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

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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 (Morimoto, 2008). Under proteotoxic stress, the constitutive chaperoning network is augmented by 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 proteins (Kampinga et al., 2009). The activity of the HSPA proteins is regulated by a number of cofactors, such as Hip (Höhfeld et al., 1995), Bag family members (Takayama et al., 1997), CHIP (Ballinger et al., 1999), and especially the DNAJ (HSP40) proteins (Qiu et al., 2006). DNAJs prime the HSPA folding machine: they supply the substrate and thereby determine the specificity of the HSPA chaperone; they also stimulate the HSPA ATPase that activates the machine (Bukau et al., 2006). The human DNAJ family has over 40 DNAJ members (Qiu et al., 2006; Kampinga et al., 2009) and can be subdivided into three subfamilies. DNAJA proteins are most closely related to E. coli DnaJ and contain, besides the N-terminal J-domain, a glycine/phenylalanine-rich domain and a cysteine-rich region. The C-terminal domain is variable. DNAJB proteins lack the cysteine-rich region. DNAJC proteins lack both the glycine/phenylalanine domain and the cysteine-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 for transfer 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 (Cheetham and Caplan, 1998). For the 13 cytosolic yeast DNAJs, both specificity and redundancy have been shown to exist (Sahi and Craig, 2007). Redundancy and specificity are 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 aging process (Morimoto, 2008). This is further emphasized by the intimate relation between the heat shock response and the IGF signaling pathway (Hsu et al., 2003), the most established pathway in both the onset of 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 aging process (Westhoff et al., 2005). However, it is not clear whether there are functional differences within the DNAJ family in this respect.

Whether the chaperone activity of DNAJ or other members of the HSP70 machine is affected by posttranslational modifications is also not known. Certain histone deacetylases (HDACs) interact with members of the DNAJ family (Dai et al., 2005; Ago...
et al., 2008), and given the role of HDACs in neurodegeneration (Dokmanovic et al., 2007), a functional link between these two machines could be envisioned.

In the current study, we compared the diverse members of the HSP70 machine for their capacity to prevent protein aggregation and related toxicity in various cellular models of protein folding diseases, as well as in a Xenopus laevis model. We identified a DNAJB subfamily in which members act as the most potent suppressors of protein aggregation and its associated toxicity. They form polydisperse oligomeric complexes that bind to and suppress aggregation of nonfoldable proteins in a HSPA-independent manner and maintain them competent for degradation, which is a HSPA-dependent process. Finally, several HDACs interact with these DNAJ proteins and can deacetylate them, and HDAC inhibition, in particular of HDAC4, as well as mutation of two acetylation-competent C-terminal lysines, results in reduced antiaggregation activity.

RESULTS

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 aggregation using HDQ119-EYFP (EYFP-tagged fragment of exon 1 of the huntingtin gene with 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 (Figure 1A), SDS-insoluble aggregates retained in cellulose-acetate filters (Figure 1B), and large cytosolic inclusions (Figure 1C). However, expression of two members of the DNAJB subfamily, DNAJB6b and DNAJB8, did do so (Figures 1A and 1B). Cells expressing these proteins showed a diffuse staining pattern of HDQ119-EYFP (Figure 1C), with less than 1% of cells containing inclusions, compared to 25%–30% in cells without chaperone coexpression (data not shown) (Rujano et al., 2006). Comparable results were obtained with nontagged constructs (Figures S1A and S1B). DNAJB6b and DNAJB8 also were effective in suppressing poly-Q aggregation in the brain-derived cell lines N2A and SHSY-5Y (Figure S1C). When a shorter poly-Q stretch of 74 glutamines was used, similar results were found, except that now DNAJB1, like DNAJB6b and DNAJB8, also nearly fully inhibited poly-Q aggregation (Figures S1D and S1E) and inclusion formation (data not shown) (Chan et al., 2000; Rujano et al., 2007). The HDQ119 data thus revealed the greater potency of DNAJB6b and DNAJB8 as suppressors of protein aggregation.

