB7 lacks K216 and this protein also has no anti-aggregation activity. Note that the deletion of the C-terminal 24 amino acids, including K216, led to a complete loss in DNAJB6/8 activity (Fig. 3B).

To evaluate the importance of the lysines K216 and K223, we substituted either single or both lysines with arginine (K216R, K223R, and K216R+K223R) to mimic the deacetylated state. All K-to-R mutants retained full activity whereas substitution with non-polar alanines (K216A, K223A, and K216A+K223A) resulted in a substantial loss of activity (Fig 4H,I). The most crucial lysine was found to be K216 as the single K216A mutant already showed significant loss of activity. The single K223A still retained most activity, but a role for K223 cannot be ruled out, as the double K216A+K223A mutant was significantly less active than the K216A single mutant (Fig. 4H,I). Although the K216A+K223A double mutant was less able to prevent poly-Q aggregation, it still bound substrates as indicated by FLIM analysis (Fig. 3D) and it also still formed complexes (Fig. 3E) of the size of the wildtype DNAJB8.
DNAJB6 a peptide chaperone

Figure 4. DNAJB8 activity is dependent on HDAC activity. (A) Co-Immunoprecipitation of HDACs (FLAG tagged) with V5-tagged DNAJB1, DNAJB6b, DNAJB8 or DNAJB8 ΔSSF-SST. (B) Reverse co-immunoprecipitation of V5-tagged DNAJB proteins with FLAG-tagged SIRT2, HDAC4 and HDAC6. (C) Western blot and (D) filter trap analysis on extracts of DNAJB8 and HDQ119-EYFP expressing cells treated with Trichostatin A, Tubacin, or Niltubacin. The activity of the inhibitors was assessed using anti-acetylated tubulin specific antibody (anti-AcTub). (E) Western blot and (F) Filter trap analysis on extracts of cells coexpressing HDQ119-EYFP and DNAJB8, and treated with siRNA against HDAC4, HDAC6 or SIRT2 compared to cells treated with non-targeting siRNA (mock). (G) FT/MS spectrum of LVSEAYEVLSKK*, triply-charged ion m/z 537.28 selected for fragmentation with high accuracy mass with TSA (left) or without TSA (right). Acetylation of Lysine K61 is indicated with an asterix (*). (H) Western blot and (I) filter trap analysis on cells extracts co-transfected with HDQ119-EYFP and tetracycline inducible, wildtype DNAJB8 or the indicated lysine single or double mutants.
So, the SSF-SST region is required for substrate binding, for the quaternary architecture of the (DNAJB8) complex, and contains a docking site for HDACs. HDAC4 is required for the full anti-aggregation activity of DNAJB6 or DNAJB8. DNAJB8 contains two C-terminal lysines that can be acetylated and that play a role in DNAJB8 activity although not in substrate binding, implying that HDAC4 functionally regulates the processing of DNAJB6/8 substrates by direct deacetylation of lysine K216 (and K223).

DNAJB6 forms stable complexes with smaller peptides and exist as oligomer in purified form DNAJB6 is expressed constitutively, whereas DNAJB8 is normally only expressed in testes (Hageman paper). When searching for posttranslational modifications of DNAJB6, proteomic analysis after in-gel digestion of the DNAJB6 28kDa band lead to a surprising result. Many of these peptides in this band were identified as not from DNAJB, but from a large variety of other proteins with entirely different molecular weights (summarized as Fig. 5). This suggested that DNAJB6 binds to peptide of many different proteins in an SDS- and reducing independent manner and in such a way that it did not affect its mobility of SDS-PAGE. Such peptides must have been generated from proteolytic cleavage of this protein and given the suggested role of DNAJB6 n protein degradation hinted towards the hypothesis of DNAJB6 being a “peptide chaperone” that prevents the aggregation initiated by peptides, which generally are aggregation-prone. Even for the aggregation of polyQ proteins, it has been demonstrated that the full length protein is in fact not very aggregation-prone and disease inducing and that actually calpains or caspases are thought to cleave them into smaller polypeptides which are aggregation prone. Though we were not able to find any specific interacting partner of DNAJB6 other than expected other heat shock proteins, proteasomal components etc.

