Cell-autonomous and non-cell autonomous protection of DNAJB6 in Huntington’s disease

Bason, Matteo

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CHAPTER 4

Neuronal expression of the chaperone DNAJB6 results in cell autonomous protection in Huntington’s disease

ABSTRACT

Neurodegenerative diseases (NDs) like Huntington’s (HD) and Spinocerebellar Ataxia type 3 (SCA-3) are characterized by protein aggregation in brain cells, due to the abnormal elongation of a Polyglutamine (PolyQ) repeat in the mutated protein. Aggregation of PolyQ huntingtin (HTT) or PolyQ ataxin-3 (ATXN3) is believed to be the main driver of pathology and degeneration. Heat Shock Proteins (HSPs) are regulators of the protein quality control system and are thought to be protective against the aggregation of disease-related proteins and thereby capable to delay the onset of these NDs. DNAJB6 is a human HSP of the DNAJ family and HSP70 co-chaperone that has been identified as possessing potent anti-PolyQ aggregation properties in different in vitro and in vivo PolyQ disease models. Here, we demonstrate that the protective cell autonomous function of DNAJB6 is conserved in a D. melanogaster ommatidia models of both HD and SCA3, and that this activity is generic for the core PolyQ-expansion of the protein. Next, we show that the selective neuronal expression of human DNAJB6 expands lifespan, reduces aggregate formation and improves overall neuronal fitness in a pan-neuronal PolyQ Htt D. melanogaster model, supporting that DNAJB6 is a potent suppressors of PolyQ aggregation within the human chaperonome and providing further evidence for the cell-autonomous protective role of this DNAJ in PolyQ diseases.

KEYWORDS

Neurodegeneration - Polyglutamine - Aggregation - Neurons - Chaperones - DNAJB6
1. Introduction

Huntington’s disease (HD, OMIM:#143100) and spinocerebellar ataxia type 3 (SCA-3, OMIM:#109150) are neurodegenerative diseases characterized by an abnormal trinucleotide CAG-expansion in a specific gene. The encoded mutant protein - huntingtin (Htt) in HD and ataxin-3 (ATXN3) in SCA-3 - contains an elongated polyglutamine (PolyQ) stretch and forms aggregates that are considered to be the main driver of neuronal degeneration and pathology. The length of the PolyQ expansion is linked with the propensity of the protein to aggregate and it is inversely correlated to the age of onset, suggesting that aggregate formation is the main driver of disease (Chapter 2, section 2.3).

PolyQ aggregates can exert their toxicity through different mechanisms, which mainly depend on the cellular localization of these toxic species and their capacity to interact and interfere with the normal functionality of different cellular components, including proteins and organelles. All together such mechanisms contribute to the functional impairment of neurons and ultimately to their degeneration. Damage of specific population of neurons in HD (i.e. medium spiny neurons in striatum and cortical neurons) and SCA-3 (i.e. neurons in brainstem and cerebellum) corresponds to the insurgence of specific symptoms in patients, including cognitive impairment and motor disabilities (i.e. chorea in HD) (Chapter 2, section 2.3 - 2.4).

Molecular chaperones, also known as Heat Shock Proteins (HSPs), play a key role in protein homeostasis (Hartl et al., 2011) and they are known to exert a protective cell-autonomous function against aggregate toxicity, hence neurodegenerative diseases characterized by protein aggregation (Hageman et al., 2011; Vos et al., 2010; Hageman et al., 2010; Kakkar et al., 2016b).

Several chaperones - defined here as canonical HSP - are up-regulated upon stress (i.e. including conditions which are known to drive protein aggregation) during a process referred as heat shock response (HSR), which is controlled by the transcription factor heat shock transcription factor-1 (HSF-1). In animal model of PolyQ diseases, the activation of HSR (Labbadia et al., 2011) or the transgenic overexpression of specific canonical HSP such as HSPA1A/HSP70 (Hansson et al., 2003; Hay et al., 2004) and HSPB1/HSP27 (Zourlidou et al., 2007) have generally not been found to delay disease onset or reduce aggregate formation. Also, the expression of PolyQ proteins does not activate the HSR (Seidel et al., 2012). These findings suggest that canonical HSP are ineffective in PolyQ diseases.