DNAJB6b and DNAJB8 Belong to a Highly Conserved Subfamily within the DNAJB Family

The human DNAJB subfamily can be further divided into two clusters, one containing the classical DNAJB1 (HSP40) and related proteins and one containing DNAJB6b, DNAJB8, and their relatives (Figures 1D and S2). Effective suppressors of HDQ119-EYFP aggregation were found only in the DNAJB6/DNAJB8-like cluster (Figures 1B and 1D). Yet some members were inactive. Of the two isoforms of DNAJB6, only the shorter isoform (DNAJB6b), present in both the nucleus and the cytosol, was effective. The longer isoform (DNAJB6a) is exclusively nuclear (Hanai and Mashima, 2003), and it was as effective as DNAJB6b when using a nuclear-targeted poly-Q protein (Figures 2A and 2B), although ineffective on cytosolic HDQ119-EYFP. The larger isoform of DNAJB2 (DNAJB2b) is targeted to the cytoplasmic face of the ER (Chapple and Cheetham, 2003) and was also inactive, suggesting that the DNAJ proteins need to be freely diffusible to prevent the aggregation of soluble poly-Q proteins. Thus, effective suppression of poly-Q aggregation appears to be a general property of the members of this DNAJB subfamily, with the possible exception of DNAJB7. Since DNAJB6b and DNAJB8 have the most potent antiaggregation capacity in the subfamily, we focused on these two proteins.

DNAJB6b and DNAJB8 Suppress Aggregation of Other Misfolded Proteins

To determine whether the antiaggregation effect of DNAJB6b and DNAJB8 is substrate specific, we tested their effect on two other poly-Q-expanded, disease-associated proteins: ataxin-3 (SCA-82Q) (Figures 2C–2E) and the androgen receptor (AR-72Q) (Figures 2F and 2G). DNAJB6b and DNAJB8 efficiently reduced the extent of aggregation of these proteins, including, in the case of the AR, the nonexpanded counterpart, which also aggregates to some extent (Bailey et al., 2002). As expected, DNAJB1 was also effective in suppression of aggregation of the shorter poly-Q stretches. DNAJB6b and DNAJB8 are thus generic suppressors of aggregation of misfolded poly-Q proteins.

DNAJB6b and DNAJB8 Also Protect against Protein Aggregation in the Absence of the Cellular Heat Shock Response

Exogenous expression of proteins can induce the stress response, which in turn can modulate protein aggregation.

Figure 1. Members of a DNAJB Subfamily Act as Strong Suppressors of Polyglutamine Aggregation

(A) Cells were cotransfected with HDQ119-EYFP and V5-tagged members of the HSPH, HSPA, or DNAJ families. Expression of chaperones was induced with tetracycline. Samples were taken 24 hr after transfection. High-molecular-weight (HMW) aggregates trapped in the stacking gel and soluble HDQ119-EYFP were assessed with anti-GFP antibodies. Expression of chaperones was detected with anti-V5 antibodies.

(B) Filter trap assay of cell extracts without and with overexpression of molecular chaperones. Serial 5-fold dilutions were loaded on cellulose ac acetate membranes and probed with anti-GFP antibody. Numbers represent the percentage of aggregation in cells with exogenous chaperones (+Tet) compared to cells without the exogenous chaperones (−Tet).

(C) Representative confocal pictures of cells cotransfected with HDQ119-EYFP (green) and V5-tagged chaperone members (red). DAPI staining is shown in blue. Bar represents 30 μm.

(D) Combined phylogram of aligned DNAJB primary amino acid sequences (left) with aggregation suppressive activity in the filter trap assay (right; efficacy indicated in percentages as above). Bootstrap values of 1000 trials on each node are given.
DNAJB6b and DNAJB8, however, did not merely act by induction of the heat shock response, as they were still effective in cells expressing a dominant-negative HSF-1, thus blocking the heat shock response (HSF379) (L. Heldens, R.P.D., S. Hensen, C. Onnekink, S.T. van Genesen, F. Rustenburg, and N.H.L., unpublished data) (Figures 3A and 3B).

(Morimoto, 2008). DNAJB6b and DNAJB8, however, did not merely act by induction of the heat shock response, as they were still effective in cells expressing a dominant-negative HSF-1, thus blocking the heat shock response (HSF379) (L. Heldens, R.P.D., S. Hensen, C. Onnekink, S.T. van Genesen, F. Rustenburg, and N.H.L., unpublished data) (Figures 3A and 3B).
Also, exogenous expression of DNAJB6b or DNAJB8 did not activate a heat shock promoter (Figure S3A). Finally, activating the heat shock response with a mild pre-heat shock did not provide protection against HDQ119-EYFP aggregation (Figure S3B). Hence, DNAJB6b and DNAJB8 do not require induction of the heat shock response for their activity.