Figure 5. DNAJB6 form stable complexes with small peptides
Proteomic analysis with DNAJB6 expressing cells using in-gel digestion. The bold red circle summarizes the results from whole lane analysis where each and every protein band was analysed through MS. The dotted circle summarizes the results obtained from analyzing DNAJB6 band only. The overlap is indicated.
Suppression of polyQ fibrillation by purified DNAJB6 protein.
To gain further insight into the mechanism of DNAJB6 and DNAJB8 in preventing polyQ aggregation, purified DNAJB6 was used to study polyQ peptide fibrillation through ThT assay (original article: Mansson, 2013). DNAJB6 was used as obvious choice because it is ubiquitously expressed including brain unlike DNAJB8 which is confined to testis in adults (original article: Hageman, 2010). In ThT fibrillation assay, the lag phase preceding fibrillation increased with increasing concentrations of DNAJB6 (Fig. 4). For Q45 peptides, fibrillation suppression occurred at a molar ratio polyQ to DNAJB6 as low as 1:0.01 (Fig. 6A). For HttEx1Q45, the fibrillation suppression was far less efficient (Fig. 6B). The t½-values for fibrillation (Table I) was increased by DNAJB6 approximately 10-fold for the Q45 peptide at a 1:0.1 molar ratio, but only by a factor of 3 for HttExQ45 at a similar molar ratio. At lower molar ratio (1:0.01), the t½ of fibrillation was increased by a factor of 2 for the Q45 peptide, whilst no significant effects were seen for HttEx1Q45.

Figure 6. Suppression of fibrillation of Q45 and HttEx1Q45 by DNAJB6.
Fibrillation was measured in triplicates as the relative ThT fluorescence increase, at 15 μM Q45 (a) or HttEx1Q45 (b) with DNAJB6 added in varying polyQ to DNAJB6 molar ratios: 1:0.1 (red), 1:0.01 (dark pink), 1:0.001 (pink), no added DNAJB6 (black), and BSA added in ratio 1:0.1 (yellow green). Note the difference in time scale between Q45 and HttEx1Q45. Molar ratios were calculated as monomer to monomer, although DNAJB6 is oligomeric.

Suppression of the formation of SDS-insoluble aggregates by DNAJB6.
As a complement to the ThT fluorescence measurements, we also assessed the effect of DNAJB6 on the formation of SDS-insoluble aggregates. Samples of Q45 peptide or HttEx1Q45 were incubated without or with DNAJB6, and aliquots were withdrawn at different time intervals and subjected to SDS-PAGE. The disappearance of the band corresponding to monomeric HttEx1Q45 and Q45 peptide (Fig. 7A) concomitant with appearance of SDS-insoluble aggregates at the top of the gel (Fig. 7B) was suppressed by DNAJB6 (Fig. 7C&D) at a 1:1 molar ratio.
Imaging and quantification of the protein bands in gels with DNAJB6 at different molar ratios showed that with substoichiometric amounts of DNAJB6 (1:0.1) there is partial aggregation suppression for HttEx1Q45, with disappearance of soluble HttEx1Q45 (Fig. 7F) occurring concomitant with the appearance of SDS-insoluble HttEx1Q45 aggregates (Fig. 7H). For the Q45 peptide disappearance of soluble Q45 occurred (Fig. 7E), without appearance of SDS-insoluble Q45 aggregates (Fig. 7G). Thus, no SDS-insoluble aggregates formed from Q45 peptides in the presence of DNAJB6, suggesting that even substoichiometric amounts of DNAJB6 affect structure, conformation and stability of the Q45 peptides.

Table I Suppression of PolyQ fibrillation by DNAJB6.

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Fibrillation suppression by DNAJB6 compared to other DNAJB protein.

To evaluate how specific the polyQ fibrillation suppression by DNAJB6 is compared to other DNAJB proteins, we added DNAJB6, its close homologue DNAJB8, and the more canonical family member, the structurally characterized homologue DNAJB1. DNAJB6 suppressed fibrillation of Q45 entirely, DNAJB8 delayed fibrillation by a factor of 4, and DNAJB1 delayed fibrillation only slightly, at a molar ratio 1:0.1 (Fig. 8 I-a; left panel). At a molar ratio 1:0.01 where DNAJB1 had no effect at all, DNAJB6 still delayed fibrillation with nearly a factor 3 (data not shown). For HttExQ45, the fibrillation was delayed 2.5-fold with DNAJB6 and, to a small extent, by DNAJB1 and DNAJB8 (Fig. 8 I-b; left panel). The suppression of polyQ fibrillation by DNAJB6 was not further enhanced in presence of HSPA1 and ATP (Fig. 8 II; right panel), confirming the finding in cellular systems that suppression of polyQ aggregation by DNAJB6 is HSPA1/ATP independent (30); original article: Hageman, 2010. Thus, DNAJB6 has unique fibrillation suppression properties, which are lacking in DNAJB1 and, probably, are located in the C-terminal and putatively substrate-binding domain which is not very conserved among the many different DNAJB proteins.
Figure 7. Suppression of the formation of SDS-insoluble aggregates by DNAJB6.

