DNAJ proteins: more than just "co-chaperones"
Kakkar, Vaishali

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Chapter 5

Ectopic expression of DNAJB6 ameliorates Huntington’s disease phenotype in R6/2 mouse model

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ABSTRACT

Protein aggregation hallmarks many neurodegenerative diseases, including polyglutamine diseases (polyQ) like Huntington’s disease (HD). Molecular chaperones are the first line of defense in maintaining protein homeostasis and are important in combating neurodegeneration. Here we show that DNAJB6, a member of DNAJ family of molecular chaperone, very efficiently inhibits aggregate formation by early acting on the initiation step. Brain specific expression of the chaperone in the R6/2 model for Huntington disease, which does not affects the general heat shock response in neurons, delays nuclear inclusion formation in the striatum and delays the onset of behavioral/motor-coordination deficits. Muscle aggregate formation and muscle wasting are unaffected, confirming that the disease is largely initiated by neuronal degeneration. The latter also implies that the effects of DNAJB6 are cell autonomous and not inducing cell non-autonomous effects in distant tissues. DNAJB6 overexpression prolongs lifespan of the R6/2 mice by 23%, meaning that DNAJB6 is the so far most potent chaperone suppressing polyQ disease and thus a target for therapy in this disease.
INTRODUCTION

The eukaryotic system is well equipped with molecular chaperones, which tightly regulate protein quality control network (PQC) and hence maintain protein homeostasis in cells. The system is vital to help proteins achieve their native conformation by preventing non-productive protein-protein interactions, which can lead to aggregate formation (1,2). Besides assisting in protein (re)folding chaperones also assist in the degradation of client proteins by either keeping them susceptible to proteolysis or even by directly transferring them to the cellular degradation machineries (3–5). Central to the PQC is the Hsp70 machinery where HSPA (Hsp70) members form the core and DNAJ (Hsp40) proteins act as their co-chaperones to drive client specificity and maybe also client fate (6). Despite such an elegant system to maintain PQC, protein aggregation can occur and in fact hallmarks almost all the major neurodegenerative diseases known, such as Alzheimer’s disease, Parkinson’s disease, Amyotrophic lateral sclerosis (ALS) and trinucleotide (CAG) repeat expansion diseases (4).

In CAG repeat expansion diseases, expanded polyQ stretch formation beyond a certain threshold in different genes like huntingtin, ataxin and androgen receptor, results in disease conditions namely Huntington’s disease (HD), Spinocerebellar ataxia (SCAs) or spinal and bulbar muscular atrophy (SBMA) respectively. Except for SBMA, CAG repeat expansion diseases are autosomal dominant, toxic-gain-of function (GOF) diseases. The expanded polyQ stretch in the corresponding disease-related proteins has an expansion length dependent propensity to aggregate (7). In line, polyQ expansion length is strongly associated with age of onset (8,9). This strongly suggests that toxic GOF resulting in aggregate formation is the basis of disease initiation. The exact cause of aggregate initiation (and hence onset of neurodegeneration) in polyQ diseases is still not fully understood. Several lines of evidence, however, suggest that it is not the full length polyQ proteins that are aggregation prone, but that protease activation (e.g. by exocitotoxic events) followed by cleavage of the polyQ proteins is required to initiate the aggregation process (10–15). Such cleavage leads to fragments containing the expansion that either directly or after further processing via the proteasome acts as aggregation seeds, which next also recruit full length proteins and other CAG repeat containing proteins such as transcription factors into the aggregates (16–18). PolyQ aggregates can also recruit many other chaperones and proteasome components (13,19), but this predominantly seems to occur at later stages (20), suggesting that chaperone and/or proteasome depletion may not be a cause for disease initiation but rather be a late consequence of the disease and hence might play a role in disease progression.