In a screen for PolyQ modifiers, our group identified two non-canonical HSP, close homologs of the DNAJ/HSP40 chaperone family, called DNAJB6 (expressed in several tissues, including the brain) and DNAJB8 (expressed in testis), that are not significantly activated by HSF-1 (Hageman et al., 2010). We found that DNAJB6 (the short nuclear and cytosolic isoform B; Hanai et al., 2003) is a very efficient suppressors of PolyQ aggregation in free-cell, cellular and animal models (Hageman et al., 2010, Gillis et al., 2013, Mansson et al., 2014, Kakkar et al., 2016). Notably, the suppression of PolyQ aggregation mediated by DNAJB6 is protective in cells (Hageman et al., 2010) and delays disease onset and significantly prolong lifespan in a mice model of PolyQ disease (Kakkar et al., 2016).
Here, we investigated whether the cell-autonomous protective function of human DNAJB6 against PolyQ aggregation was also maintained in *D. melanogaster* models of HD and SCA-3 (Chapter 3 and this Chapter). By using *D. melanogaster*, we confirmed and recapitulated the previous findings that DNAJB6 is protective against PolyQ aggregate toxicity *in vivo*, through the suppression of PolyQ Htt aggregation, resulting in a significant expansion of the lifespan and improved neuronal fitness *in vivo*. Moreover, these findings set the base to further investigate the non-cell autonomous protective function of DNAJB6 (Chapter 5). As previously explained, the use of the *D. melanogaster* model generated with the attP-site specific PhiC31 integrase system, allowed to compare how differently DNAJB6 can exert a protective function against Poly Htt toxicity in a cell-autonomous (data in this Chapter) or non-cell autonomous (data in Chapter 5) manner.

### 2. Materials and methods

**Vectors**

UAS/LexO vectors were obtained by cloning the sequences of HttQ100-mRFP (Prof. T. Littleton Group, MIT) or V5-DNAJB6 (isoform B) or eGFP (Clontech) in the multiple cloning site of pUAS *attB* or pLexO *attB* (Prof. K. Basler Group, UZH). Driver (Promoter cell-specific expression) vectors were obtained starting from the backbone of plasmids pDPP-Gal4 *attB* or pDPP-LG *attB* or pDPP-LhG *attB* (Prof. K. Basler Group, UZH). DPP promoter was substituted with the sequence of promoter *elav* (pan-neuronal, from pElav-Casper vector, Prof. Liquan Luo, Stanford University). All obtained vectors were sequenced. See table T1 of Chapter 3 for vectors list.

**Generation of new *D. melanogaster* lines**

The *D. melanogaster* lines of table T1 were obtained by injection and transformation of embryos with the above mentioned *attB* vectors, based on *attP*-site specific PhiC31 integrase system, by Best Gene Inc. injection service ([https://www.thebestgene.com/HomePage.do](https://www.thebestgene.com/HomePage.do)). *D. melanogaster* lines from Bloomington Drosophila Stock Center were also used: *gmr-Gal4* (Line BDSC #8121 in Fig.1); *gmr-Gal4* (Line BDSC #1104 in Fig.2); *alrm-Gal4* (Line BDSC #67031 in Fig.2); UAS-mCD8-GFP (Line BDSC #5130); UAS-mCD8-RFP (Line BDSC #27391); UAS-ATXN3-Q78 (Line BDSC #8150 in Fig.2); *gmr-QF2* (Line BDSC #59283 in Fig.2 was a gift from C. Potter, Baltimore, MD, U.S.A.). *gmr-QF2* and QUAS-ATXN3-Q78 are based on the Q expression system in *D. melanogaster* (Potter et al., 2010). All the lines were isogenised to remove background mutations by backcrossing each of them for 6 generations with the control stock w^{1118} line. See Chapter 3 for other details.
Genotypes

- Fig. 1: w(-); UAS HttQ100-mRFP / + (or UAS DNAJB6); gmr Gal4 / UAS mCD8-GFP (or +).
- Fig. 2: w(-); gmr-Gal4:UAS ATXN3-Q78/UAS DNAJB6 (or +); +/-.
- Fig.3A, S1A: w(-); UAS HttQ100-mRFP (or UAS eGFP) / +/-; elav Gal4/+.
- Fig 4B and S2D: 1) Control line (red): w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO eGFP. 2) Rescued line (blue): w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO DNAJB6.
- Fig. 5A-B and S4C-D: 1) Control line (red): w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO eGFP. 2) Rescued line (blue): w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO DNAJB6.
- Fig.S4A-B: w(-); UAS HttQ100-mRFP (or +) / +/-; elav Gal4/+.

