

HSPB7 is the most potent polyQ aggregation suppressor within the HSPB family of molecular chaperones

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A small number of heat-shock proteins have previously been shown to act protectively on aggregation of several proteins containing an extended polyglutamine (polyQ) stretch, which are linked to a variety of neurodegenerative diseases. A specific subfamily of heat-shock proteins is formed by the HSPB family of molecular chaperones, which comprises 10 members (HSPB1–10, also called small HSP). Several of them are known to act as anti-aggregation proteins *in vitro*. Whether they also act protectively in cells against polyQ aggregation has so far only been studied for few of them (e.g. HSPB1, HSPB5 and HSPB8). Here, we compared the 10 members of the human HSPB family for their ability to prevent aggregation of disease-associated proteins with an expanded polyQ stretch. HSPB7 was identified as the most active member within the HSPB family. It not only suppressed polyQ aggregation but also prevented polyQ-induced toxicity in cells and its expression reduces eye degeneration in a *Drosophila* polyQ model. Upon overexpression in cells, HSPB7 was not found in larger oligomeric species when expressed in cells and—unlike HSPB1—it did not improve the refolding of heat-denatured luciferase. The action of HSPB7 was also not dependent on the Hsp70 machine or on proteasomal activity, and HSPB7 overexpression alone did not increase autophagy. However, in ATG5^{−/−} cells that are defective in macroautophagy, the anti-aggregation activity of HSPB7 was substantially reduced. Hence, HSPB7 prevents toxicity of polyQ proteins at an early stage of aggregate formation by a non-canonical mechanism that requires an active autophagy machinery.

INTRODUCTION

Several inherited neurodegenerative diseases exist that are based on a CAG triplet expansion in the affected genes. This results in elongation of the glutamine (polyQ) tract present in the proteins where, in general, an extension beyond 35–40 glutamines causes disease whereby the age at onset is related to the length of the expansion (1). These diseases include, among others, polyQ extensions in the androgen receptor, the TATA-box binding protein, Huntingtin disease (HD) and several ataxia-related genes [spinocerebellar ataxia (SCA) genes]. The corresponding diseases are characterized

by protein misfolding and aggregate/inclusion formation of the affected proteins with a concurrent increase in proteotoxic stress (2).

From cellular studies, it has become clear that certain heat-shock proteins, as molecular chaperones, form a potent natural defense against proteotoxic stress induced by these protein misfolding diseases. Heat-shock proteins are divided into several main families that are both structurally and functionally highly divergent (3). Within the HSPH (HSP110), HSPA (Hsp70) and DNAJ (HSP40) families, several members have been found that can reduce aggregation and toxicity associated with polyglutamine (polyQ) containing

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proteins and other misfolded proteins (4–11). Also within the mammalian small heat-shock protein (HSPB) family, some of the 10 members, particularly HSPB1 and HSPB8, have been reported to suppress toxicity or aggregation of disease-related, mutated proteins (12,13). In cells, HSPB1 suppresses mutant huntingtin-induced toxicity without having a major effect on its aggregation; rather, it was suggested that HSPB1 suppressed cell death caused by huntingtin-related formation of reactive oxygen species (14). In a mouse model of HD, however, HSPB1 overexpression did not rescue the HD phenotype (15). In contrast, HSPB8 does reduce aggregation: it stimulates autophagy-mediated degradation of aggregates in an eIF2 alpha-dependent manner (16–18). The potency of the other eight HSPB members in reducing proteotoxic stress remains largely unknown.

Here, we present a systematic comparison of the members of the human HSPB family for their ability to modulate either HD or SCA3 aggregation or to assist in the refolding of a heat-denatured substrate. The effectiveness in preventing aggregation largely differed between members and, in part, was dependent on the type of the misfolded substrate. However, within the HSPB family, HSPB7 was overall the most potent suppressor of HD and SCA3 aggregation in cells *in vitro*. Further analysis on this most potent member showed that it was also able to reduce polyQ-related toxicity in cells. In addition, we found that HSPB7 protects against polyQ-related eye degeneration in a *Drosophila* model. Interestingly, HSPB7 lacked several characteristics of the more classical small heat-shock proteins. We demonstrate that unlike HSPB1, HSPB7 was not found in polydispersed complexes and it did not improve the refolding of heat denatured luciferase when overexpressed in cells. In addition, whereas *in vitro* data show that chaperone-like activity requires the HSPA machine for substrate release and folding (19), HSPB7 was found not to depend on the HSPA chaperone machine for its anti-aggregation activity in cells. Finally, for full HSPB7 activity, autophagy was required although, unlike HSPB8, HSPB7 did not induce autophagy.

RESULTS

HSPB7 is the most potent HSPB member in preventing polyQ aggregation

The suggestion that small HSP are molecular chaperones is mostly derived from cell-free experiments with isolated proteins in which the anti-aggregation effects seem rather general, without any clear specificity in terms of substrates (20–22); these experiments showed that small HSPs require the ATP-dependent chaperones (in particular, the HSPA machine) for substrate release and (re)folding (23). Regarding their capacity to act as anti-aggregation chaperones in mammalian cells, limited data are available and they mainly concern the HSPB1, HSPB5 and HSPB8 members (18,24–26).

To compare the effectiveness of the HSPB family in dealing with disease-related misfolded proteins, we co-expressed the various HSPB members together with either mutant Huntingtin exon 1 (Htt) or a fragment of Ataxin-3 (SCA3), both containing an expanded glutamine tract in HEK293 cells. Using the filter trap assay (27) to detect protein aggregates, we

found that aggregation of a moderately expanded HD protein (HA-HttQ43) was strongly reduced by co-expression of four HSPB members (HSPB6, HSPB7, HSPB8, HSPB9), whereas HSPB1 and HSPB4 were only marginally effective (Fig. 1A). HSPB2, HSPB3, HSPB5 and HSPB10 showed no activity at all. Using a HD fragment containing a longer polyQ tract (EGFP-HttQ74), co-expression of HSPB7 and HSPB9 resulted in a considerable reduction in aggregated material, while co-expression of HSPB1, HSPB4 or HSPB6 was hardly effective (Fig. 1B). At a glutamine length of 119, only HSPB7 was still effective in reducing the amount of aggregated material (Fig. 1C). Repeating the screen with the non-tagged HSPB library and EGFP-HttQ74 led to qualitatively similar results, i.e. that HSPB9 and especially HSPB7 are the most potent suppressors of polyQ aggregation (Fig. 1D), indicating that the V5 tag had not affected the function of most HSPB members. Under all conditions of overexpressed HSPB members, the expression levels of the soluble fraction of the various Huntingtin proteins were largely unaffected (Fig. 1E–G), meaning that different substrate concentrations could not explain differences in effectiveness of the various HSPB members. The difference in the aggregation suppressive function of the various HSPB members was also not due to differences in expression levels or higher stability of the HSPB proteins themselves; in fact, HSPB7 and HSPB9 expression levels were among the lowest of the whole HSPB family (Fig. 1E and F). This means that their effectiveness may even be underestimated here.

We next repeated the screen using another substrate, Ataxin-3, containing an expansion with 82 glutamine repeats (SCA3Q82-EYFP) (Fig. 2A). For this substrate also, HSPB7 and HSPB9 were the most effective suppressors (Fig. 2A). In accordance with data from the HD screen (Fig. 1E–G), HSPB7 effectiveness against aggregation was not accompanied by a detectable reduction in soluble SCA3Q82 levels (Fig. 2B). Because HSPB7 was the most effective member of the HSPB family in protecting against polyQ aggregation, we decided to further study its activity in more detail.

HSPB7 prevents polyQ aggregation in neuronal-like cells

To test whether HSPB7 is also effective in reducing polyQ aggregation in neuronal-like cells, we co-transfected differentiated NG-108 neuroblastoma–glioma cells with HSPB7 and EGFP-HttQ74 or SCA3Q64-EYFP. Since transfection efficiencies in NG-108 cells were very low, we could not use biochemical endpoints to test whether HSPB7 reduced the process of aggregation also in these NG-108 cells. As an alternative, we therefore used immunofluorescence analysis. Intriguingly, we noticed that unlike in HEK293 cells, in which most polyQ-related protein aggregates are found in the cytosol (28) (Fig. 3A), aggregates in these neuronal-like cells were nearly exclusively nuclear (Fig. 3B and E). But irrespective of this differential localization of the polyQ aggregates in HEK293 and NG-108 cells, there were less cells with aggregates in both cell lines when HSPB7 was overexpressed. To quantify this for NG-108 cells, we calculated which fraction of EGFP-HttQ74 or SCA3Q64-EYFP expressing cells contained visible inclusions in the presence or absence of HSPB7. Interestingly, irrespective of the nuclear localization of the mutated