Given that aggregate formation is thought to initiate diseases, repeatedly attempts were made to increase the chaperone network or the expression of single chaperones to delay aggregation and disease onset. In line with the expectation, activation of heat shock
response (HSR) which eventually leads to upregulation of various chaperones was found to be effective in delaying phenotypic changes in fly and worm models (21–23). However in sharp contrast, in mouse models of CAG repeat diseases the upregulation of HSR (24–26) or transgenic expression of individual chaperones like Hsp70 (27) or HSPB1 (28) has largely failed to delay disease onset. In fact only DNAJB2/HSJ1, was shown to have some protective effect in aggregate formation and functional endpoints in the R6/2 mouse model of HD (31). The HSR and the individual chaperones regulated by the HSR usually mostly only reduced the phenotypic effects caused by the polyQ expression and only marginally affected polyQ aggregation itself. This suggested that these chaperone-mediated effects in lower organisms were rather due to compensatory effects downstream of aggregate formation, counteracting disease consequences rather than its cause (29).

However, it became clear that the family of chaperones was much larger than originally assumed and contains many members that are not controlled by the classical HSR (30). Our previous work included several dedicated screens in search for potential better modulators of polyQ aggregation within the human chaperonome (31,32). Amongst other, this led to the discovery that several members of the DNAJB (Hsp40) subfamily (often referred to as Hsp70 co-chaperones) were most effective against polyQ mediated aggregation and toxicity (31). Since in our cell-based screens, DNAJB8 and DNAJB6 were even more potent suppressors of polyQ aggregation than DNAJB2 (31), we reasoned that these closely related members may have even greater potential in vivo. This was even further supported by findings that DNAJB6 acts at a very early stage in the aggregation process by preventing aggregate seeding of polyQ peptide in in vitro and cellular models (33,34). This prompted us to test whether single DNAJB6 overexpression might indeed protect neurons from aggregate toxicity using neuronal cell system and finally can rescue the R6/2 mouse model of HD disease.

We here show that DNAJB6 delays neurite collapse in neurons by preventing aggregate formation and maintains the overall neuronal integrity that express expanded polyQ. Most importantly, we show that modest overexpression of DNAJB6 in mouse brain substantially delays the “onset” of the disease phenotypes and prolongs lifespan by 23% in the R6/2 mouse model of HD, which is to the best of our knowledge the largest protective effect reported so far with this model.

RESULTS

DNAJB6 reduces the Htt aggregation in differentiated neuronal cell line and makes expanded polyQ fraction more mobile

We previously identified DNAJB6 as a potent suppressor of expanded polyglutamine
aggregation and associated toxicity in HEK293 cells (31). In order to evaluate the effect of DNAJB6 in neuronal like cells, NG108 precursor cells were differentiated to form neuronal processes. Expression of Htt-Q119-eYFP in differentiated NG108 resulted in aggregate formation as seen by high molecular weight (HMW) SDS-insoluble band on a western blot (Fig. 1A). Co-expression of DNAJB6 strongly suppressed the formation of aggregate formation in these cells and also concomitantly increased the soluble levels of Htt protein (Fig. 1A & 1B). Immunofluorescence (IF) imaging revealed that aggregates initially appear in and around the soma of the cells and at later time-points progress into the axon like processes (Fig. 1C). Cells expressing Htt-Q119-eYFP were found to have less neurite-like processes, even when they had no detectable aggregates yet (Fig. 1D), in line with the idea that the soluble form of Htt-Q119-eYFP lies at the origin of the toxic gain of function defects. Neurons expressing Htt-Q23-eYFP (a non-pathogenic polyQ stretch) were unaffected, demonstrating that the observed effects were truly due to the polyQ expansion stretch (Supplementary Fig. 1). DNAJB6 co-expression with Htt-Q119-eYFP not only reduced aggregate formation as detected by IF, but also preserved the formation of neurite processes under conditions of Htt-Q119-eYFP expression; even in cells without visible aggregates the number of processes was higher in DNAJB6 expressing cells (Fig. 1D). This suggests that JB6, in contrast to other chaperones acts on the soluble form of Htt-Q119-eYFP. In line, FRAP analysis revealed that the reduced mobility of non-aggregated Htt-Q119-eYFP compared to GFP control was improved by co-expression of DNAJB6 (Fig 1E), confirming that JB6 acts on the soluble, pre-aggregate form, of Htt-Q119-eYFP. A similar effect was found for a DNAJB6 H/Q mutant that is defective in its ability to interact with Hsp70 but yet inhibits aggregation (Hageman et al 2010). Together with earlier data suggesting that DNAJB6 inhibited aggregate seeding of polyQ peptides in in vitro and cellular models (33,34), these data demonstrate that DNAJB6 inhibits an early phase of the aggregation process and does not require Hsp70 at this stage (Fig. 1E).

