Longevity as evoked by different chaperone activities of small heat shock proteins

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Abstract

During aging, oxidized, misfolded and aggregated proteins accumulate in cells, whilst the capacity to deal with protein damage declines severely. To cope with the toxicity of damaged proteins, cells rely on protein quality control. In particular, heat shock proteins (HSP) have been proven to be successful in restoring cellular protein homeostasis and in boosting longevity of organisms. Cells contain many HSPs with overlapping and non-overlapping activities, but it remains unclear which functions are required to support healthy aging. In the current study, we compared the functions of the members of the Drosophila small HSP family for their ability to assist in refolding stress denatured substrates and/or prevent aggregation of disease associated, misfolded proteins. Whereas some small HSP members chaperone both substrates, we identified one member (CG14209) that exclusively assisted in HSP70-dependent refolding of stress denatured proteins. On the other hand, one member (HSP67BC) did not support protein refolding but only prevented toxic aggregation of misfolded proteins in an HSP70 independent manner. Intriguingly, both single activities supported longevity, supporting that protein homeostasis in general rather than specific chaperone activities can decelerate the negative effects of aging.
Small heat shock proteins & longevity

Introduction

Imbalances in protein homeostasis has been suggested to be a crucial factor in the development of heritable age-related neurodegenerative diseases and in normal aging (1-6). Achieving and maintaining the correct three-dimensional protein structure is a continuous struggle within cells. Firstly, folding of proteins towards an active biological state is challenged by the crowded environment within the cell which may lead to off-pathway reactions resulting in protein aggregation (7-9). Protein misfolding can further originate from direct protein damage (e.g. oxidation, thermal denaturation) but can also originate from age-related mutations in the DNA, molecular misreading (10), splicing errors (11) or errors in translation (12;13). While cells are challenged by an accumulation of oxidized, misfolded and aggregation-prone proteins, at the same time the capacity of a cell to deal with accumulated protein damage declines with aging (14-19).

As molecular chaperones, heat shock proteins (HSPs) play a central role in protein homeostasis: they safeguard protein conformation and folding, the assembly and disassembly of protein complexes and protein translocation. By their ability to bind non-native polypeptides, they maintain their clients in a state competent for subsequent folding or, when folding is not successful, for degradation by the ubiquitin-proteasome system (20) or through autophagy (21). Hereby chaperones can prevent toxic protein aggregation and as such they have been implicated as protectors against age-related protein folding diseases (22) and as supporters of healthy aging (2;23-26). Indeed, global activation of all stress-inducible HSPs, either by overexpression of the heat shock factor-1 (HSF-1) (2;25) or via caloric restriction and the accompanying insulin signaling (24) was shown to delay the onset of protein folding diseases and to induce longevity. Interestingly, overexpression of individual members of especially the small heat shock protein family was shown to support longevity both in Caenorhabditis elegans (26) and in Drosophila melanogaster (D. melanogaster) (23;27-29). All these small HSPs tested so far (HSP22, HSP23, HSP26, HSP27) share the capacity to facilitate refolding of stress denatured substrates in vitro (30), again suggesting that supporting protein homeostasis is essential for longevity. Remarkably, however, overexpression of a member of the major HSP70 chaperone family, D. melanogaster HSP70, did not result in longevity and transgenic flies even exhibited lower lifespan and higher mortality rates (31). The HSP70 family comprises a highly homologues group of (HSF-1) inducible proteins and play a rather promiscuous role in protein folding (32;33). Members of the small HSPs are much more heterogeneous (34-37) and were found to have partially overlapping but also non-overlapping functions in protein quality control (37-39). To further elucidate the role of small HSPs in aging, we cloned 10 members of the Drosophila small HSP family and compared their ability to assist in refolding of stress denatured substrates and/or in preventing aggregation of disease associated, misfolded proteins in living cells. Whereas some small HSP members chaperone both substrates, single members were identified that either exclusively assisted in HSP70-dependent refolding of stress denatured proteins or were only able to prevent toxic aggregation of misfolded proteins in an HSP70 independent manner. Therefore our combined cellular and in vivo studies unambiguously demonstrate that longevity can be achieved through separate chaperone activities.

Experimental procedures

Organisms and growth conditions

For cloning purposes and plasmid isolation, Escherichia coli DH5α was used and grown at 37 °C in LB medium (1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl), supplemented with the appropriate antibiotics when required.
Drosophila Schneider’s S2 cells, were cultured in Schneider’s Drosophila medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (Greiner), 100 units/ml penicillin and 100 g/ml streptomycin in T25 flasks at 25 °C. For exponential cell growth, cell density was kept between 3x10^5 and 3x10^6 cells/ml.

Fly stocks (Table S1) were maintained at 22 °C according to standard protocols. GAL4 driver stocks were obtained from the Bloomington Stock Centre (Indiana University, USA) (Table S1). The GMR-UAS-SCA3Q78 fly used for the eye-degeneration screen was generously provided by N. Bonini (University of Pennsylvania, USA) and maintained at 25 °C. Transgenic lines were generated by Genetic Services Inc. by injection of the pUAS vector into the W1118 genetic background.