Formation of SDS-insoluble polyQ aggregates was followed on SDS-PAGE for 20 μM Q45 (a, c) and Ex1Q45 (b, d) in the absence (a, b) or presence (c, d) of 20 μM DNAJB6. Gels were stained for 1 h (b, d) or 3 h (a, c) with SYPRO Orange. This dye is poorly bound to HttEx1Q45 and even more so to Q45, which appear as faintly stained bands on the gels, despite being present at much higher levels than DNAJB6. Protein band intensities were quantified and plotted as a relative amount of soluble monomers (e, f) or SDS insoluble aggregates (g, h) at 20μM Q45 (e, g) and HttEx1Q45 (f, h) without DNAJB6 (filled circles) or with DNAJB6 in a molar ratio of 1:0.1 (open diamonds) or 1:1 (filled squares). Molar ratios were calculated as monomer to monomer, although DNAJB6 is oligomeric. Note the difference in time scale between Q45 and HttEx1Q45. The data were fitted to the sigmoidal equation \( y = a / (1 + \exp(b(c-x))) \), where \( a \) is the proportion of aggregates at steady state, \( b \) is the slope of the exponential growth phase, and \( c \) is the time where 50% of the aggregation is reached.
Here in this chapter, DNAJB6 was identified, together with its closest homologue DNAJB8, as unique polyQ aggregation suppressors among the human HSPA, HSPH, and DNAJ family members. Surprisingly, HSPA and HSPH family members, highly efficient in stimulating protein folding, are in fact very poor suppressors of protein aggregation. DNAJ (HSP40) proteins, which control the specificity and activity of the HSPA chaperoning network, do suppress the aggregation of poly-Q proteins, but with clearly differential efficacy. The canonical DNAJ members (e.g. DNAJA1 and DNAJB1) are much less potent (< 20 fold) than the members of a DNAJB subfamily, in particular DNAJB6b and DNAJB8. The suppression was largely independent of the expression of Hsp70 members both in cells and in vitro studies with purified DNAJB6 where DNAJB6 clearly suppressed the fibrillation of Q45 peptide. The absence of a comparable polyQ aggregation inhibitory effect of DNAJB1 that shares a homologous J domain but has little sequence resemblance with DNAJB6 in its C-terminus (only 17 %) supports the crucial role for the C-terminal domain of DNAJB6 and DNAJB8 in the polyQ aggregation suppression. The DNAJB1 C-terminus lacks a serine-rich
motif (SSF-SST) and two lysines that were found to be important for the anti-aggregation function.

The anti-aggregation function of DNAJB6-type proteins is evolutionary conserved. The *Drosophila* ortholog of DNAJB6, Mrj1, was identified in a screen for genetic factors that dominantly modify poly-Q toxicity (31). Both Mrj or DNAJB6 overexpression suppresses poly-Q toxicity in various fly tissues and human cells (32,33). DNAJB6 orthologs are found in all metazoans analysed, suggesting a general need for an anti-aggregation chaperone. Homozygous DNAJB6 mutant mice die due to a failure of chorioallantoic fusion (34); DNAJB6 binds to keratin and prevents its toxic aggregation in chorionic trophoblast cells during chorioallantoic attachment in placental development (35,36). As such it mediates keratin turnover, which would agree with our observation that DNAJB6 (and DNAJB8) maintain poly-Q proteins in a degradation competent form.

Further, for anti-aggregation effect, DNAJB6b and DNAJB8 do not depend on its J-domain but on full SSF-SST domain, which is not only required for their client binding and oligomerization but also for their interaction with HDACs. The oligomerized state was further confirmed by our *in vitro* results with purified DNAJB6 where it resembles the polydisperse and dynamic oligomerization (*original article: Mansson et al. 2013*) of some small heat shock proteins for example αB-crystallin (37). The functional importance of this interaction is shown by the finding that HDAC inhibition or mutagenesis of the acetylation competent C-terminal lysines K216 and K223 resulted in a similar loss of activity as when deleting the SSF-SST domain. Neither HDAC inhibition nor lysine to alanine mutagenesis affected oligomerization and substrate binding. Together, this suggests that HDAC4 docks on the SSF-SST domains of polydispersed DNAJB6/8 already loaded with substrates and removes inhibitory acetyl groups, thereby activating substrate processing in the complex.