Characterization of DNAJB6 transgenic mice
To study whether the highly protective effects of DNAJB6 in neurons in vitro also translate into neuro-protection in vivo, we generated transgenic mice that overexpress the human DNAJB6 (hDNAJB6) specifically in the brain (Fig. 2A) with no significant expression in other tissues (Fig. 2B).

Mice were born in the expected Mendelian ratios and did not show an overt phenotype. Expression of the human transgene did not affect the expression of the endogenous mouse DNAJB6 (mDNAJB6) (Supplementary Fig. 2). Compared to wild-type animals, also the HSP70/HSPA1A levels were unaltered in brain and muscle lysates from the transgenic mice (Supplementary Fig. 3). This indicates that DNAJB6 overexpression is not inducing a general
DNAJB6 ameliorates disease phenotype in R6/2 model

Figure 1: DNAJB6 prevent polyglutamine aggregates at an early stage in neuronal cells. NG108 cells were differentiated for 48 hours with NECA and IBMX prior to transfection and next transfected with Htt-Q119-eYFP with or without V5-DNAJB6. (A) High molecular weight (HMW) aggregates trapped in the stacking gel and soluble Htt-Q119-eYFP was assessed with anti-GFP antibodies. Expression of DNAJB6 was detected with anti-V5 antibody and GAPDH was used as a loading control. (B) Quantification of aggregated and soluble fraction of Htt-Q119-eYFP with or without DNAJB6 co-transfection. (C) Representative confocal pictures of differentiated NG108 cells co-transfected with Htt-Q119-eYFP (green) and DNAJB6 (blue) with additional staining of β-III tubulin (red) at 24 and 36 hours post transfection. (D) Individual cells (approx. 60 cells per condition with or without DNAJB6) were manually counted for neurite processes (dendritic processes). Cells with and without polyQ aggregation were independently scored. (E) FRAP analysis in differentiated NG108 expressing Htt-Q119-eYFP alone or together with RFP-DNAJB6 or V5-DNAJB6 H/Q mutant. Cells were analyzed 12 hours post transfection, before the aggregates were visible.
DNAJB6 ameliorates disease phenotype in R6/2 model

Figure 2: Characterization of DNAJB6 transgenic mice.
(A) Floxed DNAJB6 transgenic mice were generated and the active transgene was generated after crossing with Nestin-Cre mice with specific expression driven in the brains of the animals. (B) mRNA levels of hDNAJB6 transcript from various tissues from both TgDNAJB6 (grey bar) and WT (black bar) animals. The values were normalized with GAPDH levels in each sample. (C) hDNAJB6 expression in the various tissue lysates (Brain, Liver, Kidney) from TgDNAJB6 and Wild-Type (WT) mice as detected by western blot. The positive control (PC) is lysates from DNAJB6 transfected cells. GAPDH was used as a loading control.

stress response and thus does not seems to interfere with the HSP70 chaperone machinery.

DNAJB6 reduces Htt aggregate levels and nuclear inclusions in R6/2 mice brain

Next, we crossed the DNAJB6 transgenic mice with R6/2 mice expressing exon-1 of the human huntingtin gene, containing 200 CAG repeats. This so-called R6/2 model is the most aggressive/severe HD mouse models and recapitulates several aspects of the human HD including (early onset) motor dysfunction associated with protein aggregation in the brain, resulting in an early death (35,36). Whole brain lysates from R6/2 mice (referred to as HTT) and double transgenic HTT/JB6 mice were compared at different ages. As expected, the huntingtin aggregates increased over time in HTT mice (Fig. 3A). This aggregation load was significantly reduced in mice co-expressing the JB6 transgene (Fig. 3A, B). Moreover,
immunohistochemical analysis on paraffin embedded brain sections revealed an age-dependent increased of nuclear inclusion formation in the striatum of the HTT mice (Fig. 3C & 3D), which was not seen in wild-type (WT) or DNAJB6 transgenic mice (Supplementary Fig. 4). Inclusion formation was strongly reduced in the double transgenic JB6/HTT mice at all-time points recorded (Fig. 3C & 3D). These results demonstrate that overexpression of DNAJB6 suppresses the formation of polyQ aggregates in mouse brains.