**Molecular techniques, bioinformatics, and plasmid generation**

Oligonucleotide primers (Biolegio) and plasmids used in this study are listed in Tables S2-4. Standard recombinant DNA techniques were carried out essentially as described by Sambrook et al. (81) Restriction enzymes were used according to the manufacturers instructions (Invitrogen, New England Biolabs). Vent DNA polymerase (New England Biolabs) was used for preparative polymerase chain reactions (PCR). DNA sequencing reactions were carried out by ServiceXS. Blast algorithms (82) were used to screen databases at the National Center for Biotechnology Information (NCBI).

Drosophila small heat shock proteins sequences were retrieved from the NCBI database using the D. melanogaster HSP27 sequence as input for a protein BLAST search. Results were analysed by ClustalX (83) and visualized by Treeview (84). A total of eleven small HSPs was found to be present in the D. melanogaster genome most of which are located at position 67B on the third chromosome (Figure S1a). Nearest neighbour analysis shows three main groups within the D. melanogaster small HSP family (Figure S1b). Group A represents the most studied classical small HSPs: HSP22, HSP23, HSP26, and HSP27 (23;29;30;85-89). Group B consists of L(2) EFL, CG4461, CG7409 and CG14207, while group C contains HSP67BC and CG13133. The Drosophila HSP plasmid library (Figure 1c) was generated using either cDNA originating from heat-shocked flies or from the Gold cDNA library (Bloomington). Primers used for isolation and amplification of individual small HSPs using are listed in Table 2. All PCR products were cloned into the pAc5.1-V5 plasmid and sequence verified. The pAc5.1-V5 was generated by annealing two oligonucleotides forming a KpnI overhang at the 5’ end (Table S2). This fragment was ligated into KpnI-EcoRV digested pAc5.1.

The L4440 RNAi feeding vector (Fire laboratory), containing two opposing T7 sequences, was modified for T/A-cloning as follows. To remove unneeded nucleotides between both T7 sequences, L4440 was digested with BglII and Xhol. Annealed oligonucleotides (Table S2), designed to provide BglII and Xhol overhangs and two internal XcmI sites, were ligated into digested L4440 leading to L4440-T/A. Upon digestion with XcmI, this plasmid contains two T-overhangs, allowing annealing of Taq DNA polymerase amplified DNA fragments (T-overhangs). To generate the dsRNA-template library, a specific part of the HSP70 and HSC70 genes was amplified using primers listed in Table S5 using Taq polymerase. Subsequently, the A-tailed PCR products were ligated into the XcmI digested L4440-T/A plasmid.

**DNA transfection**

Schneider’s S2 cells were either transfected using the CaCl2 method or Effectene (Qiagen). Transfection using the CaCl2 was performed as follows. 2.5 µg of plasmid DNA were mixed with 10 µl 2.5 M CaCl2, the volume was adjusted to 100 µl with 0.1× TE (pH 7.6) and this was added drop-wise to 100 µl 2× HEPES buffer (280 mM NaCl, 1.5 mM Na2HPO4•2H2O, 50 mM HEPES, pH 7.05) while vortexing. Precipitates were allowed to form for 30 min. and then the solution was
added to 1×10^6 cells in a 35-mm dish. Transfection using Effectene was performed according to the manufacturers instructions using 0.6 μg of plasmid DNA in combination with 5μL Effectene. HEK293 cells were transfected using Lipofectamine (Invitrogen) according to the manufacturers instructions using 1μg of plasmid DNA per 35-mm dish.

**RNA Interference**

RNA interference was performed as described previously (90). In short, DNA fragments of variable length coding for specific parts of the target genes (Table S5) were amplified using Taq DNA polymerase. These fragments were cloned into XcmI digested L4440-T/A vector. Using T7-specific primers, the T7 flanked target sequence was re-amplified. Subsequently, dsRNA was generated using the MEGASCRIPT T7 transcription kit (Ambion). S2 cells were diluted to a final concentration of 1x10^6 cells/mL in Drosophila Serum-Free Medium (GIBCO, Paisley, UK). dsRNA was added to the serum-free medium and incubated for 1h at 25 °C, followed by addition of 2 mL complete Schneider’s medium. Part of an intron sequence of the human MAZ gene was used as mock dsRNA. One day after dsRNA transfection, cells were transfected with plasmid DNA. Two days later cells were subjected to either the luciferase refolding assay or the filtertrap assay.