**Figure 3. Antiaggregation Effects of Exogenous DNAJB6-like Proteins Reflect Their Normal Physiological Function and They Do Not Disaggregate Preexisting Aggregates**

(A and B) Western blot (A) and filter trap (B) analysis on cell lysates coexpressing a tetracycline-inducible dominant-negative HSF-1 (dnHSF1; probed with an HSF-1 antibody), HDQ119-EYFP (GFP antibody), and DNAJB6b or DNAJB8 (anti-V5 antibody).

(C) Relative abundance (in %) of DNAJB subfamily transcripts in human HEK293 cells.

(D and E) Western blot (D) and filter trap (E) analysis of cell extracts expressing EGFP-HDQ74 and treated with nontargeting siRNA or siRNA directed against DNAJB6. Samples were taken 48 hr after transfection; details as in Figure 1. Numbers represent the percentage of aggregation in cells with siRNA against DNAJB6 compared to cells with nontargeting siRNA (= 100%).

(F) Western blot and (G) filter trap analysis showing effects of DNAJB8 expression on preexisting aggregates. Chaperone expression was induced at the indicated time points after transfection.

**Downregulation of Endogenous DNAJB6 Enhances Poly-Q Protein Aggregation**

DNAJB6 is expressed ubiquitously, including in brain (Figure S3C), with DNAJB6b mRNA always at somewhat higher levels than DNAJB6a mRNA (Hunter et al., 1999; Seki et al., 1999; Chuang et al., 2002). DNAJB8 transcripts were detected...
Figure 4. DNAJB6-like Proteins Reduce Toxicity and Suppress Aggregation In Vivo

(A) HEK293 cells were cotransfected with HDQ119-EYFP and chaperones as indicated. The percentage of propidium iodide (PI)-positive cells was measured in the YFP-positive fraction; data are presented as mean ±SEM (N = 2).

(B) Same as in (A) for SHSY-5Y cells.

(C) Tetracycline (µg/ml)

(D) % of aggregate containing tadpoles

(E) HEK293 cells were transfected with HDQ119-EYFP+DNAJB8 and observed under a microscope.

(F) SHSY-5Y cells were transfected with HDQ119-EYFP and observed under a microscope.

(G) % of aggregate containing tadpoles

HDAC-Dependent DNAJB Poly-Q Chaperones

Molecular Cell

DNAJB6b and DNAJB8 Do Not Disaggregate

To test whether DNAJB8 only prevents aggregation or can also disaggregate preexisting aggregates, expression of (tet-inducible) DNAJB8 was delayed until some aggregation had already occurred (Figure 3F and 3G). Inducing expression of DNAJB8 did not reduce preexisting aggregates, but further increase in aggregation was prevented (Figure 3G).

DNAJB6b and DNAJB8 Reduce Poly-Q-Induced Toxicity In Vitro and In Vivo

Most of the poly-Q aggregates were concentrated in single large aggregates adjacent to the nucleus, resembling aggresomes (Figure 1C) (Johnston et al., 1998; Rujano et al., 2006). Aggresome formation has been suggested to serve as a (secondary) mechanism to protect cells against the more toxic aggregate intermediates (Johnston et al., 1998; Arrasate et al., 2004). The inhibition of aggresome formation by DNAJB6b and DNAJB8 could thus result in increased toxicity rather than cellular protection. Using a propidium iodide (PI) exclusion assay (Figure 4A), however, we found that inhibition of poly-Q aggregation by DNAJB6b or DNAJB8 coincided with protection against poly-Q-induced cytotoxicity in HEK293 cells. Cytoprotection, albeit less efficient, was also observed in the neuroblastoma cell line SHSY-5Y (Figure 4B). Poly-Q-induced cell death was not significantly reduced by the less-effective aggregation suppressor DNAJB1 (data not shown), further emphasizing the superiority of DNAJB6b and DNAJB8. In a colony-forming assay, chronic expression of HDQ119-EYFP led to a decrease in the number of colonies present after 14 days (Figures 4C and 4D). Long-term overexpression of DNAJB8 was well tolerated and did not affect cell proliferation (data not shown) or clonogenicity (Figure 4D, inset), while its coexpression with HDQ119-EYFP largely reduced long-term toxicity associated with poly-Q expression (Figure 4D). Thus, DNAJB6b and DNAJB8 function in protein quality control at a stage prior to the formation of toxic intermediates, and boosting their expression has no deleterious effects. Expression of HDQ74 or HDQ119 under control of a muscle-specific promoter in transgenic Xenopus laevis tadpole led to developmental-related mortality (data not shown). Those tadpoles that did survive showed many inclusions throughout the body (Figures 4E and 4F). Expression of DNAJB6b or DNAJB8 resulted in predominantly diffuse and homogeneous distribution of the poly-Q-expanded proteins (Figures 4E and 4F) and strongly reduced the number of transgenic tadpoles with aggregates (Figure 4G). Coexpression of DNAJB1 did not suppress HDQ119/HDQ74 aggregation in vivo (data not shown). So DNAJB6b and DNAJB8 also protect in vivo without any deleterious effect on development.