**DNAJB6 improves the behavioral phenotype in R6/2 mouse model and also extends lifespan**

In order to investigate whether the observed reduction in aggregate formation in the brain also results in prevention of motor (dysfunction) symptoms in R6/2 model, we performed rotarod measurements as read out for motor and balance coordination (37). Consistent with earlier data, HTT mice show an early decline in rotarod performance, which progressively deteriorates with age (Fig. 4A). The decline was strongly delayed in the JB6/HTT double transgenic mice and the HTT/JB6 mice performed better on the rotarod at all-time points (Fig. 4A). Further, we performed the clasping assay as a measure of disease progression (38). Both hind limb and full body clasping as seen in the HTT mice was strongly delayed in the double transgenic HTT/JB6 mice (Fig. 4B). Finally, the health span of the HTT/JB6 mice was extended by almost 23% when compared to that of R6/2 mice (Fig. 4D).

To the best of our knowledge, the protective effects of DNAJB6 are the largest of all single chaperone over-expression used with this aggressive R6/2 model reported so far. This is even more impressive considering that DNAJB6 expression was exclusively elevated in the brain and not in other tissues (Fig. 2B) in contrast to e.g. HSPA1A or DNAJB2 transgenic mice (39,40). HD aggregates also affect skeletal and cardiac muscle (41) and aggregation-related progressive muscle atrophy was reported as a significant contributor to the motor symptoms and early death in the R6/2 model (42,43). We confirm that aggregates are indeed present in the muscles of R6/2 mice, but consistent with the brain specific DNAJB6 expression in our JB6 mice, the extent of aggregation in quadriceps skeletal muscle in the HTT/JB6 mice was similar to that in the HTT mice (Supplementary Fig. 5A and 5B). Also, the muscle wasting indicated by the muscle weight loss was similar with almost 70% muscle weight loss by 12 weeks of age in both HTT and HTT/JB6 mice (Supplementary Fig. 5C). This data also serve as a nice control for the brain-specific effects in our double transgenic HTT/JB6 mice. Moreover, and in line with the absence of an effect on the general chaperone network in the brain (Fig Supplementary 3), the tissue-specific effect of DNAJB6 implies that its upregulation does not lead to cell-non-autonomous effects unlike what has been suggested for example for effects of the HSR-regulated DNAJB1 (44).
DNAJB6 ameliorates disease phenotype in R6/2 model

Figure 3: DNAJB6 reduces HTT aggregates in the brain of the R6/2 mice

(A) Huntingtin aggregation in whole brain lysates were determined in HTT and HTT/JB6 mice and found to be reduced in double transgenic mice HTT/JB6 at all the time points. The S829 antibody was used to detect both HTT aggregates (stack) and soluble HTT (sol). (B) Relative levels of insoluble HTT was plotted using GAPDH as loading control and setting the HTT mice values at 100% (± sem) for each time-point (n=3 for each group). Statistical significance was analysed using an independent t-test. P<0.05 was considered statistically significant, *** P=0.0005, ** P=0.007.

(C) Representative immunohistochemical pictures of the striatum of HTT and HTT/JB6 mice at various time points.

(D) The percentage of inclusion positive nuclei (counted manually: mean ± SEM). Statistical significance was analysed using an independent t-test. P<0.05 was considered statistically significant, **P<0.008, *P<0.05.
Figure 4: DNAJB6 overexpression delays disease onset in R6/2 mouse model.