**Quantitative PCR**

Exponentially growing Schneider’s S2 cells were resuspended in complete Schneider’s Drosophila medium to a final concentration of 1x10^6 cells per mL. 5 mL of cell suspension was heat-shocked for the indicated temperature and timepoints using a precision waterbath. Total RNA was isolated using the Invisorb Spin Cell RNA mini kit (Westburg). First-strand cDNA was generated using M-MLV reverse transcriptase (Invitrogen) using oligo(dT)18 primers (Biolegio). Relative changes in transcript level were determined using the Icycler (Bio-Rad) in combination with SYBR green supermix (Bio-Rad). Calculations were performed using the comparative CT method according to User Bulletin 2 (Applied Biosystems) and (91). The PCR efficiencies for all primer pairs (Table S3) were between 85% and 100%. Fold induction was adjusted using RpL32 transcript levels as a standard.

**Luciferase refolding assay**

The luciferase refolding assay was performed for HEK293 cells as previously described (92). The assay was adapted for Schneider’s S2 cells as follows. S2 cells were transfected with the pAc5.1-Luc plasmid, coding for firefly luciferase together with different heat shock protein-coding plasmids in a 1:9 ratio. After two days, cells were resuspended in complete Schneider’s Drosophila medium containing cycloheximide (2 mg / 100 mL). The cell suspension was divided into 400 μL portions in 1.5 ml centrifuge tubes. Tubes were placed into custom-made acrylic glass racks (50 positions) which allowed continuous water-flow around the tubes when placed in a waterbath. After heat-treatment, the tubes were cooled down rapidly by placing them in a 25 °C waterbath followed by incubation in a 25 °C incubator. Cell lyses and luciferase measurements were performed as described (92). The experiments were performed and measured in triplicate.

**Biochemical techniques**

SDS–PAGE and Western blotting were performed by established procedures. Primary antibodies used were monoclonal anti-EGFP (Clontech) and monoclonal anti-V5 (Invitrogen) according to the manufacturers instruction. The filtertrap binding assay was performed as described previously (39).
**Lifespan analysis**
For lifespan analysis, 100-250 male flies were selected per line and maintained at non-crowding conditions at 22 °C. Dead flies were counted three times per week followed by transfer to fresh media.

**Results**

**Heat-inducibility of the Drosophila small HSPs**
Certain members of all major HSP families are known to be induced upon proteotoxic stresses, including heat-shock and exposure to heavy metals (40). This enhanced expression reflects the need of additional chaperones when protein homeostasis is compromised and has to be restored to ensure cell survival. To analyse which of the *D. melanogaster* small HSPs are heat-inducible, we heat-shocked *Drosophila* S2 cells at 40 °C for 15 minutes and analysed the small HSP mRNA levels by QPCR at various time points after heating. As controls, we measured the mRNA levels of HSP70AA, a highly heat-inducible gene, and HSC70-4 and HSC70-5, two

![Figure 1. D. melanogaster small HSP family and heat inducibility.](image-url)

Transcript levels of HSP70/HS70 (A), the classical *D. melanogaster* small HSP genes (B) and the novel small HSP members (C) were analysed directly after heat shock and one and two hours after heat shock using QPCR. Relative mRNA abundance before and after heat shock are depicted in panel D. See Appendix 4 for colour print.
constitutively expressed genes not responsive to heat-shock (41;42) (Figure 1a). The four classical small HSPs (HSP22, HSP23, HSP26, HSP27) were all found to be highly induced after a heat-shock (Figure 1b), consistent with previous findings (43;44). Apart from CG14207, all other small HSPs were also found to be heat-inducible (Figure 1c). The most strongly induced members were HSP67BC, L(2)EFL and CG4461 while HSP67BA, CG7409 and CG13133 were moderately induced (Figure 1c). Thus, CG14207 is the only Drosophila small HSP that is not responsive to heat shock.

QPCR data were subsequently used to estimate the relative contribution of each member to the total small HSP mRNA levels before and after heat shock. In S2 cells, the most abundantly constitutively expressed small HSPs are HSP23 (50% of the total pool), HSP26 (12%), HSP27 (12%) and CG14207 (25%) (Figure 1d). After heat shock HSP27 mRNA levels show the largest increase, and together with HSP23 and HSP26 constitute the predominant small HSP messengers (Figure 1d). From the less abundantly expressed group of small HSPs (HSP22, HSP67BC, CG4461, CG7409), HSP67BC and CG4461 become the most abundant messengers after heat shock treatment (Figure 1d).

**CG14207 and CG7409 are the most active members in assisting refolding of heat denatured luciferase**

It has been shown for several small HSPs that they can maintain substrates in a folding competent form both *in vitro* and *in vivo* (45-47). *In vitro*, the addition of the HSP70/HSP40 refolding machinery is required for the refolding reaction (48). This has been reproduced in living cells by using luciferase as a substrate (49;50). Here we tailored the cellular luciferase refolding assay for Drosophila S2 cells and characterized which Drosophila small HSPs could enhance luciferase refolding. The mitochondrial HSP22 was excluded from our analyses since our cellular assays were only tailored for the cytosolic and nuclear compartments. Consistent with *in vitro* data (30), overexpression of the classical small HSPs (HSP23, HSP26 and HSP27) increased luciferase refolding (Figure 2). Although less efficient, overexpression of L(2)EFL also led to improved luciferase refolding whereas HSP67BA, HSP67BC, CG4461 and CG13133 had no effect. Interestingly, overexpression of CG7409 and the non-heat shock inducible CG14207, resulted in the highest level of refolding 1 hour after heat-shock (Figure 2).