The J-Domain of DNAJB6b and DNAJB8 Is Not Absolutely Required for Antiaggregation Activity

To investigate whether DNAJB6b and DNAJB8 depend on collaboration with HSPA proteins, we coexpressed lower levels of the DNAJ Bs (Figures S4A and S4B) with HSPA family members. None of the HSPA chaperones significantly increased the antiaggregation effect of DNAJB6b (Figures S4B and S4C). 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 (Michels et al., 1997). 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 for DNAJB6b or DNAJB8. Downregulation 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 (Cheetham and Caplan, 1998). However, DNAJB6b and DNAJB8 mutants in which the histidine residue in the HPD motif was substituted by a glutamine (H/Q) (Figures 5A and 5B) were nearly fully capable of inhibiting EGFP-HDQ74 aggregation, unlike the DNAJB1 H/Q mutant (Figures 5A and 5B) (Rujano et al., 2007). 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 (Figures 5C and 5D). These data show that the J-domain is not absolutely required for the antiaggregation activity of DNAJB6b or DNAJB8, although it does contribute to full activity. We also tested whether BAG1 or CHIP, two cochaperones that antagonize the HSP40/DNAJ effect on the HSPA/HSPA ATPase cycle (Kampinga, 2006), could reverse the effects of DNAJB6b or DNAJB8 on poly-Q aggregation. Coexpression of either one of these two cochaperones with the DNAJs almost completely abolished the protective effect of DNAJB1, while the protective effects evoked by DNAJB6b or DNAJB8 were inhibited only to the same extent as when mutating in the J-domain (Figures S4D, 5C, and 5D). Hence, HSPA proteins play only a limited role in the DNAJB6b or DNAJB8 effects on poly-Q aggregation.
Molecular Cell

HDAC-Dependent DNAJB Poly-Q Chaperones

A

EGFP-HDQ74

α-GFP

α-V5

Tetracycline

B

No HSP

DNAJB1

DNAJB1 H32Q

DNAJB6b

DNAJB6b H31Q

DNAJB8

DNAJB8 H31Q

% Tet+/Tet-

55

0

57

0

1

0

12

Tet - Tet +

C

DNAJB6

H31-P32-D33

C-Domain

1 69-70 232

DNAJB6

α-J

H31-P32-D33

1 69-70 232

Construct

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% Tet+/Tet-

25 51

Tet - Tet +

D

DNAJB8

H31-P32-D33

C-Domain

1 69-70 232

Construct

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% Tet+/Tet-

23 51

Tet - Tet +

E

Control

DNAJB8

DNAJB8 H3Q

α-GFP

α-V5

Poly-Q/Chap Ratio

Tet - Tet +

F

ATG5 +/-

ATG5 -/

α-GFP

α-V5

DNAJB8

- + - +

G

ATG5+/

ATG5-/

% DNAJB8/Control

Control

DNAJB8

DNAJB8

H

MG-132

Tet

α-GFP

α-V5

α-GAPDH

Control

DNAJB6

DNAJB8

Tet +

Tet -

% Tet+/Tet-

58

60

4

24

5

12

I

MG-132

Control

Tet +

α-GFP

α-V5

α-GAPDH

Control

DNAJB6

DNAJB8

Tet -

% Tet+/Tet-

58

60

4

24

5

12
DNAJB1 and DNAJB2a suppress poly-Q aggregation via an enhancement of HSPA-dependent (proteasomal) aggregation degradation (Bailey et al., 2002; Westhoff et al., 2005). The J-domain of DNAJB8 could play a similar role. Indeed, the levels of soluble HDQ119-EYFP decreased with increasing amounts of wild-type DNAJB8, whereas such an effect was not seen for the DNAJB8-H/Q mutant (Figure 5E). This suggests that DNAJB8 keeps poly-Q-containing proteins in a soluble state, competent for degradation in an HSPA-dependent manner. We next asked whether this effect was related to autophagy or proteasomal-dependent degradation. In ATG5−/− mouse embryonic fibroblasts that are (macro)autophagy defective (Kuma et al., 2004), poly-Q aggregation was slightly higher than in ATG5+/+ cells (Figure 5G), consistent with a role of (macro)autophagy in the normal turnover of the poly-Q-containing protein (Iwata et al., 2005). However, DNAJB8 was fully functional in suppressing poly-Q aggregation in ATG5−/− cells (Figures 5F and 5G). When using MG132 to block proteasomal degradation, the effect of DNAJB6b and DNAJB8 on aggregation was reduced (Figures 5H and 5I) to the same extent as when modulating interaction with HSPA (Figures 5A–5D and S4D). These combined data suggest that DNAJB6b and DNAJB8 prevent aggregation of poly-Q independently of the HSPA machine, but do collaborate with that machine to facilitate degradation of poly-Q-containing substrates.