Antibodies and reagents

Antibodies (dilutions are indicated in brackets for western blots (WB) and immunofluorescence (IF)) against huntingtin (Chemicon, MAB2166, WB 1:5000), eGFP (Clontech-Living Colours, cat.No.632375, WB 1:5000), α-tubulin (Sigma Aldrich, clone DM1A, WB 1:2000), V5 epitope tag in DNAJB6b (Thermo Fisher Scientific, cat. No.R960-25, WB 1:2000, IF 1:50), NC-82 (DSHB, WB 1:5000) were used. DAPI for nuclei staining (cat.No.D1306) was from Thermo Fisher Scientific. 20% SDS Solution (cat.No.1610418) was from BioRad. PBS components (NaCl cat.No.S9888, KCl cat.No.P9541, Na2HPO4 cat.No.255793, KH2PO4 cat.No.V000225), Tween-20 (cat.No.P2287), Triton X-100 (cat.No.T8787), Bovine Serum Albumin (cat.No.A2058, BSA), glycerol (cat.No.G5516), 3.7% Formaldehyde (cat.No.11-0705 SAJ), Tris base (cat.No.T1503) and β-mercaptoethanol (cat.No.M6250) were from Sigma Aldrich.

D. melanogaster stocks maintenance

All stocks and experimental flies were kept in polystyrene vials 25x95 mm filled with 8 ml/vial of solidified media (17 g/l Agar; 26 g/l Yeast; 54 g/l Sugar; 1.3 mg/l Nipagin). All experimental flies were maintained in a humidified and temperature controlled incubator at 25 °C on a 12 hours’ light and 12 hours’ dark cycle (Premium ICH Insect Chamber, Snijders Labs). Experimental flies, anesthetized on a CO2 pad, were selected according to their gender and phenotype by light microscope visualization.

Lifespan curves

Parental flies (5-6 females and 5-6 males) were kept in vial for 3 days and then removed. Offspring virgin flies were collected in the same 24 hours. For each analysed group, ≈100 flies of specific gender and phenotype were collected and kept in new vials (10 flies/vial). Flies were transferred to new vials containing fresh medium every 2 days and deaths were scored daily. Statistical significance of curves differences analysed with Log rank (Mantel-Cox) test (test 1) and Gehan-Breslow-Wilcoxon test (test 2) using Graph Pad Prism Software Version 5.00. All curves comparisons were made from
flies analysed in the same experiment. T50 was defined as the time point at which 50% of the initial population has died.

Western Blotting *D. melanogaster* total head lysates preparation

30-40 *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were collected; after freezing in liquid nitrogen and vortexing of entire flies, separated heads were collected, counted and lysed in SDS-rich buffer (SDS 1.45%, Glycerol 20%; Tris Base 0.2 M. 2.5 µl of buffer/head) using sonication (3 pulses of 50 Watt for 5 seconds). Homogenized lysate was then centrifuged at 1000 x g for 3 seconds to separate cuticle debris from supernatant. Proteins in supernatant were collected and quantified using spectrophotometry (Implant NanoPhotometer UV/Vis). Protein content was equalized. Samples, supplied with β-mercaptoethanol 5% and bromophenol blue, were boiled at 99 °C for 5 minutes. Equal amounts of volume were resolved on SDS-PAGE. Flies of the same line were collected from different vials and the entire experiment was repeated at least 2 times.