So, how can our current findings of highly efficient suppression of polyQ peptide aggregation by DNAJB6 be related to its anti-aggregation and toxicity-preventing effects in cellular (33); *original article: Hageman et al. 2010*; and animal models (32) of polyQ diseases? One obvious possibility relates to the findings that proteins containing expanded polyQ stretches are not fully degraded by the proteasome (21). As a consequence, polyQ peptides that have a high propensity to aggregate into fibrillar assemblies (38) are released from the proteasome during the degradation of huntingtin (20,21) and other polyQ proteins. That free polyQ peptides are not readily detected in cells can be due to their immediate capture by DNAJB6-like chaperones and subsequent degradation by peptidases or autophagy (23). If such processing fails, polyQ peptides would, however, become enriched and immediately seed aggregation into both main and side chain hydrogen-bonded fibrillar structures (39,40). Other proteins containing Q stretches or protease-generated fragments of the polyQ
proteins next will co-aggregate with such peptides and contribute to the toxicity by either
direct aggregated toxicity or (secondary) loss of function of these sequestered proteins. The
rate and propensity of this will likely depend on the context around the polyQ stretches in
these fragments (41). If released in the cellular environment upon cell death, aggregates
can be transmitted to neighbouring cells and act as seeds promoting the aggregation of
endogenous polyQ-containing proteins (42), reminiscent of the spreading seen for the prion
PrP (43). To mimic proteasomal release of polyQ peptides in living cells, overexpression
of genetically engineered constructs of polyQ peptides was therefore performed, showing
that the polyQ peptides induced the formation of aggregates in which polyQ-containing
proteins and other heat shock proteins (HSPs) were detected, supporting the view that polyQ
peptides can act as seeds for aggregation (22). Moreover, using the same cell system, it
was recently shown that co-expression of these genetically engineered constructs of polyQ
peptides with DNAJB6 suppressed aggregation (30). Interestingly, in the few aggregates
that did form, DNAJB6 and DNAJB8 were found in the core of the aggregates, whereas other
chaperones (HSPA1) only associated with the periphery of the aggregates. This is indicating
that DNAJB6 interacts early and directly with polyQ peptides as shown in this study, and the
other HSPs interact later and more indirectly, presumably not until after the aggregates have
formed.

To summarize, the data presented in this study show that DNAJB6 is a very strong protector
against protein toxicity associated with polyQ aggregation diseases and it can directly
bind to and highly efficiently suppress polyQ peptide fibrillation initiated by soluble, pure
polyQ peptides. The view of DNAJB6 as a peptide-binding chaperone that can sweep up
polyQ peptides that are incompletely degraded by and released from the proteasome and
suppress fibrillation initiation makes DNAJB6 an interesting target for therapy against such
protein aggregation diseases.

METHODS AND MATERIALS

Cell lines, cell culture and transient transfections.
N2A (CCL-131), SH-SY5Y (CRL-2266) and HEK-293 stably expressing the tetracycline (tet)
repressor (Flp-In T-REx HEK-293, Invitrogen) were grown in DMEM (Gibco) plus 10% foetal
bovine serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and for
Flp-In T-REx HEK-293 cells, 5 μg/ml Blasticidin (Sigma) and 100 μg/ml of Zeocin (Invitrogen).
For transient transfections, cells were grown to 50-60% confluence in 35 mm-diameter
dishes coated with 0.001% of poly-L-lysine (Sigma) and/or on coated coverslips for confocal
microscopy analyses. Cells were transfected with Lipofectamine (Gibco) according to the
manufacturer’s instructions. For stable tet-inducible HDQ119-EYFP expressing cell lines,
cells were co-transfected with pcDNA5/FRT/TO HDQ119-EYFP and the Flp recombinase
expressing plasmid pOG44 and selected with 100μg/ml hygromycin. For stable DNAJB8 expressing cell lines, cells were transfected with pcDNA3 V5 DNAJB8 and selected using 500μg/ml neomycin. The double stable cell line was made by transfecting the pcDNA3 V5 DNAJB8 construct in the pcDNA5/FRT/TO HDQ119-EYFP stable cell line and selecting for hygromycin and neomycin resistant clones.

**Filter trap assay**

10, 2 and 0.4 μg of protein extracts were applied onto 0.2 μm pore Cellulose Acetate membrane prewashed with FTA + 0.1% SDS. Mild suction was applied and membranes were washed 3 times and stained with mouse anti-GFP antibody JL-8 (Clontech) at a 1:5000 dilution and HRP-conjugated anti-mouse secondary antibody (Amersham) at 17 1:5000 dilution. Staining was visualized by enhanced chemiluminescence and Hyperfilm (ECL, Amersham).