The C Termminus of DNAJB6b and DNAJB8 Is Crucial for the Antiaggregation Activity

In contrast to the J-domain, the C terminus was found to be crucial for the antiaggregation activity of DNAJB8, as demonstrated by J-domain swapping between DNAJB1 and DNAJB8: the DNAJB1-DNAJB8 chimera was fully functional, while the DNAJB8-DNAJB1 chimera was not (Figure S5A). In search for functional regions within the C terminus, we generated deletion mutants from both the C-terminal and N-terminal ends of DNAJB8ΔJ (Figure 6A). Activity was largely retained even upon deletion of the J-domain and the first 80 aa of the C-terminal region (Figure 6B). Thus, the main activity resides within the C-terminal region (aa 152–232). Within this region a short deletion of the J-domain and the first 80 aa of the C-terminal end is critical for DNAJB6/8 function.

Figure 5. The J-Domain of DNAJB6-like Proteins Facilitates the Degradation of Polyglutamine-Expanded Proteins

(A and B) Western blot (A) and filter trap (B) assay on extracts of cells coexpressing EGFP-HDQ74 and DNAJB J-domain mutants 48 hr after transfection (details as in Figure 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).
(D) Same as (C) for DNAJB8.
(E) Western blot of cells transfected with different ratios of HDQ119-EYFP to DNAJB8 or DNAJB8 H/Q expression plasmids, probed with anti-GFP and anti-V5 as indicated.
(F and G) Western blot (F) and filter trap (G) analysis on HDQ119-EYFP aggregation in ATG5+/+ and ATG5−/− MEFs with or without coexpression of DNAJB8.
(H and I) Western blot (H) and filter trap (I) assay on HDQ119-EYFP expression levels and aggregation in HEK293 cells treated with DMSO or MG-132 (10 μM, 24 hr) and with or without coexpression of DNAJB6b or DNAJB8.

The SSF-SST Domain in DNAJB6b and DNAJB8 Is Essential for Substrate Binding and Formation of HMW Complexes

Time-lapse microscopy on the dynamics of aggregate formation revealed that, in the few cells (<1%) that did contain aggregates, DNAJB6b and DNAJB8 clearly colocalized with the inclusion at a very early stage (Figure S5B and Movies S1–S3), suggesting that they interact directly with the substrate. To conclusively address this, we used fluorescence lifetime microscopy (FLIM) to measure fluorescence resonance energy transfer (FRET) between DNAJB8 and poly-Q. FRET efficiency indeed increased for YFP-HDQ119 and CFP-DNAJB8 to levels close to that of coaggregating YFP-HDQ119 and CFP-HDQ74 (Figure 6D), implying a direct interaction between the chaperones and the poly-Q substrates. Interestingly, the ΔSSF-SST mutant showed even lower FRET efficiency with poly-Q than CFP (Figure 6D), indicating its complete loss of interaction with the poly-Q substrate.

The C-terminal domain of DNAJB1 is involved in the formation of dimers, and homodimerization has been proposed to be essential for function (Hu et al., 2008). To investigate whether DNAJB8 is also dimeric, we analyzed its electrophoretic mobility on 4%–16.5% polyacrylamide native gels and compared this with the mobility of the crippled DNAJB ΔSSF-SST mutant. DNAJB8 migrated considerably more slowly than DNAJB8 ΔSSF-SST (Figure S5C), while the calculated PI was not notably changed by the deletion of the SSF-SST region (5.98 versus 5.58). On 10%–80% sucrose gradients, DNAJB8 was present in most fractions, indicating polydispersity (Figure 6E). In contrast, DNAJB1 as well as HSPA1A/B were recovered only in the first four fractions. The ΔSSF-SST mutant was also recovered only in the first fractions, indicating that loss of antiaggregation activity (Figure 6C) correlates with the loss of the ability to form large oligomeric species (Figure 6E).