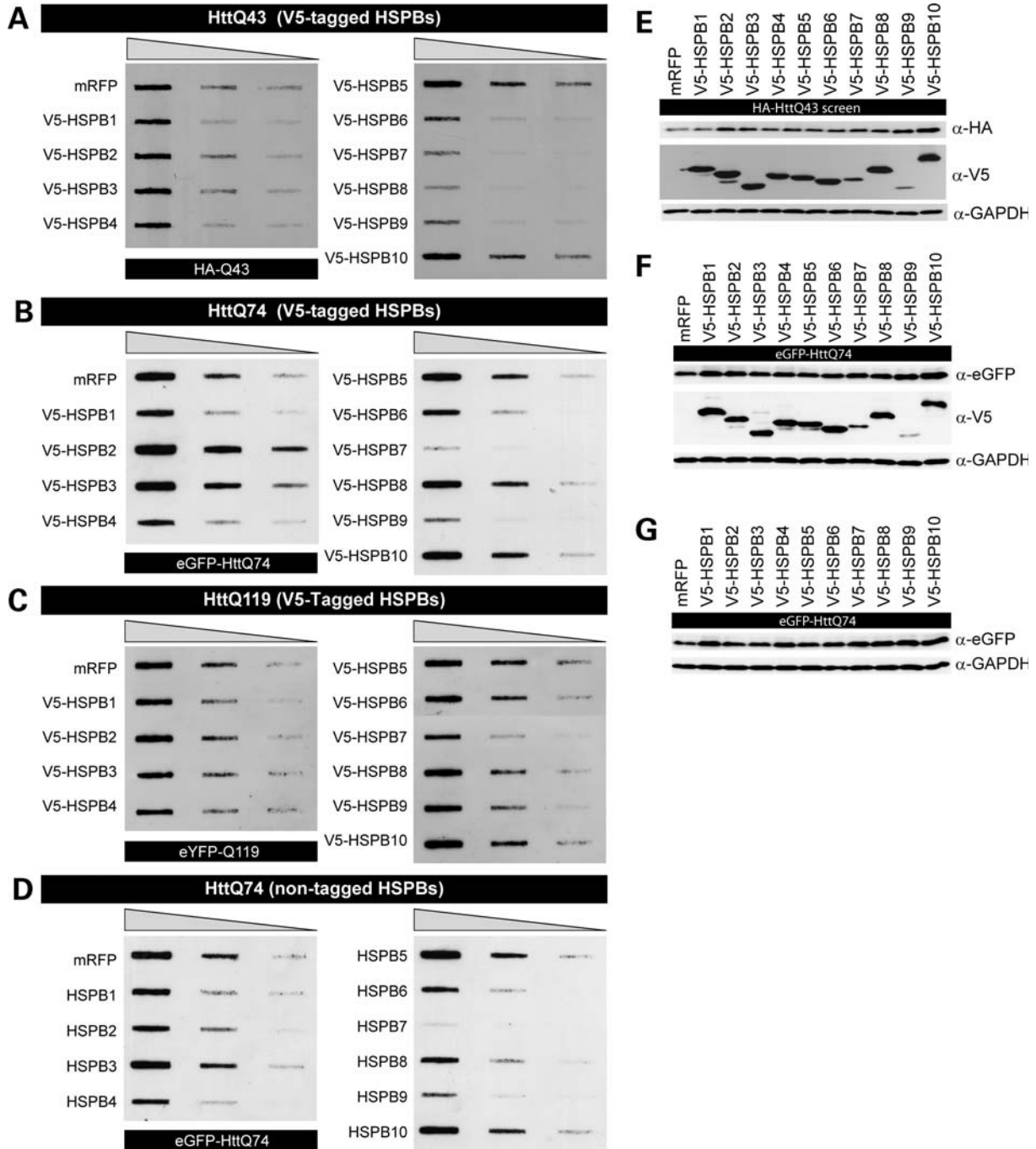


Figure 1. The human HSPB family contains potent suppressors of polyQ-expanded Huntingtin aggregation. HEK293 cells were co-transfected with V5-tagged HSPB members and polyQ encoding plasmids at a 9:1 plasmid ratio. Cells were lysed after 24 or 48 h and analysed by the filter trap-binding assay using 5-fold dilutions. Several HSPB members were able to suppress polyQ aggregation of a protein with (A) 43 glutamines, HA-HttQ43; (B) 74 glutamines, EGFP-HttQ74; and (C) 119 glutamines, HttQ119-EYFP. (D) To compare the effect of the V5 tag, also non-tagged HSPB members were co-expressed with the EGFP-HttQ74 encoding plasmid. (E–G) Expression levels of the polyQ proteins and HSPB members in 10-fold diluted cell lysates were analyzed by western blot.

aggregated protein, both V5- and non-tagged HSPB7 effectively reduced polyQ aggregation, while overexpression of HSPB5 had only a minor effect (Fig. 3C and F). This implies that HSPB7 is functional both in the cytosol (HEK293 cells) and in the nucleus (NG-108 cells). In line with this, we found that HSPB7 also prevented the aggregation of a nuclear targeted NLS-EGFP-HttQ74 in HEK293 cells (Fig. 3D).

HSPB7 anti-aggregation effects are not due to a non-specific induction of a cellular stress response

Transient overexpression can lead to non-physiologically high levels of expression per cell and hence could induce a stress response and cause non-specific phenotypes. First, we therefore titrated the levels of HSPB7 in HEK293 cells and

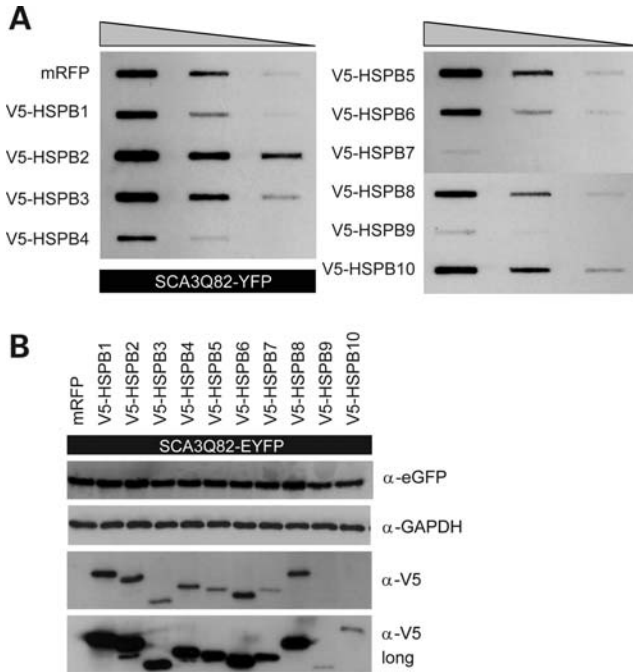


Figure 2. The human HSPB family contains potent suppressors of polyQ-expanded SCA3 aggregation. (A) HEK293 cells were co-transfected with V5-tagged HSPB members and truncated EGFP-tagged SCA3 containing a stretch of 82 glutamines at a 9:1 plasmid ratio. After 24 h, cells were lysed and analyzed by the filter trap-binding assay using 5-fold dilutions. (B) Expression levels of the polyQ proteins (EGFP-staining) and HSPB members (V5 staining) were analyzed by western blot. Since the expression levels of HSPB9 and HSPB10 were below the detection limit, a longer exposure was included.

found HSPB7-mediated protection to increase with increasing HSPB7 levels (Fig. 4A–D). To more directly exclude that HSPB7 overexpression might induce a general stress response that up-regulates other chaperones that in turn prevent polyQ aggregation, we measured levels of the stress inducible HSPA1A and DNAJB1. HSPB7 overexpression did not lead to a detectable induction of these classical heat-shock proteins (Fig. 4E). In addition, we employed cells carrying a tetracycline inducible dominant negative heat shock factor protein-1 (dnHSF-1) transgenic construct that blocks all HSF-1-dependent HSP expression (29) like, for example, HSPA6 (Fig. 4F). In these cells, HSPB7 retained its full activity to suppress polyQ aggregation (Fig. 4G and H). Thus, HSPB7 overexpression does not induce the general heat-shock response and does not require induction of other heat-shock proteins in order to be functional.

HSPB7 reduces polyQ-induced toxicity *in vitro* and *in vivo*

Since the rate of protein aggregation increases with the length of the polyQ expansion and since for all CAG repeat diseases the length of the polyQ expansion correlates with the age of onset of disease, protein aggregation has been suggested to be causative of disease (27). However, the validity of this assumption was challenged by observations in post-mortem tissues that revealed the presence of (nuclear) polyQ aggregates in non-degenerated neurons (30) and their absence in some degenerated brain areas

(31). In addition, *in vitro* studies suggested that the inclusion formation might rather be a (late) cytoprotective event against diffuse, soluble, toxic intermediates of mutant polyQ proteins (32–34). To test whether HSPB7 not only prevents inclusion formation but also exerts an (early) cytoprotective effect, we used a tetracycline-inducible HttQ119-EYFP HEK293 cell line (28). Subsequently, these cells were used to generate stable lines constitutively expressing either non-tagged HSPB5 or non-tagged HSPB7 (Fig. 5A). Tetracycline-induced expression of HttQ119-EYFP strongly reduced the colony-forming ability of the cells (28) (Fig. 5B and C). Co-expression of HSPB7 (but not HSPB5, used as ‘negative control’) led to a significant reduction in polyQ-mediated cytotoxicity (Fig. 5B and C). HSPB7 expression alone did not affect cell proliferation or clonogenicity (data not shown) suggesting that the improvement in colony-forming ability is caused by protection of HSPB7 against polyQ cytotoxicity.

We next tested whether HSPB7, besides preventing polyQ cytotoxicity *in vitro*, can also prevent polyQ-induced neuronal toxicity *in vivo*. For this purpose, we used a well-established *Drosophila* SCA3 model (35). This fly model is characterized by continuous expression of a truncated polyQ-expanded human SCA3 gene (SCA3trQ78) in the compound eyes, resulting in eye degeneration (Fig. 5D1–3), which can be visualized by the presence of dark patches, speckles and collapsed eyes. The *gmr*-GAL4-targeted expression of HSPB7 in combination with mutated ataxin-3 reduced the eye degeneration (Fig. 5D6). Scoring the eye phenotypes showed a significant reduction in the percentage of flies with degenerated eyes when HSPB7 was overexpressed (Fig. 5E). Hence, the observed effects of HSPB7 on polyQ aggregation clearly coincide with reduced toxicity *in vitro* and *in vivo*.

N-terminus of HSPB7 is indispensable for anti-polyQ aggregation activity

HSPB7 was originally discovered as a cardiovascular HSPB member (cvHSP). But, although it is indeed highly expressed in cardiac tissue (36), database analyses (37) revealed that HSPB7 is more ubiquitously expressed than originally thought. It is expressed at low levels in the brain (Supplementary Material, Fig. S1) and, at the messenger RNA level, it was found to be one of the highest expressed HSPB members in several other human tissues (37), which suggests a general function in cells. Our finding that human HSPB7 is also functional in flies further illustrates that HSPB7-like functions may be preserved in many species. Indeed, several vertebrate orthologs of HSPB7 (38) were found to be functional in preventing polyQ aggregation in human HEK293 cells (Supplementary Material, Fig. S2A and B), suggesting that a HSPB7-like function is conserved across different species. The main sequence-related features that make HSPB7 unique within the small HSP family are (i) the presence of an N-terminal serine-rich region of approximately 18 residues and (ii) a conserved C-terminal region (C-box) of 9 residues (Supplementary Material, Fig. S2C). To investigate the importance of these sequences for preventing polyQ aggregation, we co-expressed EGFP-HttQ74 with several human HSPB7 deletion mutants (Fig. 6A and B). Deletion of the entire N-terminal domain (V5-ΔN-HSPB7), which leads to