To analyse whether *D. melanogaster* small HSPs, like bacterial, plant and mammalian small HSPs (47;49;51) also require HSP70 machines for refolding, we first tested which of the *D. melanogaster* HSP70s could promote refolding of heat-denatured luciferase. Hereeto, we cloned a selection of the *D. melanogaster* HSP70 family (Supplemental Table 2) and analysed their effect on luciferase refolding. Overexpression of both *D. melanogaster* HSC70-2 and HSC70-4, but not HSC70-1, enhanced luciferase refolding in S2 cells (Figure 3a). This suggests that HSC70-1 is not required for full luciferase refolding activity. Overexpression of both HSC70-2 and HSC70-4 increased luciferase refolding by approximately 40% compared to control, while HSC70-1 increased luciferase refolding by only 10% compared to control. These results suggest that HSC70-1 is not required for full luciferase refolding activity. Overexpression of both HSC70-2 and HSC70-4 increased luciferase refolding by approximately 40% compared to control, while HSC70-1 increased luciferase refolding by only 10% compared to control. These results suggest that HSC70-1 is not required for full luciferase refolding activity. Overexpression of both HSC70-2 and HSC70-4 increased luciferase refolding by approximately 40% compared to control, while HSC70-1 increased luciferase refolding by only 10% compared to control. These results suggest that HSC70-1 is not required for full luciferase refolding activity.
either lacks the ability to assist in refolding heat denatured luciferase or that specific co-factors that are required for its activity are rate-limiting under the conditions of HSC70-1 overexpression. Subsequently, we down-regulated the individual D. melanogaster HSP70s using dsRNA molecules (Supplemental Table 5). Transfection of mock dsRNA did not affect luciferase refolding while luciferase refolding was partially inhibited by the down regulation of D. melanogaster HSP70, HSC70-1 or HSC70-2. (Figure 3b). This confirmed the role of HSP70 members in substrate refolding in S2 cells and showed that endogenous HSC70-1 does play a role in refolding of heat-denatured luciferase under normal conditions. Next, we combined overexpression of D. melanogaster HSP27 and CG14207 with down regulation of D. melanogaster HSP70 members (Figure 3c,d). Refolding of luciferase in the presence of both D. melanogaster HSP27 and CG14207 was considerably reduced by down regulating either D. melanogaster HSP70AA, HSC70-1, HSC70-2, or HSC70-4 (Figure 3c,d). Thus our results demonstrate that the refolding capacity of D. melanogaster HSP27 and CG14207 requires an intact HSP70 machine.

**HSP67BC is the most potent suppressor of polyglutamine aggregation**

Several protein folding diseases are characterized by the formation of toxic aggregates like Alzheimer’s disease, Huntington’s disease and amyotrophic lateral sclerosis. Some small HSPs have been reported to suppress the aggregation of such misfolded proteins (52;53) and we have recently found that this is not always related to their capacity to refold heat-denatured luciferase (Vos et al., in preparation). To test which of the *Drosophila* small HSPs could suppress aggregation of misfolded proteins, we co-expressed an EGFP tagged Huntingtin exon-1 containing
119 glutamines (EGFP-HDQ119) with the various *D. melanogaster* small HSPs in S2 cells. The HSP40 member MRJ, previously identified in a screen for suppressors of polyQ toxicity in flies (54) was used as a positive control and indeed also completely inhibited aggregate formation in S2 cells as demonstrated using filter trap binding (Figure 4a). While most small HSPs show only small effects on aggregate formation, HSP67BC was very active in preventing polyQ aggregation (Figure 4a). Interestingly, this small HSP did not support luciferase refolding in *Drosophila* S2 cells (Figure 2a). Inversely, the best supporter of protein refolding after heat denaturation (CG14207) did not significantly suppress polyQ aggregation. Except for CG4461, all small HSPs were expressed to equal levels (Figure 4b).

We next tested whether HSP67BC requires a functional HSP70 machine to prevent polyQ aggregation. RNAi against HSP70AA and HSC70-4, that resulted in the inhibition of the refolding promoting activity of CG14207 (Figure 3d), did not lead to an increase in polyQ aggregation (Figure 4c), indicating that the protective effect of HSP67BC against polyQ aggregation does not depend on HSP70 activity.

Our findings clearly demonstrate that HSP70-independent prevention of protein aggregation and HSP70-dependent stimulation of protein refolding are two different capacities that can be separated.