The SSF-SST Domain Interacts with HDACs, and HDAC Activity Is Required for DNAJB8 Antiaggregation Activity

Interestingly, the SSF-SST region is crucial for DNAJB8 function also partially overlaps with a region in DNAJB6

ΔSSF-SST). Combining this mutant with a deletion of the entire J-domain (ΔJ ΔSSF-SST) led to complete loss of function (Figure 6C). Strikingly, a minimal 24 amino acid C-terminal deletion combined with the J-domain deletion also led to an almost complete loss in activity (Figure 6B), indicating that the C-terminal end is critical for DNAJB8/8 function.
Figure 6. The Importance of the C-Terminal Domain of DNAJB8 for Activity, Substrate Binding, and Oligomerization

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

(D) Cells transfected with YFP-HDQ119 and CFP-tagged DNAJB8 wild-type 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 lysates of cells expressing DNAJB8 wild-type and mutant variants as indicated. DNAJB8 and its mutants were detected using anti-V5 antibody, whereas endogenous DNAJB1 and HSPA1A/B were detected with DNAJB1- or HSPA1A/B-specific antibodies.

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responsible for interaction with HDAC4 (Figure S6A) (Dai et al., 2005). Using pull-down experiments in cells coexpressing His-tagged DNAJB6b or DNAJB8 together with FLAG-tagged HDACs representative of the three well-known HDAC families, type I (HDAC1, HDAC3, HDAC8), type II (HDAC4, HDAC5, HDAC6, and HDAC7), and type III (SIRT2), we found that HDAC4, HDAC6, and SIRT2 interacted with DNAJB6b and DNAJB8 (data not shown). Coimmunoprecipitation (coIP) analyses confirmed that full-length DNAJB6b and DNAJB8 indeed bound HDAC4, HDAC6, and SIRT2, whereas DNAJB8 ΔSSF-SST did not (Figures 7A and 7B), 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 HDACs is functionally relevant, we treated cells with Trichostatin A (TSA), a general inhibitor of class I and II HDACs. 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 (Figures 7C, 7D, S6B, and S6C). We also tested the effect of Tubacin, a specific inhibitor of the α-tubulin deacetylase activity of HDAC6 (Haggarty et al., 2003), as HDAC6 has been implicated in quality control in poly-Q disease models (Pandey et al., 2007). Tubacin did not inhibit DNAJB8 activity on HDQ119-EYFP aggregation (Figures 7C and 7D), 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 downregulate HDAC4, HDAC6, or SIRT2 (Figures 7E and S6D). In cells in which HDAC6 or SIRT2 were functionally downregulated (as evidenced by increased α-tubulin acetylation [Figure S6E]), DNAJB8 could still suppress HDQ119-EYFP aggregation (Figure 7F). HDAC4 downregulation was found to be rather toxic to cells expressing HDQ119-EYFP. In the surviving cells, DNAJB8 overexpression no longer showed a protective effect (Figure 7F), 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 three peaks in the TSA-treated samples that were absent in the control sample (VEGEYEVLSDSKK*, VEEVEDIQK*LVTNGK, and SVTVNGK*EQLK) (Figures 7G and S7A–S7C). These exactly matched the predicted molecular weight of DNAJB8 fragments with acetylated lysines (marked with asterisks). 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) (Figure S7D) are in the C-terminal end of both DNAJB6b and DNAJB8. K216 is highly conserved among the DNAJB6/8 substrates by direct deacetylation of lysine K216 (and K223).

**DISCUSSION**

A subfamily of DNAJB proteins with a non-canonical mode of action was discovered during our systematic screen of the main components of the HSPA (HSP70) chaperone machinery for their ability to deal with aggregation and associated toxicity of disease-associated poly-Q proteins. 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 antiaggregation function of DNAJB6-type proteins is evolutionarily conserved. The *Drosophila* ortholog of DNAJB6, Mr1, was identified in a screen for genetic factors that dominantly modify poly-Q toxicity (Kazemi-Esfarjani and Benzer, 2000). Both Mr1 and DNAJB6 overexpression suppress poly-Q toxicity in various fly tissues and human cells (Fayazi et al., 2006; Chuang et al., 2002), consistent with our findings that human DNAJB6b and DNAJB8 were functional in *Xenopus* DNAJB6 orthologs are found in all metazoans analyzed, suggesting a general need for an antiaggregation chaperone. Homozygous DNAJB6 mutant mice die due to a failure of chorioallantoic fusion (Hunter et al., 1999); DNAJB6 binds to keratin and prevents its toxic aggregation in chorionic trophoblast cells during chorioallantoic attachment in placental development (Izawa et al., 2000; Watson et al., 2007). 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.