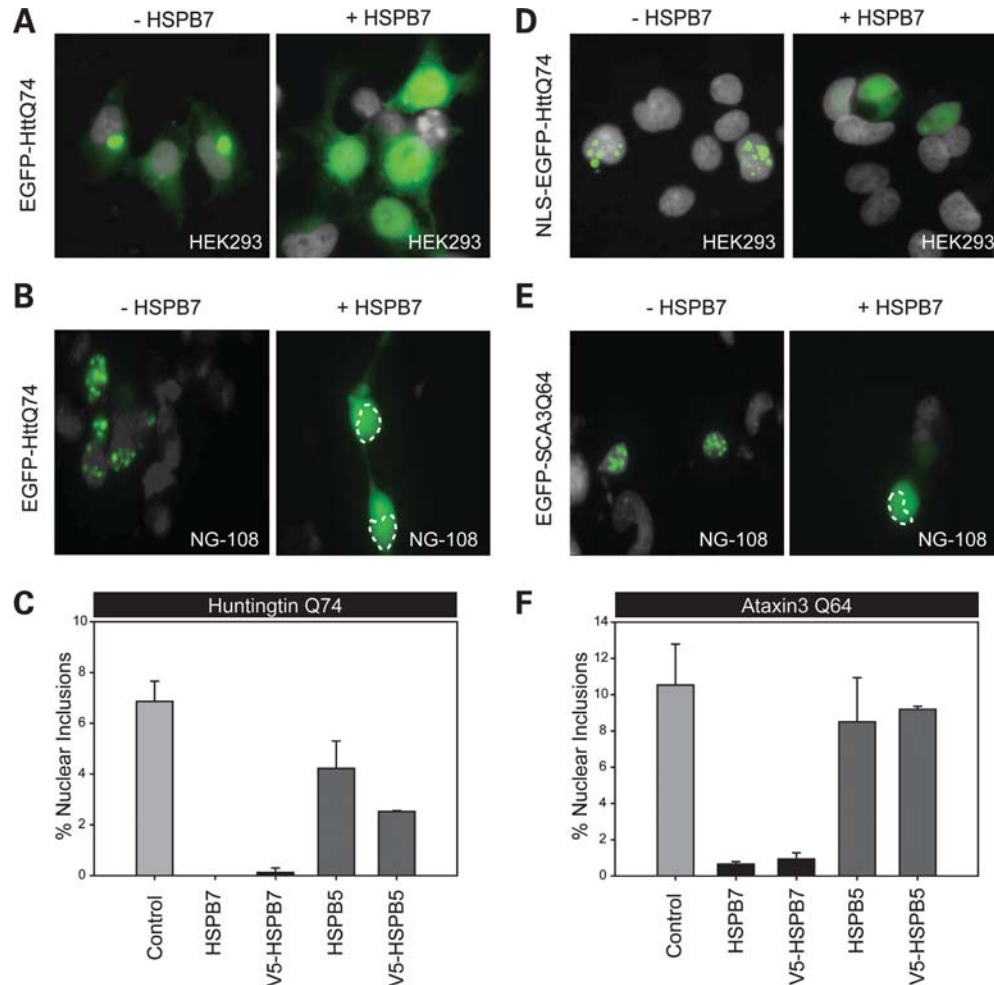


Figure 3. HSPB7 prevents polyQ aggregation in neuronal-like cells. (A) Confocal microscopy of HEK293 cells overexpressing cytosolic EGFP-HttQ74 (green signal) or NLS-EGFP-HttQ74 (D) without or with co-expression of V5-HSPB7 for 48 h. The nucleus was stained with DAPI (grey signal). (B) NG-108 cells were differentiated into neuronal cells and transfected with EGFP-HttQ74 or SCA3Q64-EYFP (E) with and without V5-HSPB7. Cells were fixed with formaldehyde and the nucleus was stained with DAPI (grey signal, or dashed white line). The number of NG-108 cells positive for either EGFP-HttQ74 (C) or SCA3Q64-EYFP (F) overexpressing HSPB7, V5-HSPB7, HSPB5 or V5-HSPB5 were counted using immunofluorescence. Graphs represent data of >100 GFP positive cells, measured in two individual experiments. Clearly, HSPB7 almost completely suppressed inclusion formation.

displacement of HSPB7 from SC35 splicing speckles (37) but which leaves the entire alpha-crystallin domain intact, almost completely abrogated the activity of HSPB7 to prevent polyQ aggregation (Fig. 6A). However, the internal deletion of the serine-rich region alone (V5-ΔS17-29-HSPB7) did not affect the activity of HSPB7 (Fig. 6A). This deletion also has been shown to have no effect on SC35 speckle localization (37). Hence, the N-terminus, but not the serine-rich stretch *per se*, seems indispensable in preventing polyQ aggregation. Deletion of the conserved C-Box (V5-HSPB7-ΔC-box) did not affect the activity of HSPB7 against polyQ aggregation (Fig. 6A) meaning that this conserved feature of HSPB7 does not edify its function in polyQ aggregation.

HSPB7 is not present in cells as oligomers

The canonical mechanism by which small heat-shock proteins are believed to function is that they form oligomeric

complexes that, via dynamic de- and re-oligomerization steps, bind unfolded substrates that are subsequently transferred to HSPA protein machines for further processing (39,40). First, we tested whether HSPB7 forms oligomers in cells, like the classical small HSPs, as HSPB1 and HSPB5 do. Hereto we expressed V5-HSPB5 or V5-HSPB7 in HEK293 cells and separated the cell lysates on sucrose gradients. While HSPB5 was found throughout the gradients, indicating formation of oligomeric species of various sizes, HSPB7 was mainly localized at low sucrose density indicative of its existence as a mono- or dimeric species (Fig. 7A). Native gel analysis confirmed these findings (data not shown). Therefore, in living cells, overexpressed HSPB7 does not seem to form the large polydispersed complexes that are so typical for many small HSPs. Yet, it is not an exception as HSPB8, when overexpressed without its binding partner BAG3, also forms mainly monomers and dimers (13,18).

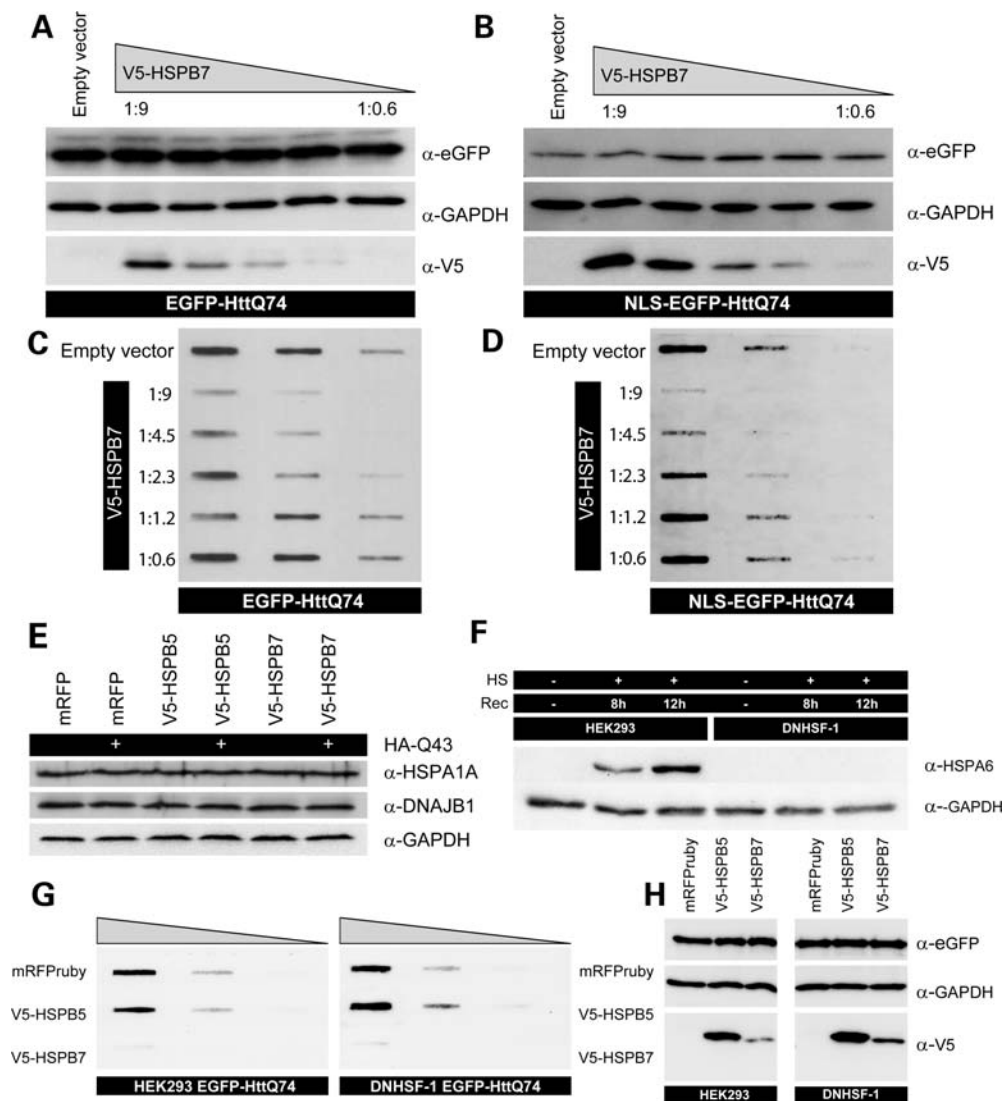


Figure 4. HSPB7 prevents aggregation in a dose-dependent manner and its protective effect is not due to an overexpression-related aspecific induction of a cellular stress response. (A and B) Expression levels of V5-HSPB7 were modified by varying the amount of transfected plasmid while keeping EGFP-HttQ74 and NLS-EGFP-HttQ74 encoding plasmid constant, showing no decrease in soluble GFP levels with increasing amounts of HSPB7. (C and D) Filter trap analysis shows a dose-dependent effect of HSPB7 on reducing polyQ aggregation of HttQ74 and NLS-HttQ74. (E) HEK293 cells were transfected with either mRFP, V5-tagged HSPB5 or HSPB7 with (+) or without (–) HA-HttQ43 encoding plasmids. Levels of the stress-inducible HSPA1A and DNAJB1 were not altered under these conditions, indicating that none of them induced a general stress response. (F) The dominant negative HSF-1 cell line lacks HSF-1 inducible gene expression as can be seen by a failure of these cells to induce the expression of strictly heat-inducible HSPA6 within 12 h after a 30 min heat shock at 43°C. (G and H) Using either HEK293 cells or the dominant negative HSF-1 cell line, V5-tagged HSPB5 or HSPB7 were co-transfected with EGFP-HttQ74 (9:1 ratio). In both cell lines, HSPB7 was found equally effective in suppressing HttQ74 aggregation (H), while total levels of EGFP-HttQ74 were similar under both conditions.

HSPB7 does not act as a chaperone in refolding of heat-denatured substrates

To test whether, despite its non-classical characteristics, HSPB7 still could act as a classical chaperone, we used heat-denatured firefly luciferase as a model substrate (35,41,42). Cells expressing luciferase with or without co-expressed HSPs were heated for 30 min at 45°C to denature luciferase and (chaperone-assisted) refolding was measured by allowing a 1 h recovery period at 37°C. Consistent with the previous data (40,43), expression of the classical (V5 tagged)

chaperones HSPB1, HSPB4 and HSPB5 increased luciferase refolding compared with cells co-expressing mRFP (a non-chaperone control) (Fig. 7B), while overexpression of the other HSPB members, including HSPB7, did not enhance luciferase refolding (Fig. 7B). Overexpression of non-tagged HSPB proteins gave similar results compared with the V5-tagged versions (data not shown), demonstrating that the V5 tag had not affected their refolding activity. This suggests that HSPB7 overexpression does not enhance protein refolding, at least not when firefly luciferase is used as a substrate.