**In vivo effects of small HSPs on polyQ toxicity and longevity**
To determine whether the different capacities of small HSPs to either prevent protein aggregation or to assist in HSP70-dependent protein refolding have an impact on polyQ-induced eye-degeneration and longevity \textit{in vivo}, we next selected CG14207 (strong refolder), HSP67BC (strong anti-aggregation), CG7409 (strong refolder and minor anti-aggregation) and CG4461 (no effect). Transgenic lines were generated (Table S1) and expression of the proteins of interest was confirmed by Western blot analysis of extracts of pUAS containing flies crossed with flies containing the actin-GAL4 driver (Figure 5a). Adverse effects of small HSP overexpression were not observed. Multiple transgenic lines per small HSP were maintained and analysed, thereby consolidating obtained results. In addition, we used RNAi lines (Table S1) targeting the four small HSPs.

Figure 5. The effect of overexpression of the \textit{D. melanogaster} small HSP \textit{in vivo} on reducing polyQ toxicity. (A) Expression levels of the V5 tagged small HSPs were examined in the individual small HSP transgenic lines with actin-GAL4 driven transgene expression. Male flies were prepared in Laemmli buffer (94) and analysed by Western blotting followed by staining with a V5 antibody. (B-E) Ataxin-3 induced eye-degeneration in flies expressing Ataxin-3 alone (SCA3-Q74) or together with (B) HSP67BC overexpression (L1, L2, L3) or HSP67BC downregulation (RNAi#1, RNAi#2) or with (C) CG7409 overexpression (L1, L2, L3) or CG7409 downregulation (RNAi) or with (D) CG14207 overexpression (L1, L2, L3, L4) or CG14207 downregulation (RNAi#1, RNAi#2) or with (E) CG4461 overexpression (L1, L2, L3) or CG4461 downregulation (RNAi#1, RNAi#2). In each panel, representative eye phenotypes are depicted for the indicated fly lines (samples for all fly lines are depicted in figure S3 and S4). Eye degeneration was quantified by microscopic classification into rough, patched, speckles or collapsed phenotypes (see also figure S2). The more severe phenotypes (patched, speckles, collapsed) were expressed relatively to the rough phenotype and set to 100% for the SCA3-Q74 expressing flies. Levels of significance (**P<0.001; *p<0.05) were determined using the Chi-square test. The numbers of flies analysed for each fly line are: SCA3-Q78 n=290, HSP67BC-L1 n=74, HSP67BC-L2 n=29, HSP67BC-L3 n=60, CG7409-L1 n=53, CG7409-L2 n=49, CG7409-L3 n=59, CG14207-L1 n=96, CG14207-L2 n=36, CG14207-L3 n=66, CG14207-L4 n=74, CG4461-L1 n=57, CG4461-L2 n=47, CG4461-L3 n=37, CG4461-L4 n=78, RNAi HSP67BC 1 n=14, RNAi HSP67BC 2 n=33, RNAi CG7409 n=145, RNAi CG14207 1 n=48, RNAi CG14207 2 n=218, RNAi CG4461 1 n=43, RNAi CG4461 2 n=40.

To determine whether the different capacities of small HSPs to either prevent protein aggregation or to assist in HSP70-dependent protein refolding have an impact on polyQ induced eye-degeneration and longevity \textit{in vivo}, we next selected CG14207 (strong refolder), HSP67BC (strong anti-aggregation), CG7409 (strong refolder and minor anti-aggregation) and CG4461 (no effect). Transgenic lines were generated (Table S1) and expression of the proteins of interest was confirmed by Western blot analysis of extracts of pUAS containing flies crossed with flies containing the actin-GAL4 driver (Figure 5a). Adverse effects of small HSP overexpression were not observed. Multiple transgenic lines per small HSP were maintained and analysed, thereby consolidating obtained results. In addition, we used RNAi lines (Table S1) targeting the four small HSPs.
As a model for polyQ diseases, we employed the Ataxin-3 fly-model (55). This fly-model expresses the Ataxin-3 gene with 78 CAG repeats under the control of the UAS/gmr-GAL4 expression system (56), resulting in eye-specific expression. Cryo-electron microscopy showed degeneration of the individual hexagonal ommatidia from flies expressing the SCA3-Q78, which was not observed in wild type flies (Figure S2a). This degeneration was also readily visible by light microscopy and used to score eye degeneration (Figure S2b). Transgenic lines overexpressing HSP67BC showed a significant reduction in eye degeneration (Figure 5b, S3b). Inversely, the two RNAi lines for HSP67BC drastically aggravated the eye degeneration (Figure 5b, S4a). This is entirely in line with the observation that HSP67BC can suppress the aggregation of polyglutamine containing proteins in cells. Interestingly, also transgenic flies overexpressing CG7409 showed a small but significant reduction in eye degeneration (Figure 5c, S3d). This small HSP had only a marginal effect on polyQ aggregation in cells (Figure 4a). Down regulation did, however, not worsen eye-degeneration (Figure 5c, S4c). Transgenic lines with either up- or down-regulated CG14207, which is not able to prevent polyQ aggregation in cells (Figure 4a), also did not show a changes in the level of eye-degeneration in flies, except for one transgenic line (UAS-CG14207-L1) (Figure 5d, S3e, S4d). Overexpression or down regulation of CG4461 had no effect in vivo (Figure 5e, S3c, S4b), which is consistent with its lack of any chaperone-like activity in cells (Figure 2,4a). In conclusion, the in vivo data suggest that only those small HSP members that show activity to inhibit poly-Q aggregation in cells (HSP67BC, CG7409) are also effective in preventing SCA3-Q78 mediated eye degeneration in vivo.