Western Blotting and Blot quantification

Following the preparation of samples, proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and processed for Western Blotting. Primary antibodies (at concentrations mentioned above) were prepared in 3% BSA/PBS-Tween 20 0.1%, secondary antibodies at concentration 1:5000 (Invitrogen, horse peroxidase conjugated to IGG or IGM) in 5% milk/PBS-Tween 20 0.1%. For visualization membranes were incubated with Pierce ECL Western Blotting substrate (cat. No. 32106) for 2 minutes and visualized using ChemiDoc Touch Imaging System (BioRad). Blots have been quantified using Image Lab Version 5.2.1 software (BioRad).

Imaging of fluorescent eyes in *D. melanogaster* and quantification

Experimental *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were kept anesthetized on a CO2 pad and their GFP fluorescent eyes were visualized using Leica MZ10 F Fluorescence stereomicroscope (GFP3 filter). 10 eyes of different flies / group were visualized and GFP fluorescence was quantified using Image J 1.48v software and expressed as corrected mean eye fluorescence (CMEF). CMEF has been calculated as: Integrated Density - (Selected Area x Mean Background Fluorescence). Statistical significance of CMEF differences analysed with 1-way ANOVA test using Graph Pad Prism Software Version 5.00.

Analysis of eye degeneration at light microscope

For each condition, at least 80 fly eyes were checked. The fraction of the eyes that showed degeneration phenotype were calculated as previously (Vos et al., 2010). For each analysed line, eyes of at least 40 flies were scored. The results were average of at least three independent experiments.
3. Results

In order to test whether and how the expression of HSPs in astrocytes might be relevant for neuroprotection in PolyQ diseases, we decided to use DNAJB6, one of the most potent human chaperones in providing protection against these diseases in different in vitro and in vivo models (Hageman et al., 2010; Kakkar et al., 2016).

In the set of experiments presented in this Chapter, we mainly aim to verify that the co-expression of the chaperone in the same neurons expressing the toxic PolyQ protein provides cell-autonomous protection in the D. melanogaster model of HD.

We verified whether such protective function of human DNAJB6 against PolyQ aggregation was also maintained in D. melanogaster. To do so, we first used the ommatidia integrity of transgenic D. melanogaster lines as readout for PolyQ-mediated degeneration. Hereto, the construct HttQ100-mRFP (encoding for human PolyQ-HTT exons 1-12; Weiss et al., 2012) was expressed in fly ommatidia together with human DNAJB6 (short nuclear and cytosolic isoform B; Hanai et al., 2003) and the membrane-targeted mCD8-GFP (a quantifiable fluorescent reporter of internal ommatidia integrity, established and validated in different D.melanogaster ommatidia-models of PolyQ diseases; Burr et al., 2014), using the gmr promoter driven by the Gal4-UAS expression system (Brand and Perrimon, 1993). The sole expression of HttQ100-mRFP caused a significant reduction in mCD8-GFP fluorescence in ommatidia (Fig.1A, B) and in total mCD8-GFP protein levels (Fig. 1C, D), indicating the degeneration of ommatidia. Both endpoints were alleviated by co-expression of DNAJB6 in the same cells (Fig. 1A-D), which implies a cell-autonomous protective effect of this HSP against HttQ100-mRFP toxicity. Notably, HttQ100-mRFP aggregation and mCD8-GFP levels are inversely correlated, further validating the use of this reporter, and confirming previous mechanistic findings that DNAJB6 can directly prevent the aggregation of PolyQ proteins in a cell-autonomous manner (Hageman et al., 2010; Kakkar et al., 2016). In line with the protective action against cell degeneration, DNAJB6 expression also resulted in a reduction in the amount of HttQ100-mRFP aggregates in total head lysates (Fig. 1C, D).
**Figure 1: Protective activity of DNAJB6 against PolyQ-HTT-mediated degeneration in D. melanogaster ommatidia.**

**A)** Representative images of eyes in 48 hours old adult male flies expressing the indicated transgenes in ommatidia. mCD8-GFP is a reporter for ommatidia integrity. Genotypes in Materials and Methods.

**B)** Quantification of the corrected mean eye fluorescence (CMEF) for mCD8-GFP of Fig.1A. Statistical significance was assessed using 10 eyes/group with 1-way ANOVA test (SEM, ***, p<0.001).