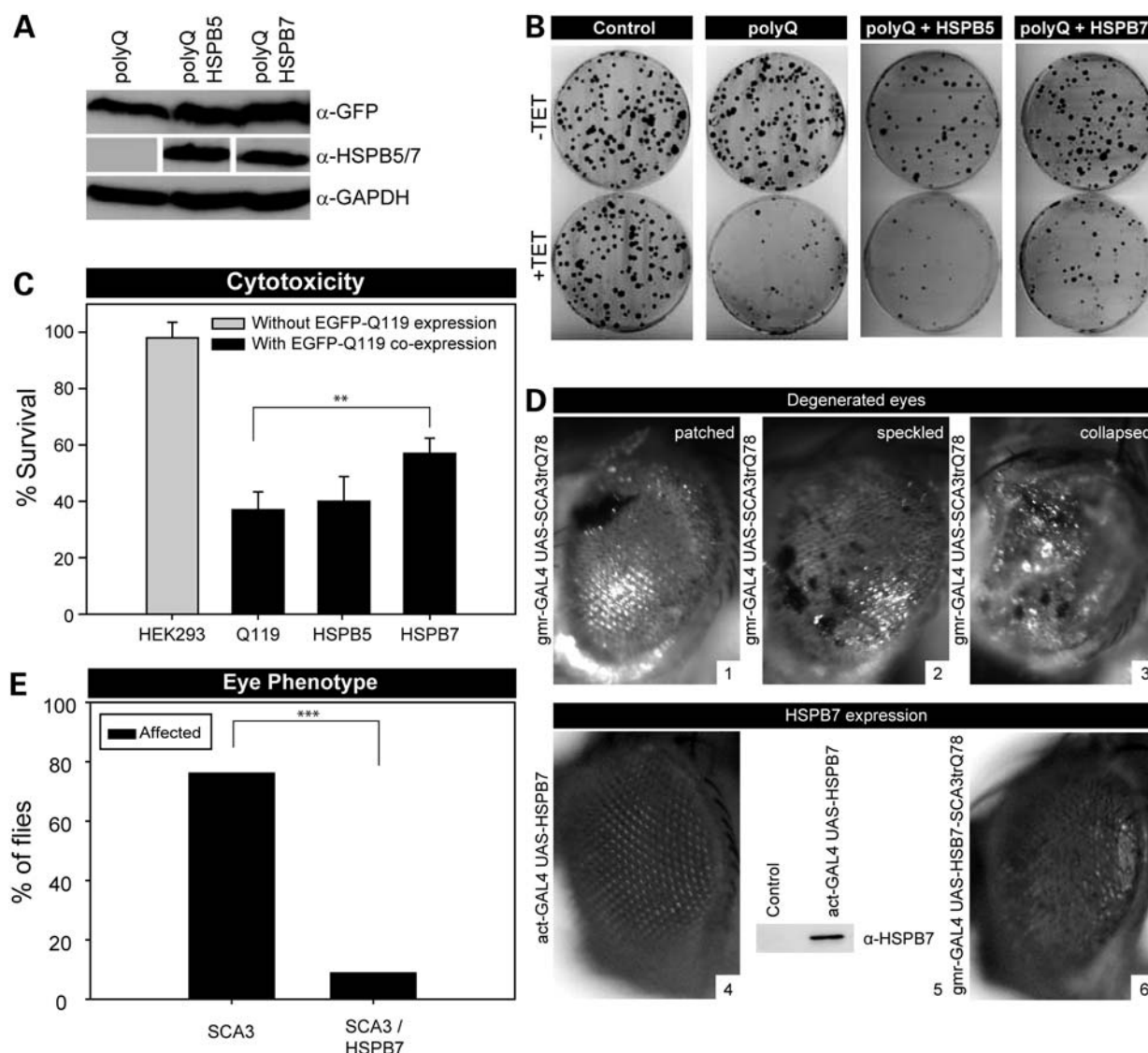


Figure 5. HSPB7 reduces polyQ-induced toxicity in cells and *in vivo*. PolyQ-induced cellular toxicity was analysed using a HEK293 cell line containing a tetracycline inducible HttQ119-EYFP construct (60) that stably expressed either non-tagged HSPB5 or HSPB7. (A) Addition of tetracycline to induce the expression of HttQ119-EYFP (A, upper lane). (B) Long-term toxicity of polyQ expression was evaluated using the colony formation assay showing reduced colony formation (within 2 weeks) upon polyQ expression. (C) Quantitative analysis of colony formation reveals that expression of HttQ119-EYFP alone or together with HSPB5 results in a major reduction in colonies. Co-expression of HSPB7 results in a significant protection against polyQ toxicity. (D) The *Drosophila* SCA3 model (74) was used to evaluate the effect of HSPB7 expression on eye degeneration. *Gmr-UAS-SCA3trQ78* eye degeneration showed different phenotypes ranging from patched (moderate), speckled (severe) and collapsed (extremely damaged) (D1–3). UAS-HSPB7 transgenic flies were generated as described in the Materials and Methods section. Expression of the HSPB7 transgene was confirmed by crossing the UAS-HSPB7-TM3 line with the *act-GAL4* driver line. This caused no phenotypic changes and these flies had normal eye morphology (D4). Equal amounts of proteins were analysed by western blotting and detected with the HSPB7 antibody (D5). A dramatic reduction in eye degeneration upon co-expression of HSPB7 was observed (D6). For each line, 100 eyes were scored and grouped in affected (patched, speckled, collapsed) and unaffected eyes. HSPB7 overexpression significantly improved the degeneration of SCA3trQ78 eyes (chi-square analysis, $^{**}P = 0.002$, $^{***}P = 0.0001$) (D6, E).

HSPB7 is not dependent on a functional HSPA machine to prevent polyQ aggregation

In vitro data have shown that many small HSPs, as ATP-independent chaperones, can prevent aggregation itself, but depend on collaboration with ATP-dependent chaperones like the HSPA machines for release and folding of their clients (44). To explore whether the action of HSPB7 on polyQ aggregation also depends on collaboration with HSPA

members, we initially aimed at downregulating HSPA8 and HSPA1, the endogenously expressed HSPA members in HEK293 cells. However, this turned out to be rather toxic to these cells (Hageman and Kampinga, unpublished data). We therefore used an indirect approach to address this question and co-transfected the HEK293 cells with HSPB7 and EGFP-HDQ74 with or without BAG1. BAG1 is a nucleotide exchange factor that accelerates the ATPase cycle of HSPA that requires to be present in precise stoichiometry with

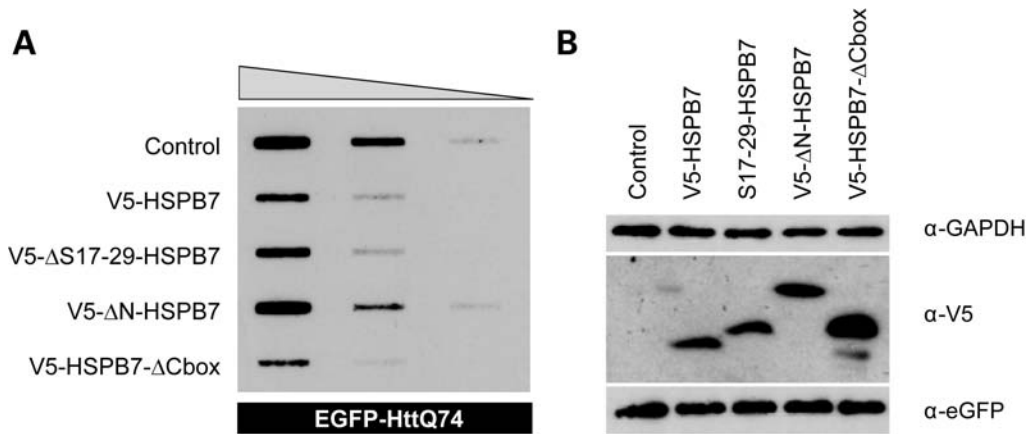


Figure 6. HSPB7 activity in preventing polyQ aggregation is independent of two conserved motifs. (A) HEK293 cells were co-transfected with EGFP-HttQ74, wild-type HSPB7 or deletion mutants (1:9 ratio, for topology see Supplementary Material, Fig. S2). Deletion of the conserved serine stretch or the C-box did not abolish effects of HSPB7 on polyQ aggregation. Deletion of the complete N-terminus did abrogate anti-polyQ aggregation activity. (B) Expression levels of the polyQ proteins (EGFP-staining) and HSPB7 deletion mutants (V5 staining) were analyzed by western blot.

HSPA proteins in order to form an adequate chaperone complex (45). In fact, both *in vitro* (46) and cellular data (47) have revealed that elevated BAG1:Hsp70 ratios poison the functionality of the Hsp70 machine and inhibit refolding reactions. Consequently, Hsp70-dependent refolding reactions in cells such as supported by HSPB1 are also inhibited (40). Similarly, BAG1 could negate the HSPA1A-dependent effects of DNAJB1 on polyQ aggregation (28). Also under the experimental conditions used here, BAG-1 reduced the refolding activities of HSPA1A on heat-inactivated cytosolic or nuclear luciferase (Fig. 7C). However, this BAG1 overexpression did not significantly affect the HSPB7 activity on polyQ aggregation (Fig. 7D), indicating that HSPB7's major route of anti-aggregation does not require HSPA activity.

HSPB7 interacts with polyQ proteins

Under physiological conditions, HSPB7 is distributed diffusely throughout the cell in HEK293 (Fig. 8Aa–c) and NG-108 cells (Fig. 8Ag–i), with some speckled nuclear staining as shown before (37). When co-expressed with polyQ proteins, the nuclear and cytoplasmic inclusions that do form under such conditions also stain positive for the V5 antibody detecting the V5-tagged HSPB7 (Fig. 8Aj–o). Interestingly, HSPB7 shows a ring-like staining pattern suggesting that it only associates with the periphery of the inclusions in both cell types. This is not related to a staining artifact by an incomplete penetration of the antibody in the inclusion since an mRFP-ubiquitin-HSPB7 construct showed similar staining patterns (Fig. 8Ad–f). Moreover, we recently found that DNAJB8, another powerful suppressor of polyQ aggregation, can be detected inside the core of the inclusions (28).

To test whether HSPB7 indeed interacts directly with the inclusions, we used fluorescence lifetime microscopy (FLIM) to measure fluorescence resonance energy transfer (FRET) between an ECFP-tagged HSPB7 and EYFP-polyQ. This ECFP tagging did not affect the suppressive effect of HSPB7 on polyQ aggregation (Fig. 8B). The FRET efficiency between EYFP-polyQ and ECFP alone (negative control) was

indeed low (Fig. 8C). The FRET efficiency for ECFP-HSPB7 and EYFP-polyQ was close to levels of co-aggregating ECFP-HttQ74 and EYFP-polyQ (positive control) strongly suggesting that HSPB7 can bind polyQ proteins.