**Gene cloning and generation of mutants**

Detailed information about the (construction of) plasmids used in this study can be found in original article on which this chapter is based (Hageman et al., 2010; Mansson et al., 2013)

**FLIM**

FLIM experiments were performed on an inverted Nikon TE2000 microscope using the LIFA frequency domain lifetime attachment (Lambert Instruments) and LI-FLIM software. CFP was excited with light from an 1W 445 nm LED, using the AQUA filter cube (EX 436/20, DM 455, BA 480/30), modulated at 40 MHz and emission was collected using an intensified CCD camera. Lifetimes were referenced to a 10 mM solution of Fluorescein in saline pH10 that was set at 4.00 ns lifetime. The measured lifetimes (calculated from phase differences) of CFP in the absence of acceptors were 2.5 ns. FRET efficiency E was calculated as E=1-(measured lifetime of FRET pair)/(measured lifetime of donor). FRET efficiencies are means of 9-23 ROI’s per construct.

**Mass Spectrometry**

After immunoprecipitation of DNAJB8 and SDS-PAA electrophoresis, ingel digestion was performed. The digested material was analyzed with a LTQ–Orbitrap-XL mass spectrometer (ThermoFisher Scientific, San Jose, CA) (see supplemental information for details).

**Recombinant expression and purification of DNAJB6/8.**

Detailed information regarding the purification of DNAJB6 and determination of particle size can be found in original article (Mansson et al., 2013).
**Fibrillation assay based on thioflavin T fluorescence increase**

The constructs of purified His-tagged MBP-TEV-HttEx1Qn and MBP-TEV-Qn of varying lengths were used to monitor fibril formation by continuous measurement of thioflavin T (ThT) fluorescence increase (44). The final volume was 100 μl per well in a 96-well, low-binding, half-area microplate (Corning 3881; Corning Incorporated Life Sciences, Acton, MA) kept on ice, while constructs from 30 μM stock solutions were mixed to a final concentration of 15 μM (or as else stated) with filtered and degassed buffer (Tris–HCl, pH 7.4 and 150 mM KCl); the final concentration of ThT was 10 μM, and the final concentration of DNAJB6, DNAJB8, and DNAJB1 (obtained from Sigma-Aldrich, Stockholm, Sweden) was varied as stated in Fig. legends. All molar ratios between proteins are calculated on a monomer-to-monomer basis, although the same oligomeric DNAJB6 protein was used in all assays. HSPA1 (Sigma-Aldrich, Stockholm, Sweden) was added in a molar ration to DNAJB of 2:1, together with 2 mM ATP and 5 mM MgCl2.

**Fibrillar assembly monitored as formation of SDS-insoluble material**

The assembly of purified HttEx1Qn and Qn peptides into fibrils was monitored also by SDS-PAGE as disappearance of the band corresponding to monomeric, SDS-soluble HttEx1Q45 and Q45 and concomitant appearance of SDS insoluble material accumulated at the top of the gel using 13% Tris–tricine (Q45) or 12 % Tris–glycine (HttEx1Q45) gels stained with SYPRO Orange (Invitrogen, Paisley, UK) followed by imaging with a LAS-3000 (Fujifilm, Tokyo, Japan) using the software Multigauge (Life Science Systems), for quantification of protein bands. Experiments were performed two to six times, and the data presented are representative of the experiments we performed. After adding TEV protease (molar ratio protein to protease 50:1), the microplate plate was sealed with a plastic film to avoid evaporation and immediately inserted into the plate reader FLUOstar Omega (BMG Labtech, Offenburg, Germany) and incubated at 37 °C without agitation. ThT fluorescence was recorded every 150 s through the bottom of the plate with excitation and emission filters of 440 and 480 nm, respectively. Fibrillation under seeded conditions was performed by adding preformed fibrils, from a fibrillation assay experiment on the previous day, corresponding to an additional 5 or 20 % of the monomer concentration (10 μM).
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DNAJB6 a peptide chaperone


SUPPLEMENTARY INFORMATION – CHAPTER 3

EXPERIMENTAL PROCEDURES

siRNA mediated knockdown.
siGENOME SMARTpool DNAJB6 siRNA (M-013020-00), HDAC-4 siRNA (M-003497-03), HDAC-6 siRNA (M-003499-00) and SIRT2 siRNA (M-004826-02) were purchased from Dharmacon and transfected in a final concentration of 50 nM using lipofectamine 2000 (Invitrogen). Cells were transfected with siRNA 96 hours before analysis and once again were transfected with siRNA in combination with DNA 48 hours prior to analysis. Non-targeting siRNA (Dharmacon siGENOME® Non-Targeting siRNA Pools/pool #1 D-001206-13-20, which is a mix of 4 non-targeting siRNA molecules) was used negative control.