**C)** Western Blots of total head lysates of 48 hours old adult female flies expressing the indicated transgenes in ommatidia. Anti-huntingtin antibody used for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Anti-GFP antibody used for mCD8-GFP detection. Anti-V5 antibody for (V5 tagged) DNAJB6 detection. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.

**D)** Quantification of HMW aggregates of HttQ100-mRFP (signal in stacking gel) and mCD8-GFP of data of Fig. 1C (signal normalized on tubulin; a.u.: arbitrary units).

Comparable data were found, in a different set of experiments, when co-expressing DNAJB6 with a truncated ataxin-3 (ATXN3) carrying an expanded PolyQ (ATXN3-Q78) (Warrick et al., 1998), responsible for spinocerebellar ataxia type 3 (OMIM:#109150) (Costa and Paulson, 2012) (Fig.2). This confirms our previous findings that the protective action of DNAJB6 is generic for the core...
PolyQ-expansion (Månsson et al., 2014) and independent from regions flanking the PolyQ expansion for which it is known that they can affect the aggregation propensity of the PolyQ-containing protein (Kuiper et al., 2017). Interestingly, the neuronal expression of DNAJB6 leads to a near to complete eye protection (Fig. 2), whilst expression of DNAJB6 in astrocytes of the same SCA-3 D. melanogaster model does not suffice to prevent depigmentation, but did attenuate progression into necrosis (Fig. 3 of Chapter 5).

**Figure 2: Cell autonomous protective activity of DNAJB6 against PolyQ-ATXN3 mediated degeneration in D. melanogaster ommatidia. A)** Cell autonomous protection by neuronal expression of DNAJB6. Top panels show representative images of eyes of 1-day old adult female flies expressing the indicated transgene. Eye degeneration is quantified as the percentage of eyes showing either depigmentation (mild degeneration) or black necrotic spots (dotted line, arrowhead) (severe degeneration). Data are compared using an unpaired t-test (SD; **: P ≤ 0.01. ***: P ≤ 0.001). Genotypes in Materials and Methods.

Next, we generated a D. melanogaster line expressing HttQ100-mRFP in all neurons using the validated and well-characterized pan-neuronal promoter elav-Gal4 (Yao et al., 1993). Western blots of total head lysates showed abundant levels of HTT monomers, cleaved products, aggregating intermediates and high molecular weight (HMW) HTT-aggregates in 5-day-old adult male (Fig. 3A) and female (Fig. 3C) flies. In 15-day-old adults, monomers and other intermediate species were remarkably decreased and HMW aggregates were still present, indicating progressive aggregation and a worsening of the HD-phenotype. Lifespan analysis showed a significant reduction in lifespan of HttQ100-mRFP expressing males (Fig. 3B), with a T50 decrease of 55% (Fig. S1A); a comparable effect has been also observed in females (Fig. 3D and Fig. S1A).
Figure 3: Pan-Neuronal expression of PolyQ-HTT causes aggregate formation and reduces lifespan in D. melanogaster. A) Western Blots of total head lysates of 5 and 15-day-old adult male flies expressing the indicated transgenes in all neurons. Anti-huntingtin antibody for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods. B) Lifespan of the same isogenised males flies expressing HttQ100-mRFP or control transgene (eGFP or only promoter) in all neurons. Lifespan of HttQ100-mRFP-expressing line (red curve) is significantly reduced compared to the eGFP-expressing control line (green curve). Detailed statistics, comparisons and genotypes are provided in Fig. S1A. C) Western Blots of total head lysates of 5 and 15-day-old adult female flies expressing the indicated transgenes in all neurons. Anti-huntingtin antibody for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods. D) Lifespan of the same isogenised females flies expressing HttQ100-mRFP or control transgene (eGFP or only promoter) in all neurons. Lifespan of HttQ100-mRFP-expressing line (red curve) is significantly reduced compared to the eGFP-expressing control line (green curve). Detailed statistics, comparisons and genotypes are provided in Fig. S1A.