To test whether HSPB7 binding to polyQ proteins affects the nature of the aggregates in cells, we analyzed the distribution of HSPB7 and EGFP-HttQ74 when expressed alone or together. As shown earlier (Fig. 7A), HSPB7 was primarily found in the low-sucrose density fraction and this was not affected upon co-expression of EGFP-HttQ74 (Fig. 8D, upper two panels). Inversely, EGFP-HttQ74 distribution was polydispersed and this did not change upon co-expression of HSPB7 (Fig. 8D, lower two panels). Hence, the presence and binding of HSPB7 in a ring around the polyQ inclusion may suggest a late-stage co-aggregation as a result of a failed function in an early stage of interaction.

HSPB7 does not target polyQ proteins for proteasomal degradation

Heat-shock proteins such as HSPA1A, DNAJB1 and DNAJB2 have been shown to be able to promote degradation of certain misfolded proteins through the ubiquitin–proteasome system (28,48,49).

To test whether HSPB7 is a general stimulator of proteasomal degradation, we co-expressed it with the proteasome activity reporter Ub-R-EGFP (50). The relative EGFP expression increases when using the proteasomal inhibitor MG132 and decreases upon co-expression of DNAJB1, known to be able to support and accelerate proteasomal degradation (33) (Fig. 9A and B). Co-expression with HSPB7, however, had no effect, neither when expressed alone (Fig. 9A and B) nor when co-expressed together with polyQ proteins (HA-HttQ43) (Fig. 9B). Although HSPB7 did not promote overall proteasomal activity, it is still possible that HSPB7 depends on an active proteasome to exert its protective effects against polyQ proteins. To test this, we co-transfected cells with HSPB7 together with HttQ43 and treated cells with the proteasome inhibitor MG132. Under such conditions,

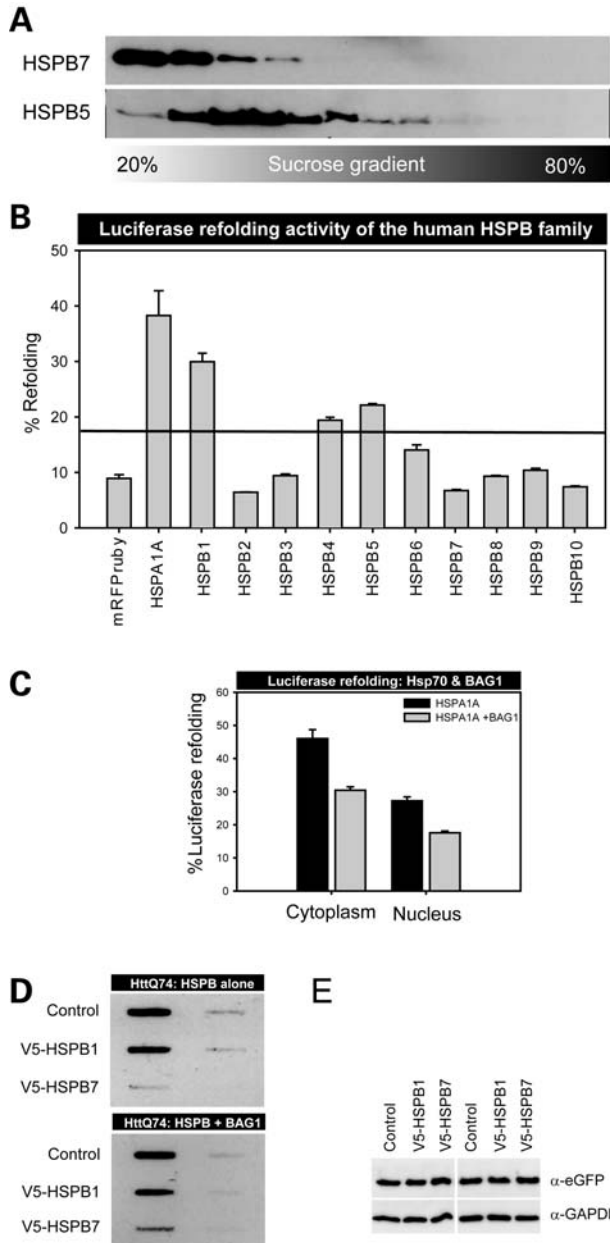


Figure 7. HSPB7 is not a canonical small heat-shock protein and is not dependent on a functional Hsp70 machine to prevent polyQ aggregation. (A) HEK293 cells were transfected with either V5-tagged HSPB5 or HSPB7. Cell lysates were separated on a sucrose-gradient. Whereas HSPB5 oligomers can be detected almost throughout the sucrose gradients, indicative of a hetero-oligomeric complex formation, HSPB7 is found only in the less dense fractions (mainly mono- and dimers). (B) Cells were co-transfected with firefly luciferase and the indicated chaperones (1:9 ratio) or mRFP:ruby as a control. Cells were heat-shocked at 43°C for 30 min and allowed to recover for 1 h at 37°C. The percentage of reactivated luciferase relative to the un-heated controls is depicted. Error bars indicate the SD of three independent experiments, threshold for positive refolding is set at two times the relative refolding of the negative control. (C) HSPA1A was co-transfected with BAG1, cytoplasmic luciferase or nuclear luciferase (4.5:4.5:1 ratio). Cells were heat-shocked at 43°C for 30 min and allowed to recover after which luciferase activity was determined. In both cellular compartments, co-expression of BAG1 inhibits the HSPA machine. (D) Co-expression of BAG1 with HSPB7 and EGFP-HttQ74 shows that BAG1 mediated inhibition of the Hsp70 machine only marginally reduced the effect of HSPB7 in preventing polyQ aggregation. (E) Total levels of EGFP-HttQ74 were similar under both conditions.

HSPB7 was still active in preventing aggregate formation, whereas the protective action of DNAJB1 was clearly reduced (Fig. 9C), consistent with the finding that the anti-aggregation effect of DNAJB1 but not HSPB7 is through stimulating proteasomal degradation (48).

HSPB7 needs a functional autophagosomal machine for its function

Evidence is emerging that rather than degradation via the proteasome, autophagy-mediated degradation may be the mechanism of defense against toxic aggregation of polyQ containing proteins (51,52). At least one member of the small HSPB family (HSPB8) acts in concert with its physiological partner BAG3 to reduce polyQ aggregation specifically by stimulating phosphorylation of eIF2 α , causing transcriptional shutdown and stimulating autophagy (16–18). To explore whether HSPB7 also may inhibit polyQ aggregation via an effect on autophagy, we tested the capacity of HSPB7 to suppress polyQ aggregation in autophagy deficient (ATG5 $^{-/-}$) or -proficient (ATG5 $^{+/+}$) murine embryonic fibroblasts (MEFs) (53). In ATG5 $^{+/+}$ cells, HSPB7 inhibited polyQ aggregation (Fig. 10A); like in all other cell lines tested, this occurred without any detectable changes in soluble EGFP-HttQ74 levels (Fig. 10C). ATG5 $^{-/-}$ cells show more polyQ aggregation, consistent with the role of constitutive autophagy in clearance of polyQ aggregates (54,55). Also in these, ATG5 $^{-/-}$ expression of HSPB7 reduced aggregation of HttQ74 but the effects were reduced by approximately 40% compared with the effects of HSPB7 in ATG5 $^{+/+}$ cells (Fig. 10A and B, quantified in E). Suppression of polyQ aggregation by BAG3 overexpression, which leads to stabilization of endogenous HSPB8 (17), is also reduced in ATG5 $^{-/-}$ cells [(16); Fig. 10A, B and E]. However, various lines of evidence suggest that HSPB7 works by a different mechanism from the HSPB8–BAG3 complex. First, in contrast to HSPB8, HSPB7 does not form a stable complex with BAG3 [Supplementary Material, Fig. S3A; (16)]. Second, unlike HSPB7, the HSPB8–BAG3 complex does reduce total levels of polyQ proteins [(56), Fig. 10C], an effect that could be attributed to an inhibitory effect of HSPB8–BAG3 on translation, via induction of eIF2 α phosphorylation. Consistently, we found that HSPB7 had no effect on the phosphorylation status of eIF2 α (Supplementary Material, Fig. S3B). Moreover co-expression of GADD34, which maintains eIF2 α in the de-phosphorylated state (56), abolished the protective effect of HSPB8 (16) but not that of HSPB7 (Supplementary Material, Fig. S3C–F).

To test whether HSPB7 induces autophagy via an alternative, eIF2 α independent route, we analyzed its effects on the autophagy marker LC3. The ratio between the LC3 and the lipidated form LC3II is considered to be a good indicator of macroautophagy (57). Whereas we could confirm the accumulation of LC3II upon overexpression of the HSPB8–BAG3 complex, HSPB7 overexpression had a moderate effect on the formation of LC3II (Fig. 10F). Together, these results indicate that HSPB7 functions via a different mechanism than the HSPB8–BAG3 complex and by a yet unknown mechanism