Unlike the refolding-competent DNAJB1-type proteins (Terdada and Mori, 2000; Michels et al., 1997), DNAJB6-type proteins do not support refolding, although they can bind to stress-unfolded substrates (J.H., M.A.W.H.v.W., A. Zyliec, and H.H.K.,
unpublished data), suggesting that their interactions with substrates differ. DNAJB1 is active as a dimer, and dimerization is thought to increase the substrate-binding affinity (Stirling et al., 2006). DNAJB8 forms large polydisperse oligomeric complexes, which could enlarge its substrate-binding area and affinity. The finding that deletion of the SSF-SST region leads to loss of substrate interaction, loss of complex formation, as well as substantial loss of function supports the functional relevance of the oligomerization. Surprisingly, and also unlike DNAJB1, DNAJB6 and DNAJB8 retain activity as suppressors of poly-Q aggregation even when mutated in the HSPA interaction domain. For full activity, however, the J-domain is required and functions as a molecular bridge for HSPA-dependent degradation.

The DNAJB6-like proteins do also differ in the way in which they deal with nonfoldable substrates. DNAJB2, which uses the proteasomal route, interacts with ubiquitin moieties on substrates through its ubiquitin interaction motifs (UIMs). This UIM motif is specific to DNAJB2 and not found in other members of the DNAJB6 subfamily (Westhoff et al., 2005). Unlike DNAJB6 and DNAJB8, DNAJB2 fully depends on its J-domain and lacks the full SSF-SST domain (Figure S2) present in DNAJB6/B8 that is required not only for their client binding and oligomerization but also for their interaction with HDACs. 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 loss of activity similar to 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 polydispered DNAJB6/8 already loaded with substrates and removes inhibitory acetyl groups, thereby activating substrate processing in the complex.

The connection between HDAC activity and activity of DNAJB6 and DNAJB8 is highly intriguing given the suggested roles of HDACs in protein quality control (Pandey et al., 2007) and in regulation of HSF1 activity (Westerheide et al., 2009). Our inhibitor and siRNA data point to HDAC4 as the regulator of DNAJB6-type proteins. Interestingly, HDAC4 is highly expressed in brain, and overexpression was found to protect neurons from cell death. Mice lacking HDAC4 showed cerebellar abnormalities, including progressive loss of Purkinje cells (Majd-zadeh et al., 2008). Furthermore, HDAC4 regulates the survival of retinal neurons in mice (Chen and Cepko, 2009). HDAC4 also interacts with DNAJB5 (Ago et al., 2008). This interaction is quite distinct from that between HDAC4 and DNAJB8, DNAJB5, which lacks the SSF-SST domain, acts as chaperone for nucleocytosolic shuttling of HDAC4 during oxidative stress: DNAJB5 thus “regulates” HDAC4 activity. Our data show a reversed mode of interaction between HDAC4 and DNAJ proteins: here, HDAC4 activity functionally regulates protein homeostasis via interaction with and deacetylation of molecular chaperones of the DNAJ family. This interaction could well explain the neuroprotective activity of HDAC4. Intriguingly, also mutant huntingtin is acetylated; in this case, acetylation controls its nucleocytoplasmic shuttling and is required for its targeting to autophagosomes (Jeong et al., 2009). These combined data point to an intricate but complex role for (de)acetylation in protein quality control.

Overall, our data suggest that DNAJB6-type proteins act like small HSP proteins, but with a J-domain. Like shHSPs, formation of oligomeric assemblies results in complexes with high substrate affinities that shield the nonfoldable clients during their lifetime. But unlike the shHSPs that have no known domains to recruit HSPA for further processing, DNAJB6-type proteins can recruit HSPA via their J-domain to link the substrate-chaperone complex to protein degradation machines. Through this dual mode of action, the DNAJB6-type proteins are among the strongest protectors against protein toxicity associated with protein aggregation detected thus far. This makes DNAJB6 an interesting target for therapy against protein folding diseases either through functional modulation (HDACs) or by increasing its expression. Our siRNA-mediated downregulation showed that DNAJB6b was rate-limiting for aggregation prevention and that boosting DNAJB6 expression to increase the capacity of a cell to deal with aggregating proteins did not require simultaneous upregulation of HSPA members, the latter being associated with oncogenesis (Dai et al., 2007). Since chronic overexpression of DNAJB6 had no deleterious effects on cell growth, cell morphology (data not shown), and development of Xenopus tadpoles, upregulation of DNAJB6 could be a powerful and also safe approach to ameliorate the progressive neuropathological effects of aging-related protein folding diseases.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Cell Culture, and Transient Transfections**