Subsequently, we used the GAL4-UAS and LexA-LexO (Yagi et al., 2010) expression systems that can drive expression of transgenes in a completely independent and non-overlapping manner (Chapter 3). We generated lines co-expressing HttQ100-mRFP regulated by GAL4-UAS and DNAJB6 by LexA-LexO, solely in neurons (using the elav promoter). Neuronal expression of DNAJB6 extended the lifespan of flies co-expressing neuronal HttQ100-mRFP, increasing the T50 by 43% (Fig. 4A, Fig. S2A, S2C). The line co-expressing neuronal eGFP and HttQ100-mRFP showed a lifespan comparable to that of flies expressing neuronal HttQ100-mRFP only (compare Fig. 3B, Fig. S1A with Fig.4A, Fig.
This indicates that combined use of multiple promoters and eGFP expression was without any biological consequences, meaning that the lifespan reduction in the line co-expressing neuronal eGFP and HttQ100-mRFP of Fig.4A is primarily due to the expression of the toxic PolyQ protein. Expression of DNAJB6 in neurons of control flies did not result in lifespan modulating effects (Fig. S2B, S2C), indicating that the protection in the PolyQ model is not due to a non-specific fitness-enhancing effect of DNAJB6.

Again, in line with the previous observations (Hageman et al., 2010; Kakkar et al., 2016) and the data on D.melanogaster eyes-degeneration (Fig.1), we found that neuronal expression of DNAJB6 also reduced the amount of HttQ100-mRFP aggregates in total head lysates (Fig.4B, Fig. S2D). Together these data imply and confirm that DNAJB6 has a specific and cell-autonomous protective activity against HttQ100-mRFP mediated toxicity in neurons that is associated with its ability to reduce PolyQ aggregate formation (Månsson et al., 2014). Notably, brain cell degeneration due to HttQ100-mRFP toxicity might lead to reduce expression of the various transgenes during the time and disease progression, but this does not change the overall conclusion of the above described experiments.

Interestingly, we also found that neuronal expression of DNAJB6 at lower levels than those in the previous experiments in Fig.4, (using for DNAJB6, the weaker promoter elav-LG instead of elav-LHG; Yagi et al., 2010), did not lead to a cell autonomous protection in terms of lifespan and PolyQ-HTT aggregate formation in the pan-neuronal HttQ100-mRFP D. melanogaster model (Fig. S3A-D). These data suggest that the in vivo cell-autonomous protection of DNAJB6 also depends on the level of the expression of the protective chaperone, in line with previous in vitro findings indicating that the quantitative level of DNAJB6 is a key factor for protection and anti-aggregation activity against PolyQ proteins (Månsson et al., 2014).
**Figure 4: Effect of neuronal DNAJB6 expression on PolyQ-HTT aggregate formation and lifespan in a pan-neuronal HttQ100-mRFP D. melanogaster model.**

A) Lifespan of isogenised male flies co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) DNAJB6 or eGFP. Lifespan of DNAJB6-expressing line (blue curve) is significantly expanded compared to the control line (red curve). Additional control lines, comparisons, statistics and genotypes are provided in Fig. S2A, C.

B) Western Blots of total head lysates of 5 and 15-day-old adult female flies (with equal transgenes expression of flies in Fig. 4A). Anti-huntingtin antibody used for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Anti-V5 antibody for (V5 tagged) DNAJB6 detection. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. HMW aggregates of HttQ100-mRFP quantification (signal in stacking gel normalized on tubulin signal; a.u.: arbitrary units) for each line is shown. Number of fly heads per each lysate sample and genotypes in Materials and Methods. An independent repeat of the experiment is shown in Fig. S2D.