Previous studies have shown that overexpression of HSP23, HSP26 and HSP27 can extend lifespan in flies (27-29). In this study, we show that these proteins have intermediate activities both in assisting refolding of stress denatured substrates and in preventing aggregation of disease associated, misfolded proteins. Moreover, we have demonstrated that these two functional properties can be separated and can be found in individual small HSPs. To more specifically ask which one of these chaperone activities is the most enviable in supporting longevity, we determined lifespan of flies overexpressing CG14207 (strong refolder), HSP67BC (strong anti-aggregation), CG7409 (strong refolder and intermediate anti-aggregation) or CG4461 (no effect) using elav-GAL4 or ey-GAL4. Elav-GAL4 drives expression in the central and peripheral nervous systems (57), while ey-GAL4 was used to drive ubiquitous expression throughout development and adulthood (FlyAtlas http://www.flyatlas.org). All control lines (W1118, elav-GAL4, ey-GAL4) showed comparable aging kinetics (Figure 6a), meaning that the drivers had not integrated into areas affecting normal lifespan. From the UAS transgenic lines, only UAS-CG7409-L2 and UAS-HSP67BC-L2 showed a small trend towards a shorter lifespan (Figure 6b). Expression of HSP67BC by elav-GAL4 resulted in a 5,8% mean lifespan increase (Figure 6c, 7), while expression by ey-GAL4 resulted in a 16,3% mean lifespan increase (Figure 6c, 7). Elav-GAL4 driven expression of CG7409, did not change the mean lifespan of the flies (Figure 6d, 7). However, when expressed under the control of ey-GAL4 a 16,3% increase in the mean lifespan was observed (Figure 6d, 7). The largest effect on mean lifespan was achieved by overexpression of CG14207. This led to a 17,8% and 20,4% mean lifespan increase by elav-GAL4 or ey-GAL4 driven expression respectively (Figure 6e, 7). Flies expressing CG4461 did not show any beneficial effects on mean lifespan (Figure 6f, 7). Thus, overexpression of small HSPs with chaperone activities either on heat unfolded proteins and/or on preventing polyQ aggregation were both able to extend the mean lifespan of flies.

**Discussion**

For the first time various *Drosophila* small HSPs were functionally compared for heat-inducibility, their ability to assist protein refolding and their ability to prevent polyQ aggregation. Next, we have linked these activities to in vivo effects on poly-Q mediated neurodegeneration and longev-
Figure 6. The effect of overexpression of the *D. melanogaster* small HSP in vivo on lifespan. Cohorts of 100-250 male flies were analysed for their lifespan. Every two days, dead flies were counted, and the percentage of flies alive at each time point is plotted. (A) Wiltype (W1118) and both driver lines (elav-GAL4, ey-GAL4). As they differ only marginally in their lifespan characteristics, the data from these lines was combined to plot the control lifespan curve in the panels C-F. (B) All UAS-sHSP carrying transgenic lines without GAL4 induced transgene expression. Except for line UAS-HSP67BC-2 and line UAS-CG7409-L2, these lines show comparable lifespan characteristics. (C) Effect of overexpression of HSP67BC using the elav-GAL4 (left-hand panels) or ey-GAL4 (right-hand panels) driver in 2 fly-lines. The individual lines are depicted separately as they were analysed in two individual lifespan experiments.
Figure 6. Continued

(D) Effect of overexpression of CG7409 using the elav-GAL4 (left-hand panel) or ey-GAL4 (right-hand panel) (E) Effect of overexpression of CG14207 using the elav-GAL4 (left-hand panel) or ey-GAL4 (right-hand panel) (F) Effect of overexpression of CG4461 using the elav-GAL4 (left-hand panel) or ey-GAL4 (right-hand panel). See Appendix 4 for colour print.
Intriguingly, we identified two small HSPs that were not previously studied, which exclusively supported either refolding (CG14207) or prevented aggregation (HSP67BC). CG14207 was the only small HSPs that was not heat-inducible and in addition depended on HSP70 for its refolding activity. HSP67BC, on the other hand, was clearly heat-inducible, showed strongest but HSP70 independent activity in preventing polyQ aggregation, while its overexpression did not increase the cellular ability to refold heat-denatured luciferase. Although diverse in action, both small HSPs were shown to increase lifespan in various degrees.