Site directed mutagenesis and generation of deletion mutants
Site-directed mutagenesis was done using the QuickChange Site-Directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer. Primers used are listed in original article (Hageman et al., 2010) Deletion mutants were constructed as follows. First, the whole plasmid except the fragment to be deleted was amplified by inverse PCR. Thereafter, the parental DNA was cleaved by DpnI, the PCR product was phosphorylated by T4 polynucleotide kinase (Invitrogen), self-ligated by T4 ligase (Invitrogen) and transformed. All mutations were verified by sequencing and expression was verified by western blot analysis of transfected cell extracts.

Cell extracts and sample preparation
24 or 48 hours after transfection cells were recovered by trypsinization, pelleted and resuspended in 1 ml of PBS. The cell suspension was centrifuged at 6000 rpm for 5 min at RT and the pellet was resuspended in 75-100 ul of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 2% SDS plus protease inhibitors) and sonicated. Protein content was determined with the DC protein assay (Bio-Rad). Western blot samples were prepared at a final concentration of 1ug/ul in SDS-PAGE loading buffer and heated for 5 min at 100 °C. Filter trap samples were prepared at a final concentration of 100 ng/ul, 20 ng/ul and 4 ng/ul in FTA buffer (10 mM Tris- Cl pH 8.0, 150 mM NaCl and 50 mM dithiothreitol) + 2% SDS and heated for 5 min at 100 °C. Samples were used immediately or kept frozen at -20°C.

Western blot analysis
Equal amounts of protein were loaded on 10% or 12.5% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and probed with mouse anti- GFP antibody JL-8 (Clontech) at a 1:5000 dilution, mouse anti-V5 antibody (Invitrogen) at a 1:5000 dilution
or rabbit AR (N20) antibody (Santa Cruz) at a 1:2000 dilution. Anti-HSP40 (SPA 400) and anti-HSPA (SPA 810) were purchased from Stressgen and used at a 1:1500 dilution. Anti-HDAC4 (H-92), anti-HDAC6 H-300) and anti-SIRT2 (H-95) were obtained from Santa-Cruz and used at a 1:400 dilution. Anti-DNAJB6 was obtained from Abnova and used at a 1:2000 dilution. Both anti-acetylated Tubulin and anti-FLAG antibodies were obtained from Sigma and used at a 1:1000 dilution. Anti-1 HSF-1 was purchased from Stressgen and used at 1:15000 dilutions. GAPDH was used as a loading control and was detected with a mouse antibody (RDI Research Diagnostics) at 1:10000 dilution. Blots were subsequently incubated with HRP-conjugated secondary antibodies (Amersham) at 1:5000 dilution. Visualization was performed with enhanced chemiluminescence and Hyperfilm (ECL, Amersham).

**Immunolabelling and confocal microscopy**

16-24 hours after transfection indirect immunofluorescence of the V5 tag was performed to detect the exogenously expressed chaperones. Cells were fixed with 3.7% formaldehyde for 15 minutes, washed three times with Phosphate-Buffered Saline (PBS), permeabilized with 0.2% Triton-X100 and blocked during 30 minutes with 0.5% BSA and 0.1% glycine in PBS. Incubation with mouse anti-V5 monoclonal antibody (Invitrogen) 1:100 dilution was performed overnight at 4°C followed by a 1 hour incubation with CY5-conjugated anti-mouse secondary antibody (Jackson) at 1:200 dilution. Aggregates were visualized using the EYFP or EGFP tag. To visualize nuclei, cells were stained 10 minutes with 0.2 μg/ml 4',6-diamidino-2-phenylindole (DAPI). Cover slips were mounted in Citifluor. Images of EYFP, EGFP, CY5, and DAPI fluorescence were obtained using the Leica confocal laser scanning microscope (Leica TCS SP2, DM RXE) with a 63X/1.32 oil lens. The captured images were processed using Leica Confocal Software and Adobe Photoshop.

**Immunoprecipitation assay**

Cells were trypsinized and washed with PBS. Cell pellets were harvested in 0.5 ml of ice cold lysis buffer containing 150 mM NaCl, 50 mM NaH2PO4, 10 mM imidazole, 0.5% NP-40 (Igepal), 1.5 mM MgCl2, 3% glycerol 0.9 mM DTT and protease inhibitors, pH 8.0. Cells were lysed by passage through a 26G needle for 5 times and subsequently centrifuged twice at 14000 rpm for 15 minutes. Subsequently, the supernatant was pre-cleared with 10 ul of Sepharose-A/G beads (Santa Cruz Biotechnologies) and incubated for 1 hour at 4 °C with slow agitation. Next, 50 μl of lysisbuffer containing 1 ug prebound antibody to 10 ul Sepharose-A/G beads was added and incubated at 4 °C for 1 hour with slow agitation. Thereafter, the beads were washed 4 times with wash buffer containing 300 mM NaCl, 50 mM NaH2PO4, 20 mM imidazole, 0.5% NP-40 (Igepal), 1.5 mM MgCl2, 3% glycerol, pH 8.0. The proteins were eluted from the pelleted beads in 30 ul lysis buffer and 30 ul 2X Laemli samplebuffer and boiled. 10 ul was subsequently loaded for western blot analysis.
Sucrose Density Gradient Fractionation.