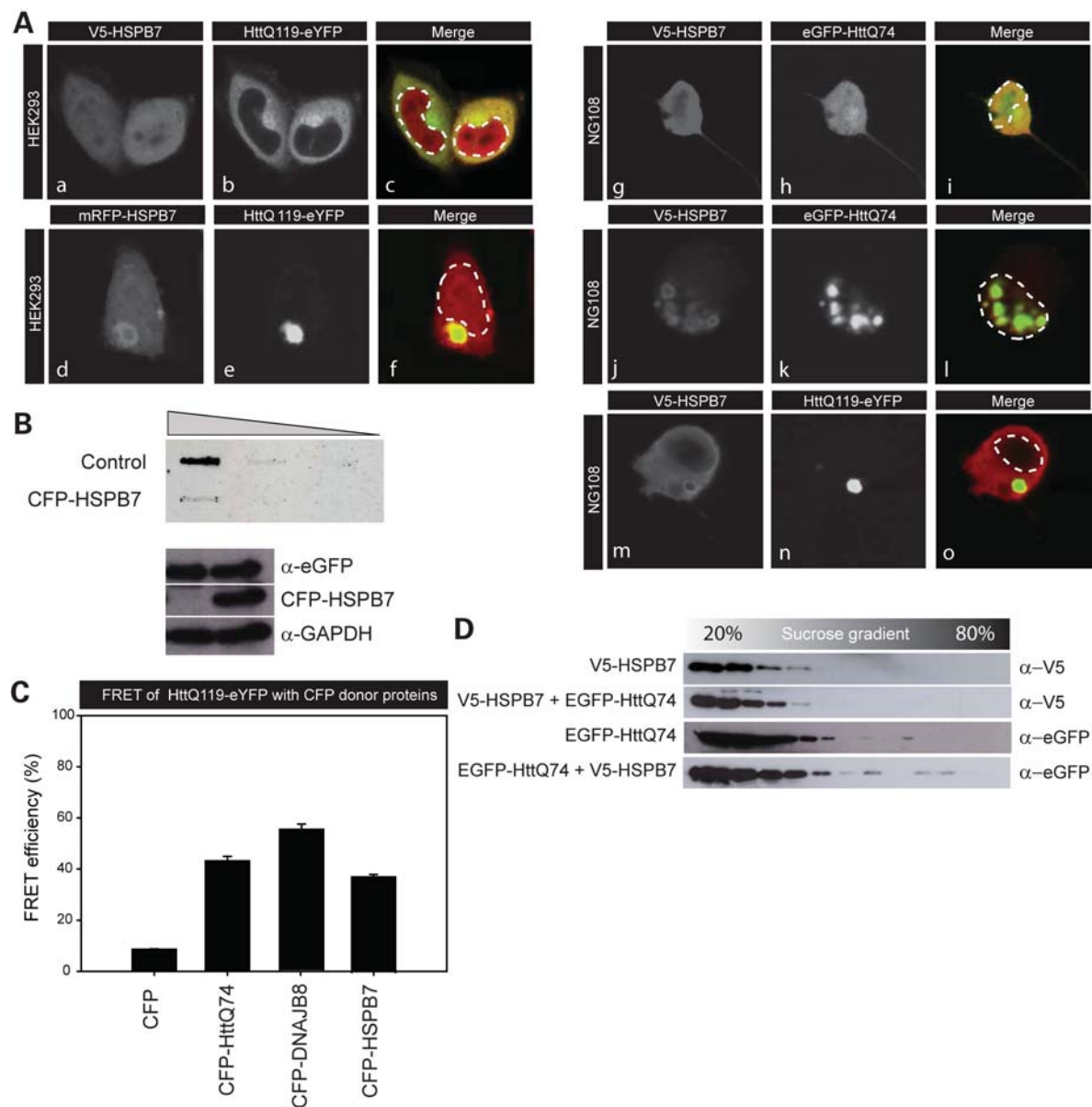


Figure 8. HSPB7 interacts with polyQ proteins. (A) Confocal microscopy of HEK293 or differentiated NG-108 cells. HEK293 cells overexpressing (a) diffusely distributed V5-HSPB7 (grey/red signal) and (b) cytosolic HttQ119-EYFP (grey/green signal). (c) Overlay of (a) and (b), dashed white line represents the nucleus. (d) mRFP-HSPB7 co-localizing with the ring of the (e) perinuclear HttQ119-EYFP inclusion. (f) Overlay of (d) and (e). (g) NG-108 cells with diffusely distributed V5-HSPB7 with (h) diffusely distributed EGFP-HttQ74. (i) Overlay of (g) and (h). (j) V5-HSPB7 co-localizing with the ring of the (k) intranuclear EGFP-HttQ74 inclusions. (l) Overlay of (j) and (k). (m) Diffusely distributed V5-HSPB7 co-localizing with the (n) perinuclear HttQ119-EYFP inclusion. (o) Overlay of (m) and (n). (B) Filter trap assay showing that CFP-HSPB7 is functional, with below the corresponding western blot showing that the protein levels are equal. (C) Cells transfected with HttQ119-EYFP and CFP-tagged HttQ74, DNAJB8 and HSPB7 and CFP (as a control) were analyzed by FRET using fluorescence lifetime (FLIM) analysis; data are mean $\pm$ SD ($n > 26$ individual cells). (D) Sucrose density gradient fractionation of lysates of cells expressing combinations of V5-HSPB7 and EGFP-HttQ74 as indicated. HSPB7 was detected using the anti-V5 antibody (upper two lanes), whereas HttQ74 was detected with an anti-GFP antibody (lower two lanes).

utilizes but does not induce autophagy for its protective action against polyQ.

DISCUSSION

When comparing the human HSPB members for their ability to prevent polyQ aggregation, we identified HSPB7 as the most active member. HSPB7 was found to be a non-canonical

HSPB protein: unlike the more classical HSPB1 and HSPB5, HSPB7 did not form high-molecular weight oligomeric complexes in cells and HSPB7 was unable to chaperone heat-denatured luciferase. Also, unlike the refolding activity of HSPB1 and HSPB5, the anti-aggregation activity of HSPB7 was found not to be dependent on a functional Hsp70 machine. For its anti-aggregation activity, HSPB7 does not enhance nor does it require proteasomal activity. Unlike

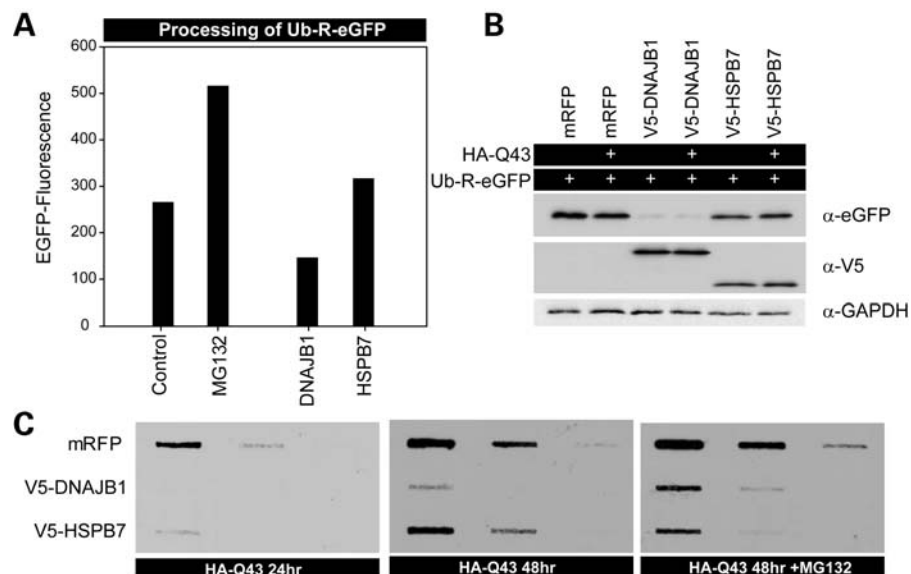


Figure 9. HSPB7 is not involved in proteasomal degradation. To monitor the activity of proteasomal degradation, HEK293 cells were co-transfected with the N-end rule proteasomal substrate Ub-R-GFP and V5-tagged DNAJB1 or HSPB7. GFP intensity was measured by fluorescent activated cell sorting measurements (FACS) (A) showing that DNAJB1, but not HSPB7, reduces the Ub-R-EGFP fluorescence. Graph is showing data from one typical experiment. (B) Western blot analysis confirming the FACS data. Co-expression of HA-HttQ43 did not influence Ub-R-EGFP clearance. (C) HEK293 cells were co-transfected with V5-tagged DNAJB1 or HSPB7 together with HA-HttQ43 at a 9:1 plasmid ratio. After 24 and 48 h, samples were generated and analysed by the filter trap assay. In addition, cells were treated for 12 h with the proteasome inhibitor MG132.

HSPB8, HSPB7 does not interact with BAG3, not induce the phosphorylation of eIF2 α or seem to induce autophagy (18) (this study). However, in ATG5 knockout cells that lack the classical autophagosomal degradation route, the anti-aggregation effect of HSPB7 is greatly impaired. HSPB7-like activities are evolutionarily conserved and also prevent aggregate formation in neuronal-like cells. Moreover, HSPB7 reduces polyQ-related cytotoxicity in cells and in a *Drosophila* SCA3 model.

As described before, we found that the polyQ length is directly correlated with the kinetics of aggregation and formation of inclusion bodies (58,59). This allowed us to compare the relative ability of the diverse members of the HSPB family to prevent polyQ aggregation. Whereas especially HSPB6, HSPB7, HSPB8 and HSPB9 showed suppressive activities against polyQ aggregation of relatively short repeat lengths (Q43), HSPB9 and especially HSPB7 were the only members capable of delaying the formation of aggregates by polyQ proteins with long repeats (Q119). This indicates that although these different HSPB members all can act on polyQ substrates, consistent with a presumed rather promiscuous substrate recognition ability of small HSPs, the effectiveness of the various members to handle (the same) substrates may differ substantially. The well-studied HSPB1, HSPB4 and HSPB5 were all found to be able to assist in refolding of heat-denatured luciferase in cells, an activity that was found to depend on a functional Hsp70 machine (40). These three proteins were largely ineffective in preventing polyQ aggregation (13). This might suggest that they either do not recognize polyQ proteins (or their small aggregates) as clients or that the interaction with these polyQ proteins is too transient to be effective, because of a rapid, Hsp70-mediated release. Intriguingly, for reducing

polyQ aggregation, HSPB7 does not require a functional Hsp70 machine and HSPB7 cannot assist in the (Hsp70-dependent) refolding of heat-denatured luciferase. In line with this, HSPB8 (16) and HSPB9 (Zijlstra *et al.*, manuscript in preparation) also do not require Hsp70 for the anti-aggregation activity, and both members were also found to be unable to support luciferase refolding (this study). The data are also strikingly in parallel with recent findings on the family of DNAJ proteins: here it also is found that DNAJ proteins that suppressed polyQ aggregation acted in a mere Hsp70-independent manner and were not able to stimulate luciferase refolding and vice versa (60). It is still possible that the ability of the above-mentioned HSPB and DNAJB members to support refolding is substrate dependent. Alternatively, it can be speculated that the members of these DNAJB and HSPB families have evolved to function in either one of the two fundamentally different sections of the protein homeostasis, i.e. Hsp70-dependent refolding or Hsp70-independent chaperoning of non-refoldable substrates for degradation (see below).

Besides the (in)dependency on Hsp70, another striking feature of HSPB7 is that under the conditions used here, it is not found in (dynamic) oligomeric complexes in cells as has been described for many small HSP both in cells and in cell-free experiments (21,61) and as confirmed here for, for example, HSPB5. Similar to HSPB7, HSPB8 also does not form large oligomers, but unlike HSPB7, HSPB8 interacts with BAG3 in cells (17). How far this is related to substrate specificity or/and Hsp70 dependency remains to be elucidated.