To confirm that the expression of neuronal DNAJB6 indeed resulted in the actual and specific cell autonomous protection of neurons, we analysed the level of the neuronal marker NC82 (Wagh et al., 2006) in total fly head lysates. NC82 (Bruchpilot) is a key synaptic protein for the activity and integrity of the pre-synaptic zone in *D. melanogaster* brain (Wagh et al., 2006). NC82 is critical for a normal-evoked neurotransmitter release at the chemical synapses and, notably, the RNAi-induced-reduction of neuronal NC82 expression leads to an alteration of the normal synaptic components, to locomotor inactivity, and to unstable flight in adult *D. melanogaster* (Wagh et al., 2006). The expression level of NC82 provides therefore a direct measure of the functional fitness of the neuronal population. We found NC82 levels to be strongly decreased in 15-day-old flies solely expressing neuronal HttQ100-mRFP (Fig. S4A and S4B). This NC82-decline was alleviated in flies co-expressing neuronal DNAJB6 (cell autonomous protection, Fig. 5A, B and S4C, D), implying an overall improvement of the neuronal fitness.
**Figure 5:** Effect of neuronal DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on overall neuronal fitness. **A)** Western blots of NC82 from total head lysates of 5 and 15-day-old adult female flies co-expressing neuronal (Neur.>) HttQ100-mRFP and neuronal (Neur.>) or astrocytic (Astr.>) DNAJB6 or eGFP. Anti-NC82 antibody for NC82. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods. An independent repeat of the experiment is shown in Fig. S4C. **B)** Quantification of NC82 of data in Fig. 5A at day 15 (signal normalized on tubulin; a.u.: arbitrary units).

### 4. Discussion

Here, we showed that the cell-autonomous protective function of human DNAJB6 against PolyQ aggregation are also maintained in *D. melanogaster* models of HD and SCA-3 (Chapter 3 and this Chapter).

We recapitulated and confirmed the previous findings that DNAJB6 is protective against PolyQ aggregate toxicity *in vivo*. The cell-autonomous protective effects of DNAJB6 are strongly associated with its ability to prevent the initiation of PolyQ aggregation by the core-PolyQ fragment, irrespective of regions flanking the expansion (Hageman et al., 2010; Månsson et al., 2014), for which it is known that they can affect the aggregation propensity of the PolyQ-containing protein (Kuiper et al., 2017). The suppression of PolyQ Htt aggregation via overexpression of DNAJB6 in the affected neurons of *D. melanogaster* results in a significant expansion of the lifespan and improved neuronal fitness. Noticeably, we also found that the capacity of cell-autonomous protection of DNAJB6 against PolyQ aggregation is also dependent by the expression level of the chaperone. Our and previous findings confirm that DNAJB6 is among the strongest protectors against toxicity associated with PolyQ protein aggregation. Importantly, the cell-autonomous protective capacity of DNAJB6 mainly resides in its preventive ability to suppress aggregation of PolyQ proteins and not only in alleviating the downstream consequences of aggregation (Hageman et al., 2010, Kakkar et al., 2016). All together makes DNAJB6 an interesting target against neurodegenerative protein folding diseases such as HD.

Previous findings provide insights in the mechanism of action of DNAJB6 against PolyQ aggregation that is also pertinent for the rescue that we observed in our HD and SCA3 models. Notably, DNAJB6 exerts its function by forming oligomeric complexes and by direct interaction with the PolyQ client (Kakkar et al., 2016). DNAJB6 strongly inhibits the primary nucleation step and also
perturbs the secondary nucleation in the aggregation process (Kakkar et al., 2016). Whilst the J-domain is not absolutely required, the C-terminus of the chaperone is crucial for the anti-aggregation activity (Hageman et al., 2010). Particularly, a serine/threonine (S/T)-rich region in the C-terminus of DNAJB6 is fundamental in the substrate binding (Kakkar et al., 2016, Söderberg et al., 2018). The hydroxyl groups in the side chains may reduce the primary nucleation rate of PolyQ species by competing with the hydrogen bonding necessary for formation of amyloid fibrils and beta-hairpins (Hoop et al., 2016). The J-domain of DNAJB6 allows the interaction and cooperation with HSPA/HSP70. This might link the substrate-chaperone complex to protein degradation pathways or initiate aggregate sequestration (Kumar et al., 2018).