N2A (CCL-131), SH-SY5Y (CRL-2266), and HEK293 stably expressing the tetracycline (tet) repressor (Flp-In T-REX HEK293, Invitrogen; Breda, The Netherlands) were grown in DMEM ( Gibco; Breda, The Netherlands) plus 10% FBS (Sigma Aldrich Chemie BV; Zwijndrecht, The Netherlands), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), and for Flp-In T-REX HEK293 cells, 5 μg/ml Blasticidin (Sigma) and 100 μg/ml of Zeocin (Invitrogen). For transient transfections, cells were grown to 50%–60% confluence

**Figure 7. DNAJB8 Activity Is Dependent on HDAC Activity**

(A) CoIP of HDACs (FLAG-tagged) with VS-tagged DNAJB6b, DNAJB8, or DNAJB8 ΔSSF-SST.

(B) Reverse coIP of VS-tagged DNAJB proteins with FLAG-tagged SIRT2, HDAC4, and HDAC6.

(C and D) Western blot (C) and filter trap (D) 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 and F) Western blot (E) and filter trap (F) analysis on extracts of cells coexpressing HDQ119-EYFP and DNAJB8 and treated with siRNA against HDAC4, HDAC6, or SIRT2 compared to cells treated with nontargeting siRNA (mock).

(G) Fourier transform mass spectrometry (FT/MS) spectrum of LVSEAYEVLSDSKK*, triply charged ion m/z 537.28 selected for fragmentation with high-accuracy mass with TSA (left) or without TSA (right). Acetylation of K61 is indicated with an asterisk (*).

(H and I) Western blot (H) and filter trap (I) analysis on extracts of cells cotransfected with HDQ119-EYFP and tet-inducible, wild-type DNAJB8 or the indicated lysine single or double mutants.
in 35 mm diameter dishes coated with 0.001% 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-EYPF-expressing cell lines, cells were co-transfected with pcDNAs/FRT/TO HDQ119-EYPF and the Flp recombinase expressing plasmid pOG44 and selected with 100 μg/ml hygromycin. For stable DNAJB8-expressing cell lines, cells were transfected with pcDNA3 V5 DNAJB8 and selected using 500 μg/ml neomycin. The double stable cell line was made by transfecting the pcDNA3 V5 DNAJB6 construct in the pcDNA5/FRT/TO HDQ119-EYPF stable cell line and selecting for hygromycin- and neomycin-resistant clones.

**Gene Cloning and Generation of Mutants**

Detailed information about the (construction of) plasmids used in this study can be found in the Supplemental Information and Tables S1 and S2. Primers used for site-directed mutagenesis are listed in Table S1.

**Filter Trap Assay**

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

**FLIM**

FLIM experiments were performed on an inverted Nikon TE2000 microscope using the LIFa frequency domain lifetime attachment (Lambert Instruments; Roden, The Netherlands) and Li-FLIM software. CFP was excited with light from an 1 W 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 (pH 10) 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 $E = 1 - (\text{measured lifetime of donor})/\text{(mesured lifetime of donor)}$. FRET efficiencies are means of 9–23 regions of interest (ROIs) per construct.

**Mass Spectrometry**

After immunoprecipitation of DNAJB8 and SDS-PAA electrophoresis, in-gel 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).

**Stable Transgenesis of Xenopus laevis**

Cloning details can be found in the Supplemental Information. Expression cassettes consisting of the Cac promoter, poly-Q/DNAJ protein coding region, and SV40 polyadenylation signal were excised from the pCS2+ vector backboned using SalI and NotI. Transgenesis of *Xenopus laevis* was performed according to Kroll and Amaya (Kroll and Amaya, 1996) with modifications according to Sparrow et al. (2000). Embryos at the four-cell stage were transferred to 6% Ficoll/0.1x MMR and incubated overnight at 17°C. Gastrulae (Nieuwkoop stage 12) were transferred to 0.1x MMR and incubated at 22°C. YFP-positive tadpoles were immobilized with MS-222 and photographed using an M2 FLIII fluorescence stereomicroscope provided with a DC200 camera (Leica Microsystems; Rijswijk, The Netherlands).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, seven figures, two tables, and three movies and can be found with this article online at doi:10.1016/j.molcel.2010.01.001.

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**REFERENCES**

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