How the polyQ suppressive, non-canonical branch of the HSPB family handles its clients remains to be elucidated in detail. But, HSPB7 and HSPB8 seem to play mechanistically distinct roles in protein quality control. HSPB8 forms a

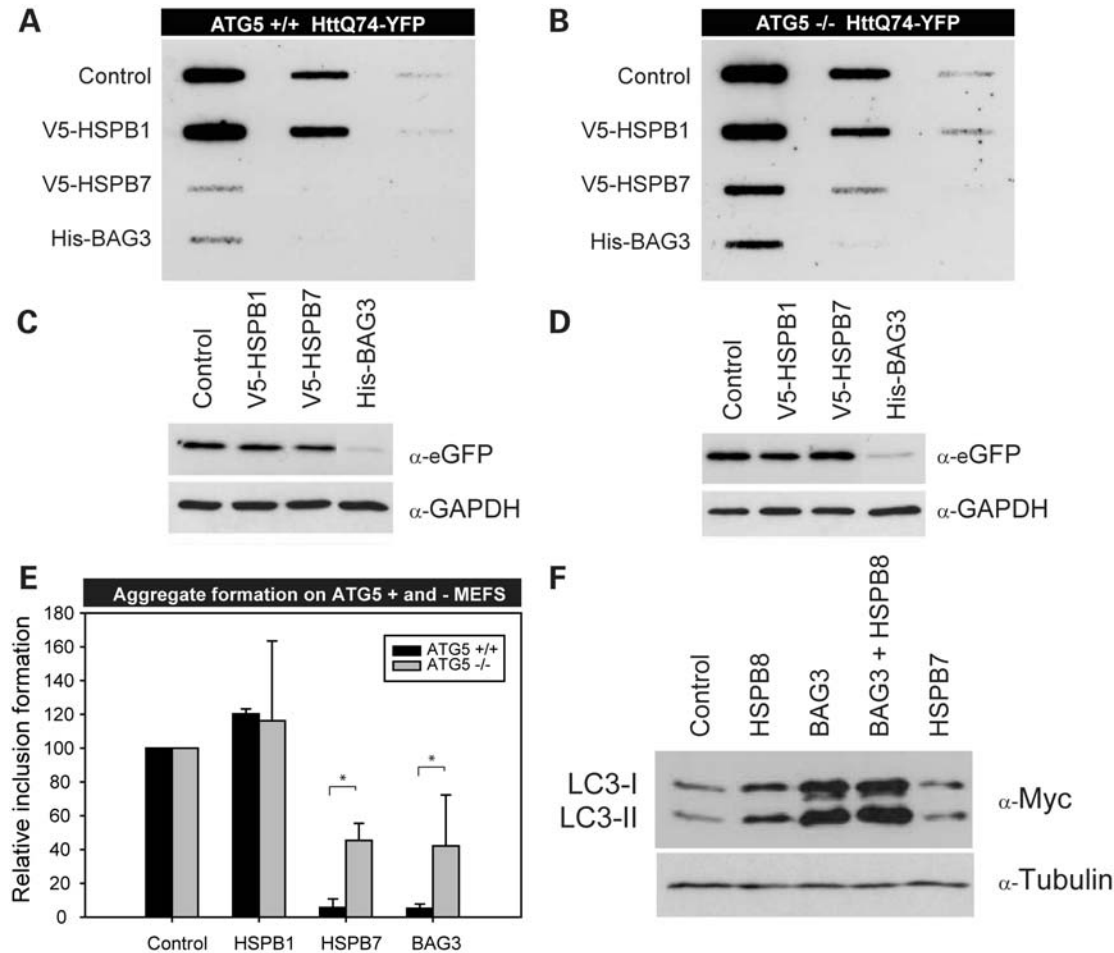


Figure 10. HSPB7 efficacy is reduced in autophagy-deficient *ATG5*^{-/-} cells. (A) Wild-type MEF cells (*ATG5*^{+/+}) and (B) MEF cells lacking the *ATG5* gene (*ATG5*^{-/-}) were co-transfected with V5-tagged HSPB1, HSPB7 or His-tagged BAG3 together with EGFP-HttQ74 (9:1 ratio). Cell lysates were analysed by filter trap assay 48 h after transfection. (C and D) Western blot analysis shows that BAG3, but not HSPB7, reduces the soluble pool of EGFP-HttQ74 in *ATG5*^{+/+} (C) as well as in *ATG5*^{-/-} (D). (E) Quantification of three individual filter trap assays showing that the HSPB7 and BAG3 anti-aggregation capacity is decreased in *ATG5*^{-/-} cells (**P* < 0.05; Mann-Whitney U-test). (F) HEK293T cells were transfected with Myc-LC3 and HSPB or BAG3 proteins and analyzed by western blot.

stoichiometric complex with BAG3 (2:1). This complex induces autophagy (17) and inhibits protein synthesis in an eIF2 α -dependent manner (16). Although HSPB7 in part also depends on active autophagy, its mechanism of action seems distinct from the HSPB8-BAG3 complex: HSPB7 does not interact with BAG3, does not act via eIF2 α , and does not up-regulate autophagy. Together these data imply that the evolution of HSPB members has not only occurred for gene regulatory or compartmentalization purposes only, but also for functional divergence in client specificity and client handling.

With regard to the details of the mechanisms by which HSPB7 works, we so far only have a number of deterministic observations. HSPB7 was found to be associated with polyQ protein aggregates as revealed by FLIM analyses, and immunofluorescent analysis revealed HSPB7 to be present at the periphery of inclusions. This apparent association of HSPB7 with inclusions does not affect its main distribution when analyzed on sucrose gradients, suggesting its presence at inclusion could reflect a failed function at an earlier stage of interaction with the client. By interacting with early polyQ intermediate

complexes, HSPB7 might prevent the nucleation reaction and allow a larger time window to clear the other toxic intermediates without the requirement of boosting proteasomal or autophagic clearance.

The lack of reduction in overall levels of the expressed polyQ proteins by HSPB7 is likely due to technical limitations. Under the conditions used, only a relatively small fraction (5–10%) of the expressed polyQ protein is aggregated. Even if 50% of the aggregates would have been removed by HSPB7-assisted autophagy, this would result in a loss of only 2.5–5% of the total amount of protein, an effect that will not be detected by western blotting. For HSPB8, the situation is different since this protein, besides inducing autophagy, also affects translation (16) and therefore HSPB8 expression does affect total polyQ protein levels under such conditions.

The finding that the activity of HSPB7 is only decreased by 40% in cells lacking *ATG5* suggests that HSPB7 either acts upstream of the autophagic pathway and/or can be, in part, uncoupled from the autophagy or that it is partly mediated via another autophagic pathway. In support for the latter,

it became recently clear that mouse cells lacking ATG5 are able to form autophagosomes/autolysosomes, and still can perform autophagy-mediated protein degradation by an ATG5/ATG7-independent alternative pathway (62). The possibility that macro-autophagy is not completely shutdown in ATG5^{-/-} cells may underestimate the importance of autophagy for the function of HSPB7.

Another yet poorly understood feature of HSPB7 is its association with nuclear splicing (SC35) speckles (37). The main component of these nuclear bodies, SC35, was proposed to be an RNA-binding protein that facilitates transcriptional elongation (63). Binding of speckle components to RNA was suggested to stabilize RNA and prevent RNA misfolding and/or to loosen tertiary RNA structures. Triplet expansion diseases such as myotonic dystrophy (MD) and (most likely) Huntington disease-like 2 are caused by expansions in the 3'-untranslated region of the gene encoding MD protein kinase and the juncophilin-3 gene, respectively (64,65). Both disorders are characterized by the presence of RNA foci containing the mutant transcript (66) in which the disease-associated mRNAs have entangled other mRNA species. In turn, this might disrupt cellular homeostasis leading to a concurrent sensitivity for internal and external stressors. In line with this, it was recently shown that untranslated RNAs containing CAGs can cause retinal degeneration and neuronal dysfunction in *Drosophila* (67). Whether CAG RNA toxicity also contributes to the toxicity in polyQ diseases remains controversial (68). But if so, HSPB7 might act as an SC35-speckle resident RNA chaperone hereby preventing polyQ toxicity. This would be supported by our data on HSPB7 mutants where we find a correlation between HSPB7 speckle association (37) and ability to suppress polyQ aggregation (this study). How such a mechanism would (in part) depend on autophagy remains unclear. Clearly, this hypothesis requires much more experimental evidence.

Despite the original identification of HSPB7 in cardiovascular tissue (36), HSPB7 messenger RNA is more widely expressed throughout the body (37) and also in the brain albeit at lower levels than the more abundant HSPB1, HSPB5 and HSPB8 (Supplementary Material, Fig. S1). Interestingly, gene arrays to identify genes that are altered by expression of mutant Huntingtin in HeLa cells pointed to HSPB7 as one of the 70 transcripts that were increased and preventing HSPB7 up-regulation by siRNA led to an increased accumulation of Q103-CFP (69). These data indicate that there is a possible role for endogenous HSPB7 in combating polyQ protein aggregation. Whereas under the experimental conditions used here, we did not detect HSPB7 expression in the HEK293 nor NG108 cells, irrespective of polyQ expression (Supplementary Material, Fig. S4), all the data combined suggest that boosting the expression of HSPB7, for example by calcium panthotenate (70), can provide a possible therapy against polyQ disorders.

MATERIALS AND METHODS

Reagents and antibodies

Chemicals were obtained from Sigma (Sigma-Aldrich, St Louis, MO, USA) and beetle luciferin from Promega.

Antibodies against the V5 tag (Invitrogen, Carlsbad, CA, USA), EGFP (JL-8) (BD Bioscience, Franklin Lakes, NJ, USA), GAPDH (RDI-TRK564-6C5, Fitzgerald Industries International Inc., Concord, MA, USA), HA tag (Covance, Princeton, NJ, USA), HSPB7 (Abcam, Cambridge, UK) and HSPA1A (SPA-800), HSPA6 (SPA-754, Stressgen Biotechnologies, Victoria, Canada) were mouse monoclonal. The antibodies against DNAJB1 (SPA-400) (Stressgen Biotechnologies), HSPB5 (Abcam) and HSPB8 (18) were rabbit polyclonal. Reagents for western blotting were obtained from Amersham (GE Healthcare, Uppsala, Sweden). Secondary antibodies used for immunocytochemistry were CY5 anti-mouse and FITC anti-mouse (Jackson, Immunoresearch, West Grove, PA, USA) and CY3 anti-mouse (Amersham, GE Healthcare).

Molecular techniques

Standard recombinant DNA techniques were carried out essentially as described by Sambrook *et al.* (71). Oligonucleotide primers were obtained from Biolegio (Nijmegen, The Netherlands). Restriction enzymes and DNA polymerases (Invitrogen, and New England Biolabs, Ipswich, MA, USA) were used according to the manufacturer's instructions. DNA sequencing reactions were carried out by ServiceXS (Leiden, The Netherlands).

Plasmid generation

The SCA3Q82-YFP construct was described before (28). The truncated SCA3Q64-EYFP was PCR amplified from cDNA from human skin fibroblasts of a heterozygous SCA3 patient with 64 CAG repeats present in the ataxin-3 gene with (5'-ATAAGCGCCGCTCAGAAGAGCTTCGGAAGA-3', forward)

5'-ATCGCTCGAGGGACCCTATGCTGTAATCAC-3' reverse) primers and cloned into pCDNA5.1-FRT/TO-EYFP, in frame with EYFP. Plasmids containing either HDQ43 or HDQ74 were kindly provided by Dr David Rubinshtein (Cambridge, UK). Generation of the human HSPB plasmid library was described before (43) as well as the HSPB7 deletion mutants (37). For retroviral transduction, the pQCXIN vector (Clontech, Mountain View, CA, USA) was used for cloning. The Ub-R-EGFP construct was kindly provided by Dr Nico Dantuma (Karolinska Institutet, Sweden). The constructs encoding His-BAG3 and HA-BAG1 have been described before (18,47). The Myc-LC3 construct was described before (72).