(A) Rotarod measurement for HTT (red) and HTT/JB6 (green) mice at different time points. The time to stay on the rotating rod before falling is provided. The animals were recorded for 3 trials per day for 3 consecutive days for each group. The mean ± sem of the trials at each day is plotted. HTT mice had to be sacrificed at 13 weeks and HTT/JB6 at 17 weeks due to >20% weight loss. (B) Hind limb clasping (circle) and full body clasping (triangles) for HTT and HTT/JB6 mice. (C) Kaplan-Meier survival plot for WT (purple), TgDNAJB6 control (gray), HTT (red) and HTT/JB6 (green) animals.
FINAL REMARKS

Our current findings further strengthen our previous observation that DNAJB6 is one of the most potent suppressors of polyQ aggregation within the human chaperonome. It not only prevents phenotypic changes associated with expression of polyQ proteins but actually prevent aggregation both in \textit{in vitro} i.e. in cells (31) and neurons (this report) as well as in \textit{in vivo} i.e. \textit{Xenopus} (31) and mice (this report). The high anti-aggregation potency of DNAJB6 is thought to rely in the fact that it can chaperone polyQ peptides (33,34) that likely initiate the aggregation process as seeds (13) upon proteolytic cleavage of the full length proteins that is activate by extracellular stresses like exitotoxic glutamate stimulation (14). DNAJB6 alone (i.e without the need of Hsp70 or any other chaperones) extremely efficiently binds to these polyQ peptide at substoichiometric concentrations and thus maintains them in a soluble state (33). How these complexes are next processed and if or how DNAJB6 is recycled, remains to be elucidated.

The brain specific overexpression of DNAJB6 (so far) is without any deleterious phenotypic effects, which suggest it could be a realistic therapeutic target in polyQ diseases. Since DNAJB6 is expressed in the brain, one could aim at increasing its basal expression levels. The promotor of DNAJB6 is however complex and no pathways have yet been established that strongly regulate its activity. However, unbiased compounds screens are ongoing to identify DNAJB6 expression enhancing compounds. Alternatively, one could try to directly target DNAJB6 itself to increase its specific activity toward polyQ aggregation prevention. We previously showed that DNAJB6 exists as hetero-oligomeric complexes in cells (31) and \textit{in vitro} (33). Mutagenesis of DNAJB6 revealed that especially the larger oligomeric complexes are needed for polyQ protection (31). Thus, one could screen for compounds that increase DNAJB6 oligomerization. Also, DNAJB6 can act in a non-canonical way in “preventing” the polyQ mediated aggregation where it doesn’t require Hsp70 for its anti-aggregation effect. Thus, up-regulating or boosting DNAJB6 levels or activity may be favorable over changing the entire chaperone network as the latter has been associated with malignancy (45,46).

METHODS/MATERIALS

\textit{Production of DNAJB6 transgenic mice}

DNAjb6 transgenic mice were generated as described by van Ree \textit{et al} (47). PCR-amplified human DNAJB6 cDNA with a N-terminal Flag tag was cloned into a unique BglII and XhoI site of the Z/EG expression vector, and verified by sequence analysis. Sca-I linearized vector transfected into ES cells by electroporation and G418-resistant colonies with high expression levels of Flag-DNAJb6 were selected as previously described (47). ES cells were karyotyped and injected into C57BL/6 blastocysts to generate chimeric animals. Chimeric males were
used for breeding and offspring were screened for the presence of the transgene by PCR. Mice were backcrossed to C57BL/6 for at least 6 generations. To specifically excise the _-geo-stop cassette in neurons, DNAJB6 transgenic mice were bred to Nesting-Cre mice (B6. Cg-Tg(Nes-cre)1Kln/J ; Jackson’s Lab, #003771).

The R6/2 HD mice (B6CBA-Tg(HD exon1)62Gpb/3J; Jackson’s Lab, #006494) were from the colonies of Gillian Bates.