Classical HSP70 activity & anti-aggregation
Chaperone-like actions of small HSPs have generally been suggested to depend on HSP70 activity. In protein refolding assays in vitro (58-60), aggregation prevention by the ATP-independent small HSPs has been shown to occur independent of the ATP-dependent HSP70 machinery, but for efficient refolding, substrate transfer to the HSP70 machine is required (47;51). Also, in living cells, refolding assistance by HSPB1 was found to depend on a functional HSP70 machinery (49) and in this study, a similar scenario was found to be true for the best refolding stimulating D. melanogaster small HSP; CG14207. Strikingly, CG14207 was in effective in preventing polyQ aggregation, while its overexpression did not increase the cellular ability to refold heat-denatured luciferase. Although diverse in action, both small HSPs were shown to increase lifespan in various degrees.
binding and release which may slightly delay aggregation but not prevent it. Indeed, in cells overexpression of HSP70s only marginally affects polyQ aggregation (61;62) whereas in vivo HSP70 mediated rescue of polyQ toxicity only occurs in the presence of semi-soluble nuclear polyQ aggregates (63-65). So, the different small HSPs with different affinities to substrates and HSP70s may have evolved to serve adequate processing of a broad spectrum of clients. The low HSP70 and high substrate affinity may serve to prevent aggregation of unfoldable substrates and provide a longer time window for ubiquitination and normal proteasomal turnover and/or autophagic processing.

**In vivo anti-aggregation**

Using the *in vivo* Ataxin-3 fly model, we could show that the cellular data nicely predict the effects of the small HSP actions in terms of preventing poly-Q aggregation. The transgenic lines for CG7409 and especially HSP67BC showed reduced eye-degeneration. Inversely, the lines transgenic for CG14207 and CG4461, being ineffective in cells, also did not show reduced Ataxin-3 induced eye-degeneration, except for one line (line UAS-CG14207-L1). Strikingly, CG14207 has recently been reported as a modifier of Ataxin-3 neurodegeneration also using eye-degeneration as endpoint (55). However, it was shown that CG14207 did not directly interfere with aggregate formation (55) consistent with our cellular data. As we only find a similar effect in one out of the four CG14207 transgenic lines tested, the combined data suggest that CG14207 may not only prevent degeneration by a non-direct, compensatory mechanism but also depend on other factors (see also below).

**Inducing longevity**

Several screens have been performed as an attempt to elucidate the molecular events underlying aging (66;67). This has revealed changed expression of several genes involved in many cellular pathways, including members of heat shock protein families. Instead of looking at physiologically-induced aging effects on gene expression, we tried to determine which molecular functions of small HSPs may contribute to enhancing lifespan. By screening the *D. melanogaster* small HSP family, we were able to show for the first time that longevity can be the result of two different chaperone-like activities; HSP70 dependent assistance of (re)folding reactions (CG14207) and HSP70 independent prevention of polyQ aggregation (HSP67BC). This is in line with the theory that overall protein homeostasis is important for healthy aging (68). Whereas previous data have demonstrated a role of other members of the *D. melanogaster* small HSP family protein refolding (30), we now show that these proteins have partial activities on the two substrates investigated here. Furthermore, other studies have already pointed out that a multitude of molecular chaperones of which the expression is regulated via HSF-1, a transcriptional regulator of stress-inducible gene expression, is vital in both the protection against protein folding diseases and aging (25;69;70). We show that overexpressing CG14207, a non HSF-1 regulated gene, can also provide longevity. This provides a means to boost longevity and avoid the adverse effects that HSF-1 activation and down-stream events may have in the development of cancer (71-73).

It is rather surprising that especially small HSPs have been reported to induce longevity, while overexpression of HSP70 members, including *D. melanogaster* HSP70, one of the most strongly induced HSF targets (41) has no effect on lifespan (31). Interestingly, we show that overexpression of this member did not result in increased refolding activity (Figure 3a) and also did not affect polyQ aggregation (data not show). Yet, this HSP70 member does contribute to endogenous and small HSP-mediated refolding (Figure 3b,c,d) and deletion of *D. melanogaster* HSP70 genomic copies does translate in higher levels of neurotoxicity in flies (74). This suggests that a lack of co-factors, when HSP70 is overexpressed, may explain why it does not
contribute to longevity in *Drosophila*. Alternatively, HSP70 overexpression may have deleterious side-effects; in flies HSP70 overexpression has been associated with reduced growth and development (75) and developmental abnormalities (76) that are not seen upon overexpression of small HSPs. In fact, HSP70 overexpression may allow stabilization of a greater variety of metastable proteins (2;77) than is the case at physiological levels. This could mask hidden mutations, allowing malformation effects. Physiological activity in buffering of genetic variation and masking hidden mutations has also been shown to be the case for another ATP dependent chaperone HSP90 (78;79). In view of this, employing small HSP activity to deal with adverse affects of protein misfolding seems rather a safer choice.