Cell lines, cell culture, transfections and transductions

NG-108-15, mouse embryonic fibroblasts (MEF), human embryonic kidney 293 (HEK293) cells expressing the tetracycline repressor (Flp-In T-Rex HEK293, Invitrogen Carlsbad, CA, USA), DNHSF-1 HEK293 (29) and HEK293T cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Greiner Bio-one, Longwood, FL, USA) at 37°C under a humidified atmosphere containing 5% CO₂. The generation of HEK293-HDQ119, with integration of a tetracycline regulatable expression

vector coding for EGFP-HDQ119, has been described before (28). Gene expression in Flp-In T-Rex HEK293 cells was induced with a final concentration of 1 µg/ml tetracycline. HEK293 and MEF cells were transfected using Lipofectamine and Lipofectamine 2000, respectively (Invitrogen) according to the manufacturer's instructions using 1 µg of plasmid DNA per 35 mm dish. NG-108 cells were differentiated into neuronal-like cells by the addition of 2 ml differentiation medium [DMEM containing 1 mM sodium pyruvate, 50 µM 3-isobutyl-1-methylxanthine, 10 µM 5'-(N-ethylcarboxamido) adenosine, 0.5% FCS] to 35 000 cells in a 35 mm dish. Seven days later, cells were transfected using Jetpei (Polyplus Illkirch, France) according to the manufacturer's instructions using 3 µg of plasmid DNA per 35 mm dish. For transduction, retroviral particles were generated by co-transfection of HEK293-T cells with pCL-ampho and pQCXIN encoding either HSPB5 or HSPB7 using Fugene (Roche, Basel, Switzerland) according to the manufacturer's instructions. The supernatant was collected after 48 h and filtered using a Millex-HV filter (Millipore, Billerica, MA, USA). Cells were transduced by adding 1.5 ml of filtrated medium containing 4 µg/ml of polybrene. The medium was replaced after 24 h and transduced cells were selected using puromycin selection.

Drosophila

Fly stocks were maintained at 22°C according to the standard protocols. *GAL4* driver stocks were obtained from the Bloomington Stock Centre (Indiana University, USA). The *GMR-UAS-SCA3Q78* flies used for the eye-degeneration screen were generously provided by Dr N. Bonini (University of Pennsylvania, USA) and maintained at 25°C. HSPB7 transgenic lines were generated by Genetic Services Inc. (Sudbury, USA) by injection of the pUAS transformation vector into the W1118 genetic background. The UAS-HSPB7 transgenic line was crossed with the *act-GAL4* driver (Bloomington Stock Center) line to test for transgene expression (Fig. 5D). To analyse effects on eye degeneration, the *GMR-UAS-SCA3trQ78* transgenic line was crossed with either control flies (*w1118*) or with the UAS-HSPB7 transgenic line. The resulting offspring was analysed for eye degeneration. Severity of eye degeneration was scored by counting eyes with visible degeneration as illustrated in Figure 5D.

Western blot analysis

Samples were prepared in Laemmli sample buffer (250 mM Tris-Cl, 20% glycerol, 4% SDS, 0.001% bromophenol blue and 10% β-mercaptoethanol). Equal amounts of protein were separated on 10 or 12.5% SDS-PAGE gels followed by transfer onto nitrocellulose membranes. Primary antibodies were used at the following dilutions: V5 (1:5000), EGFP (1:5000), GAPDH (1:10000), HSPA1A (1:5000), HSPA6 (1:5000) and DNAJB1 (1:5000). After 1 h of incubation with the primary antibody in 0.1% PBS-Tween 20, membranes were incubated with HRP-conjugated secondary antibodies at a 1:5000 dilution. Detection was performed using enhanced chemiluminescence and Hyperfilm (ECL, Amersham, GE Healthcare).

Filter trap assay

The filter trap assay was performed basically as described by Carra *et al.* (13). Briefly, cells grown in a 35 mm dish were lysed in 200 µl FTA buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl) containing 2% SDS. 100 µg, 20 µg and 4 µg samples were diluted in FTA, 2% SDS with 50 mM dithiothreitol (DTT), boiled for 10 min and applied onto a pre-washed (FTA, 0.1% SDS) 0.2 µm cellulose acetate filter (GE Water and Process Technologies, Trevose, PA, USA) with 2 Whatman papers (Bio-Rad, Hercules, CA, USA) in a Bio-Dot microfiltration apparatus (Bio-Rad). Gentle suction was applied to filtrate the samples followed by one washing step using FTA, 0.1% SDS. Trapped material was probed with anti-GFP (JL-8) or anti-HA at a 1:5000 and 1:2000 dilution, respectively, followed by incubation with HRP-conjugated secondary antibodies at a 1:5000 dilution. Detection was performed using enhanced chemiluminescence and Hyperfilm (ECL, Amersham, GE Healthcare).

Microscopy

For microscopy, HEK293 cells were plated on poly-L-lysine (0.001%, Sigma-Aldrich) coated cover slips, and transfected the next day with V5-tagged HSP plasmids and EYFP- or EGFP-polyQ constructs or RFP-HSPB7 using Lipofectamine (Invitrogen) and incubated for 24 h/48 h depending of the polyQ constructs used (Q119 24 h, other constructs 48 h). NG-108 cells were plated on non-coated cover slips, differentiated for 7 days and transfected with Jetpei (Polyplus Illkirch). For fixation, the cells were washed once with PBS and fixed with 3.7% formaldehyde (Merck, White House Station, NJ, USA) in PBS for 15 min. After three washes with 0.1%, PBS-triton cells were permeabilized in blocking solution (100 mM glycine, 3% BSA, 0.1% triton) for 1 h followed by 1 h incubation with the primary antibody (V5 anti-mouse, 1:200). After three washing steps, cover slips were incubated with CY3-conjugated anti-mouse secondary antibody (Jackson, Immunoresearch) at 1:200 dilution for 1 h, followed by a 10 min incubation with 0.2 µg/ml 4',6-diamidino-2-phenylindole (DAPI). After three washing steps, the cover slips were mounted using Citifluor mounting medium (Citifluor Ltd, London, UK). Images were obtained using an inverted confocal laser scanning microscope (TCS SP2, DM RXE, Leica, Wetzlar, Germany) with a 63×/1.32 NA oil objective.

Fluorescence resonance energy transfer

HEK293 cells were plated on poly-L-lysine (0.001%, Sigma-Aldrich) coated cover slips and transfected the next day with CFP-tagged HSP plasmids and HttQ119-EYFP using Lipofectamine (Invitrogen) and incubated for 24 h. For fixation, the cells were washed once with PBS and fixed with 3.7% formaldehyde (Merck, White House Station) in PBS for 15 min. After three washes, the cover slips were mounted using Citifluor mounting medium (Citifluor Ltd). 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 a 1 W 445 nm LED using the AQUA filter cube (EX 436/20, DM 455, BA 480/30) and 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 lifetime (calculated from phase differences) of CFP in the absence of acceptors was 2.45 ns. FRET efficiency E was calculated as $E = 1 - (\text{measured lifetime of FRET pair})/(\text{measured lifetime of donor})$. FRET efficiencies are means of 26–55 regions of interest per construct.

Luciferase refolding assay

Chaperone activity of HSPB members was assessed by using the luciferase refolding assay (35). Briefly, HEK293 cells were co-transfected with luciferase (Superluc-EGFP) (73) together with HSPB encoding plasmids (1:9 ratio). Two hours after transfection, expression was induced by addition of 1 µg/ml tetracycline. After 24 h, cells were re-suspended and divided into 1 ml portions in tissue-grade 10 ml tubes. The following day, cells were given a heat shock (30 min at 43°C) in the presence of cycloheximide (20 µg/ml) and 4-morpholinepropanesulfonic acid (MOPS; 20 mM, pH 7.0) in order to inhibit protein synthesis and increase the buffer capacity of the medium, respectively. After heat treatment, cells were allowed to recover before luciferase activity was determined (3 h at 37°C). Luciferase activity measurements were performed using a Berthold Lumat 9510 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Sucrose gradient centrifugation

Cells from a 35 mm dish were scraped in 200 µl cold lysis buffer (150 mM NaCl, 50 mM NaH₂PO₄, 10 mM imidazole, 0.5% NP-40, 1.5 mM MgCl₂ and 3% glycerol, pH 8). Cells were lysed by five passages through a 25-gauge needle, followed by centrifugation at 300g for 15 min. The cell lysate was loaded on top of a 10–80% sucrose gradient (10 mM Tris–HCl, pH 8, 5 mM EDTA, 50 mM NaCl) and centrifuged for 18 h at 100 000g using a Sorvall Discovery 90SE ultra centrifuge and the SW55 rotor. Fractions of 400 µl were precipitated by adding an equal volume of 25% (w/v) trichloroacetic acid. After 30 min of incubation on ice, proteins were pelleted at 17 000g for 15 min. The pellet was washed twice with 80% acetone (–20°C) and allowed to dry. The pellet was dissolved in 20 µl of 0.1 M NaOH containing 1% SDS. An equal amount of Laemmli buffer was added and samples were boiled for five minutes followed by western blot analysis.

Clonogenic assay

The clonogenic assay was used to determine cellular survival by using a tetracyclin-inducible HttQ119-EYFP HEK293 stable cell line without or with HSPB members. Exponentially growing cells were plated in triplicate in 10 cm dishes at a density of 200 cells per dish. HttQ119-EYFP expression was activated by the addition of tetracycline. Cells were allowed to grow and form colonies for 2 weeks, after which they were fixed and stained (0.1% Coomassie Brilliant Blue, 50%

methanol, 10% glacial acetic acid) followed by washing (10% methanol, 7.5% glacial acetic acid). The effect of HttQ119-EYFP expression and co-expression of HSPB5 and HSPB7 was determined by comparing colony reduction in the presence of HttQ119-EYFP expression.

Proteasome inhibition, proteasome activity measurement and autophagy analysis

Proteasomal activity was blocked by the addition of 10 µM MG132 to the culture medium for 12 h. Proteasomal activity was monitored by co-transfection of Ub-R-EGFP followed by western blot and FACS analyses. The role of autophagy in reducing polyQ aggregation was analysed by filter trap and western blot analysis using mouse embryonic fibroblasts lacking the *ATG5* gene, which were kindly provided by Dr Mizushima (Okazaki, Japan). The induction of autophagy was measured by transfecting HEK293T cells with Myc-LC3 constructs (72) together with HSPB proteins or BAG3, followed by western blot analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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