Mice handling and maintenance
All procedures were performed with approval of the University of Groningen Ethical Committee for Animal Experiments, which adheres to the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Experiments were carried out on R6/2 mice (kindly received from Gillian Bates lab, King’s College London, UK) and DNAJB6 transgenic mice (generated at Mayo Clinic, USA), housed individually with ad libitum access to food and water. Hemizygous R6/2 mice were bred by backcrossing R6/2 males to (CBA x C57Bl/6) F1 females (B6CBAF1/OlaHsd, Harlan Olac). The genotyping and CAG repeat measurements were done as explained in Mangiarini et al. 1996 (35) with primers R6/2 fwd/rev & HDAC4 fwd/rev used for genotyping R6/2 mice and 40256 fwd–40261 rev for CAG repeat sizing (Supplementary Table I). DNAJB6 transgene was checked by using hDNAJB6, NEO and CRE primer pairs. The sequences of all the primers used in this study are listed in Supplementary Table I.

NG108 cell culture and transient transfections
NG108 cells were cultured according to the standard protocols in DMEM (Invitrogen, 41966-052) supplemented with 10% fetal bovine serum (Greiner Bio-One, 758093) and penicillin/streptomycin (Invitrogen, 15140-163). Differentiation was induced using 1% NECA (Sigma) and 0.1% IBMX (Sigma). Cells were transfected with HDQ119-eYFP, pcDNA5/FRT/TO DNAJB6-V5 48 hours post differentiation, using Lipofectamine 2000 (Gibco) reagent according to the manufacturer instructions. Immunocytochemistry was performed as described earlier (31). For immune-staining, differentiated cells were additionally stained for βIII-tubulin to visualize neurite processes.

Cell extract, tissue extracts, sample preparation and western blot
For NG108 cells, 24hrs post transfection samples were prepared as explained before for HEK293 cells (31). For mouse tissue extracts for Htt aggregate detection, tissues were snap froze in liquid nitrogen immediately after dissection and homogenized (with pellet pestle for brain tissues and beat beater for muscle tissues) in RIPA buffer with NaCl (150mM), Igepal Cholate (1%), Deoxycholate (0.5%), SDS (0.01%) and Tris-HCl (50mM). Protease inhibitors cocktail, 0.1M PMSF and 0.5M DTT were added fresh to the solution. Samples
were sonicated and left on ice for 20 min to ensure proper lysis of the cells and release of proteins. Samples were spun at high speed (13000rpm) for 15 min and pellet was discarded.

Protein content was determined using DC protein assay (BioRad). Western Blot samples were prepared in SDS-PAGE loading Laemmeli buffer and heated for 5 min at 100°C. Equal amount of protein (5ug for NG108 cell extract and 50ug for tissue extract) was loaded on 8% (Htt detection) and 12.5% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and probed with anti-GFP (JL-8 Clontech), 1:5000, anti V5 (Invitrogen), 1:5000; anti-GAPDH (RD research Diagnostics), 1:10000; antibodies. Anti-Htt antibody S829 was kindly provided by Gillian Bates Lab, UK. Blots were subsequently incubated with appropriate HRP-conjugated secondary antibodies (Amersham) at 1:10000 dilutions. Anti-goat HRP (Dako) for S829 was used at 1:5000 dilutions.

Confocal microscopy for NG108 cell line
Confocal images were obtained using a confocal laser scanning microscope (Leica TCS SP8) with a 63X/1.32 oil objective. Images were processed using ImageJ software (http://rsb.info.nih.gov/ij/). As minor manipulation, background correction was applied to all parts of the image.

FRAP analysis
Differentiated NG108 cells were transfected with Htt-Q119-eYFP and with or without mRFP-DNAJB6b construct and V5-DNAJB6 H/Q mutant; plasmid description as described in (31). 12 hours post transfection, cells were observed and images were collected using Zeiss LSM780 Confocal 65 Laser Scan Microscope, 63x/1.3Imm, equipped with incubation chamber with CO2 and temperature control (all images were recorded at 37C). A strip spanning the cytoplasm was recorded using minimum laser intention at frame rate of 1 second (50 numbers of pre-bleach recordings), subsequently the strip was bleached (one iteration at full laser intention) and recorded for 10 seconds. The values were analyzed using Zeiss software. Briefly, values were normalized to the pre-bleach pulse. Only the post bleach curves are displayed.