Improving protein homeostasis has also been suggested to lead to resistance to age related protein folding diseases such as polyQ related diseases (68). So, why does a general increase in protein homeostasis mediated by CG14207 that does lead to longevity increases, not also delays Ataxin-3 mediated eye degeneration? Our data suggest that dealing with high polyQ overexpression, produced within a short time both in cells and in the Ataxin-3 flymodel, is only effective by chaperones capable of modifying aggregate formation directly. Dealing with polyQ induced misfolding of meta-stable proteins (80) by CG14207 seems not effective enough to resist polyQ toxicity. However, at normal physiological circumstances either dealing with polyQ aggregation itself or dealing with the side-effects, might turn out to be equally effective in resisting neurodegeneration.

**Acknowledgements**

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**References**

Small heat shock proteins & longevity


Chapter 5


Small heat shock proteins & longevity

### Table S2 Primers used for molecular cloning

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### Table S4 Primers used for molecular cloning

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General

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Small heat shock proteins & longevity
Table S5 Primers used for the generation of dsRNA and specificities of the dsRNA sequences

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Transgenic lines

- UAS-HSP67BC-RNAi Obtained from VDRC 322 HSP27 0 1 6
- UAS-CG4461-RNAi Obtained from VDRC 284 none
- UAS-CG7409-RNAi Obtained from VDRC 306 none
- UAS-CG14207-RNAi Obtained from VDRC 340 none

* This pool of siRNA’s targets both the HSP70A and HSP70B members.

On-target and off-target siRNA’s were determined using dsCheck located at http://dscheck.mai.jp

Figure S1. The Drosophila family of small heat shock proteins. (A) The Drosophila small HSP family consisting of eleven individual members. (B) Bootstrap analysis (bootstrap value: 10000) shows three main groups within the small HSP family, (I) HSP22, HSP23, HSP26 and HSP27; (II) CG7409, CG4461, L(2)EFL and CG14207; (III) HSP67BC and CG13133. (C) Molecular cloning of the Drosophila small HSP plasmid library was performed using the pAc5.1 vector and derivatives thereof containing either no tag, a V5 tag or EGFP tag.
a visible disturbed eye morphology and degeneration that can be categorized into several morphological stages: rough and the more severe degenerative phenotypes referred to as patched, speckles, collapsed. Pictures indicate representative examples of either phenotype. The effect of small HSP expression on eye degeneration was determined by variation in the amount of patched, speckles and collapsed eyes. The amount of patched, speckles and collapsed eyes were expressed relatively to the amount of rough eyes and set to 100% for the SCA3-Q74 expressing flies. Flies with either sHSP overexpression or dsRNA mediated sHSP downregulation were scaled accordingly.

**Figure S2. Scoring system for eye-degeneration.** The efficacy of HSP67BC, CG4461, CG7409 and CG14207 on ameliorating polyQ toxicity was determined using the Ataxin-3 fly model (ATX3-trQ78) (55). (A) Cryo-electron microscopy clarifies the nature of the SCA3 eye-degeneration. Wildtype eyes show a symmetrical alignment of smooth hexagonal shaped ommatidia, whereas overexpression of SCA3-trQ78 leads to loss of the hexagonal structure and smooth surface. (B) The UAS-SCA3-trQ78, without SCA3 expression, shows normal eye morphology which is also the case for the gmr-GAL4 activator line. Gmr-GAL4 driven expression of SCA3-trQ78 in the eyes results in a visible disturbed eye morphology and degeneration that can be categorized into several morphological stages: rough and the more severe degenerative phenotypes referred to as patched, speckles, collapsed. Pictures indicate representative examples of either phenotype. The effect of small HSP expression on eye degeneration was determined by variation in the amount of patched, speckles and collapsed eyes. The amount of patched, speckles and collapsed eyes were expressed relatively to the amount of rough eyes and set to 100% for the SCA3-Q74 expressing flies. Flies with either sHSP overexpression or dsRNA mediated sHSP downregulation were scaled accordingly.

**Figure S3. Overview of eye phenotypes upon overexpression of small HSPs.** (A) The SCA3-trQ78 expressing flies show various degrees of eye-degeneration. Representative examples of eye morphology are shown for co-expression of (B) HSP67BC, (C) CG4461, (D) CG7409 or (E) CG14207. In each panel the top row indicated morphology without SC3-trQ78 expression (L(x)) while the bottom row indicates morphology with co-expression of small HSPs and SCA3-trQ78 (L(x) x SCA3).
Figure S3. Continued.

Figure S4. Overview of eye phenotypes upon down regulation of small HSPs. Representative examples of eye morphology are shown for dsRNA mediated downregulation of (A) HSP67BC, (B) CG4461, (C) CG7409 or (D) CG14207. In each panel the top row indicated morphology without SC3-trQ78 expression (RNAi#(x)) while the bottom row indicates morphology with downregulation of small HSPs and SCA3-trQ78 overexpression (RNAi#(x) x SCA3).