Cells from a 35 mm dish were trypsinized, washed and lysed in 200 ul lysis buffer pH 8 containing 150 mM NaCl, 50 mM NaH2PO4, 10 mM imidazole, 0.5% NP-40, 1.5 mM MgCl2 and 3% glycerol. After passing through a 25G gauge needle 5 times, the lysate was centrifuged at 300xg for 15 min. Thereafter 4.5 ml sucrose gradients containing 10-80% sucrose in 10 mM Tris-HCl pH 8, 50 mM NaCl, 5 mM EDTA were loaded with 150 ul of the lysate and centrifuged at 100,000xg for 18 hours in a Sorvall Discovery 90 SE ultra-centrifuge using the SW55 rotor. Next, fractions of 400 ul were taken and precipitated with an equal volume of 25% (w/v) trichloroacetic acid. After 30 min incubation on ice, the precipitate was pelleted at 14000 rpm for 15 min. The pellet was washed twice with 80% acetone, air dried and dissolved in 30 ul 1% SDS/0.1M NaOH and an equal volume of Laemmli buffer. 10 ul of the sample was subsequently loaded on SDS-PAGE gels for western analysis.

HDAC inhibition

For type I and II HDAC inhibition, trichostatin A (TSA) (Sigma) was added to a final concentration of 0, 0.1 or 0.5 μM. The HDAC6 specific inhibitor Tubacin or the inactive analog Niltubacin were a kind gift from Dr. Schreiber and described before (Hideshima et al., 2005). Both were used at a final concentration of 0, 3 or 5 μM. All HDAC inhibitors were added for 24 hours. Thereafter, cells were lysed and processed as described above.

Mass Spectometry

After immunoprecipitation of DNAJB8 or DNAJB6 and SDS-PAA electrophoresis, in-gel digestion was performed according to protocol of (Shevchenko et al., 1996). Trypsin (sequencing grade modified trypsin, # V5111, www.promega.com) was used (10μg/mL) for digestion overnight at 37°C with shaking at 450 rpm (Eppendorf Thermomixer). Digestion with Endoproteinase Asp-N (# 11420488001, Roche), Endoproteinase Lys-C (# 11420429001, Roche), Endoproteinase Glu-C (# 11420399001, Roche), was done at the same conditions except the temperature for digestion with Glu-C (25°C). The digested material was analysed by nanoLC–MS/MS on an Ultimate 3000 system ( Dionex, Amsterdam, The Netherlands) connected on-line with a LTQ–Orbitrap-XL mass spectrometer (ThermoFisher Scientific, San Jose, CA). Samples were loaded onto a 5 mm x 300 μm i.d. trapping micro column packed 15 with C18 PepMAP100 5 μm particles (Dionex) in 0.1% FA at the flow rate of 20 μL/min. Upon loading and washing, peptides were back-flush eluted onto a 15 cm x 75 μm i.d. nanocolumn, packed with C18 PepMAP100 3 μm particles (Dionex). The following gradient was used at the flow rate of 300 nL/min: 5–50% of solvent B (30 min), 50–90% B (5 min), 90% B (7 min), and to 5% B (3 min). Solvent A: H2O/acetonitrile/formic acid (v/v); 100:0:1, solvent B:10:90 H2O/acetonitrile/formic acid (v/v); 10:90:1. Peptides were infused into the mass spectrometer via dynamic nanospray probe (ThermoElectron Corp.) with a stainless steel
emitter (Proxeon, Odense, DK). Typical spray voltage was 1.6 kV with no sheath and auxiliary gas flow; ion transfer tube at temperature 200°C. Operation of mass spectrometer was done in data-dependent mode. The automated gain control (AGC) was 5 x 10^5 charges and 1 x 10^4 charges for MS/MS at the linear ion trap analyzer. DDA cycle consisted of the survey scan within m/z 300–1600 at the Orbitrap analyzer with target mass resolution of 60,000 (FWHM, full width at half maximum at m/z 400) followed by MS/MS fragmentation of the five most intense precursor ions under the relative collision energy of 35% in the linear trap. Singly charged ions were excluded from MS/MS experiments, and m/z of fragmented precursor ions were dynamically excluded for further 90 s. Ion selection threshold for triggering MS/MS experiments set to 1000 counts. Protein identification was performed using the SEQUEST algorithm in the BioWorks™ 3.1 software (Thermo Electron) and the uncompressed human database (Swiss Institute of Bioinformatics, Geneva, Switzerland). The following HUPO SEQUEST criteria were selected for high confidence peptide identification: 1- charge state versus crosscorrelation number (XCorr) and that is XCorr > 1.9 for singly charged ions, XCorr > 2.7 for doubly charged ions, and XCorr > 3.75 for triply charged ions, 2- deltaCn 0.1, 3- peptide probability 0.001, 4-RsP 4- and 5-final score (sf) 0.85.
Figure S1. Aggregation suppressive activity of untagged chaperone members is similar to that of tagged proteins. 