Nonetheless, further investigations are needed to better understand the mechanisms by which DNAJB6 protects cells in a cell-autonomous manner. Insights may come by studies regarding the physiological functions of DNAJB6. As previously said, it is expressed ubiquitously and DNAJB6 orthologs are found in all metazoans analysed so far (Hageman et al., 2010). Data suggests that DNAJB6 is important for protein degradation (Izawa et al., 2000; Watson et al., 2007) and expression network analysis indicate that its expression is strongly associated with catabolic processes (Kakkar et al., 2016). DNAJB6 may therefore have a role in preventing amyloid formation due to the fragmentation of full-length proteins during such catabolic events (as it occurs in the Htt aggregation process). Consistently, DNAJB6 mutations cause a dominant heritable form of limb-girdle muscular dystrophy (Harms et al., 2012; Sarparanta et al., 2012) associated with amyloid formation in muscle biopsies (Suarez-Cedeno et al., 2014) and the downregulation of endogenous DNAJB6 enhances PolyQ aggregation in vitro (Hageman et al., 2010). Moreover, cell autonomous protection of DNAJB6 has been shown not only in PolyQ diseases (Hageman et al., 2010, Kakkar et al., 2016) but also against the toxicity of other disease-causing proteins in Alzheimer’s (Månsson et al., 2014; Månsson et al., 2018) and Parkinson’s (Aprile et al., 2017, Kakkar et al., 2016b). This supports the hypothesis that DNAJB6 serves as strong generic inhibitor of aggregation in cells.

Further insights into the DNAJB6 protective mechanisms will provide insights in understanding the aggregation process of these proteins and how to inhibit such process, leading to future therapeutic strategies against neurodegenerative diseases.
REFERENCES


✓ Kakkar V, Kuiper EFE, Pandey A, Braakman I, Kampinga HH (2016b) Versatile members of the DNAJ family show Hsp70 dependent anti-aggregation activity on RING1 mutant parkin C289G. SciRep, 6: 34830.

✓ Kakkar V, Kuiper EFE, Pandey A, Braakman I, Kampinga HH (2016b) Versatile members of the DNAJ family show Hsp70 dependent anti-aggregation activity on RING1 mutant parkin C289G. SciRep, 6: 34830.


**SUPPLEMENTARY DATA**

**A**

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<th>LINE</th>
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<th>TEST 2</th>
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*Figure S1: Statistics, comparison and genotypes of lifespan curves (male and female flies) shown Figure 3B and 3D. A) Genotypes of lines, comparisons, and statistical analysis of lifespan curves in Fig. 3B and 3D. Statistical significance analysed using ≥100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).*
Figure S2: Lifespan analyses of control lines in Fig. 4. A) Lifespan of isogenised male flies (additional control lines of Fig. 4A) co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2C. B) Lifespan of isogenised male flies expressing neuronal (N>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2C. C) Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig. 4A and S2A, S2B. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2). D) Independent repeat of experiment shown in Fig. 4B.
Figure S3: Effect of neuronal DNAJB6 moderate expression on PolyQ-HTT aggregate formation and lifespan in a pan-neuronal HttQ100-mRFP D. melanogaster model. A) Lifespan of isogenised male flies co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) DNAJB6 or eGFP. Data shown are for moderate expression of LexO-DNAJB6/eGFP using elav-LG promoter. Genotypes: 1) HttQ100-eGFP line (red): w(-); UAS HttQ100-mRFP / elav-LG; elav Gal4 / LexO eGFP. 2) HttQ100-DNAJB6 line (blue) w(-); UAS HttQ100-mRFP / elav-LG; elav Gal4 / LexO DNAJB6. Additional control lines, comparisons, statistics and genotypes are provided in Fig. S3C. B) Lifespan of isogenised male flies (additional control lines of Fig. S3A) co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) moderate DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S3C. C) Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig. S3A-B. Statistical significance analysed using ≥100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2). D) Western Blots of total head lysates of 5 and 15-day-old adult female flies (with equal transgenes expression of flies in Fig.S3A). Anti-huntingtin antibody used for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Anti-V5 antibody for (V5 tagged) DNAJB6 detection. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. HMW aggregates of HttQ100-mRFP quantification (signal in stacking gel normalized on tubulin signal; a.u.: arbitrary units) for each line is shown.
Figure S4. Supplemental experiments to Fig.5. A) Western Blots of NC82 from total head lysates of 15-day-old adult female flies with or without neuronal HttQ100-mRFP. Anti-NC82 antibody for NC82. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods. B) Quantification of NC82 for data of Fig. S4A (signal normalized on tubulin; a.u.: arbitrary units). C) Independent repeat of experiment shown in Fig. 5A. D) Quantification of NC82 for data of Fig. S4C at day 15 (signal normalized on tubulin; a.u.: arbitrary units).

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