(A) Western blot analysis of cell extracts prepared 24 hours after transfection. High molecular weight (HMW) aggregates trapped in the stacking gel and soluble HDQ119-EYFP were assessed with anti-GFP antibodies. (B) Filter trap assay in samples expressing HDQ119-EYFP without and with coexpression of molecular chaperones prepared 24 hours after cotransfection. Serial five-fold dilutions were loaded on cellulose acetate membranes and probed with anti-GFP antibody. Numbers next to the lanes represent the percentage of aggregation in cells with exogenous chaperones (+Tet) compared to cells without the exogenous chaperones (-Tet).

DNAJB6a and DNAJB8 also suppress protein aggregation in neuronal like cell lines (C) N2A and SHSY-5Y were cotransfected with expression plasmids for HDQ119-EYFP and the chaperones HSPA1A, DNAJB1, DNAJB6b and DNAJB8. Western blot analysis for chaperone expression in N2A cells using the anti-V5 antibody (left panel) and filter trap assay (right panel). Serial five-fold dilutions were loaded on cellulose acetate membranes and probed with anti-GFP antibody. Numbers next to the lanes represent the percentage of aggregation in cells with exogenous chaperones compared to cells without the exogenous chaperones for both cell lines.
Figure S2. DNAJB6-like aggregation suppressive effects are dose dependent.

(A) Western and filter trap assay on cells co-expressing HDQ119-EYFP and DNAJB6b or DNAJB8. Dose dependent expression was induced by the addition of increasing amounts of tetracycline as indicated.

DNAJB6 or DNAJB8 do not synergize with overexpressed HSPA proteins.

(B) Western blot analysis of cell extracts prepared 24 hours after transfection. The expression of chaperones was assessed using the anti-V5 antibody.

(C) Filter trap assay in samples without and with overexpression of HDQ119-EYFP together with DNAJB1, DNAJB6b or DNAJB8 and various HSPA chaperones. Samples were prepared 24 hours after cotransfection. Serial five-fold dilutions were loaded on cellulose acetate membranes and probed with anti-GFP antibody.
Figure S3: The chaperones colocalize with HDQ119-EYFP early during aggregate formation and dose dependent relationship between Trichostatin A and reduction of DNAJB6b or DNAJB8 mediated aggregation suppression.

(A) Time lapse imaging of aggregate formation and co-localization with DNAJB6b or DNAJB8. For each panel, confocal pictures were taken at the beginning (upper row) or at the end of the formation of the aggregate (lower row). Arrows indicate the positions of the aggregate.

(B) Western analysis of cells cotransfected with expression constructs for HDQ119-EYFP and DNAJB6b or DNAJB8 and treated with various doses of Trichostatin A as indicated. Samples were prepared 24 hours after transfection and addition of Trichostatin A.

(C) Filter trap analysis of cells cotransfected with expression constructs for HDQ119-EYFP and DNAJB6b or DNAJB8 and treated with various doses of Trichostatin A as indicated. Samples were prepared 24 hours after transfection and addition of Trichostatin A. Serial five-fold dilutions were loaded on cellulose acetate membranes and probed with anti-GFP antibody.

(D) Specific siRNA mediated knockdown of Flag-tagged HDAC4, HDAC6 or SIRT2 verified by western blot analysis.

(E) Knockdown of HDAC6 results in enhanced acetylation of Tubulin as demonstrated by detection of acetylated tubulin with an antibody specific for acetylated tubulin.
Figure S4: DNAJB8 is acetylated on K61, K216 and K223
Schematic representation of lysine groups in DNAJB8, which are acetylated upon incubation with TSA.