Quantitative PCR
Total RNA was extracted from tissue samples Trizol method. 1 μg of total RNA was transcribed in first strand cDNA using M-MLV reverse transcriptase (Invitrogen, 28025-013). The cDNA synthesis was performed with oligo (dT) 12-18 (Invitrogen, 18418-012). Relative changes in transcript levels were determined on the Icycler (Bio-Rad) using SYBR green supermix (Bio-Rad, 170-8885RK). Calculations were done using the comparative CT method according to User Bulletin 2 (Applied Biosystems). For each set of primers, the PCR efficiency was determined. Primer sequences used in this study are listed in Supplementary Table I.
**Immunohistochemistry**

Dissected mouse brain and muscles were fixed in 4% buffered formaldehyde, followed by dehydration and then embedded in paraffin and sliced into 5 μm sections. The sections were de-waxed and labelled with the following antibody: EM48 for Htt aggregates (MAB5374, Millipore), secondary biotin carrying antibodies (Dako, Carpinteria, CA), an avidin–biotin–horse radish peroxidase complex (ELITE ABC Kit, Vector Laboratories, Burlingame, CA) and the AEC chromogen were added for visualization under bright field microscopy. Nuclear counterstaining was performed with hematoxylin. Control sections without primary antibodies did not show positive immunostaining.

**Behavioural analysis**

RotaRod analysis was performed on an accelerating RotaRod (n ≥ 10 for HTT group; n ≥ 9 for HTT/JB6, Wt & JB6 group) as explained in Hockly et al. (37) with few adaptations i.e. top speed of 40rpm over a period of 300 seconds. To maintain a gap of 4 weeks between the recordings (in order to avoid animal learning and hence naïve situation), two cohorts of animals were maintained. The recordings from 4, 8, 12, 16 weeks served as one cohort and from 6, 10, 14 weeks of animals another cohort. At each time point, the mice were tested on three consecutive days, for three trials per day. The data is plotted as an average of all the trails for each day. Clasping of limbs was performed as described in Chou, 2008 et al. (38) with n = 9 for each animal group. The mice were scored for either hind limb clasping (referred as stage 1: where one of the hind limb is clasped for over 50% of the time) or full body clasp (referred as stage 2: where both the limbs were clasped around abdomen for more than 50% of time) over different age groups. Lifespan was assessed by Kaplan-Meier analysis.

**ACKNOWLEDGEMENTS**

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38. Orth M, Cooper JM, Bates GP, Schapira AHV. Inclusion formation in Huntington’s disease R6/2 mouse
SUPPLEMENTARY INFORMATION – CHAPTER 5

Figure 1: Differentiated NG108 cells co-transfected with Htt-Q23-GFP and V5-DNAJB6.
NG108 cells were differentiated for 48 hours with NECA and IBMX prior to transfection. Representative confocal pictures of differentiated NG108 cells co-transfected with Htt-Q23-GFP (green), β-III tubulin (red) and DNAJB6 (blue) at 24 and 36 hours post transfection. β-III tubulin was used as a visualizing marker for neurite processes.

Figure 2: Transcript levels of endogenous levels of DNAJB6 from transgenic and WT mice
mRNA levels of endogenous mDNAJB6 transcript from brain and muscle lysates from both TgDNAJB6 (Gray bar) and WT (Black bar) animals. The values were normalized with GAPDH levels in each sample.
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Figure 3: HSPA1A levels remained unaltered in the transgenic DNAJB6 mouse as compared to WT animal  
Brain or Muscle lysates from 12 week old animals were immune-blotted for Hsp70 levels using anti-HSPA1A/Hsp70 (Stressgen, SPA-810) antibody. GAPDH was used as loading control.

![Figure 3: HSPA1A levels remained unaltered in the transgenic DNAJB6 mouse as compared to WT animal](image)

Figure 4: TgDNAJB6 mouse show same staining pattern as WT with no aggregates present even at 12 weeks of age  
Representative immunohistochemical pictures of striatum of WT and TgDNAJB6 control animals at 12 weeks of age. Hematoxylin was used nuclear counterstain (blue stain) and EM48 to visualize HTT aggregates (brown staining). Scale: 100um. No aggregate (absence of brown staining) was visible in any of these groups for the late time-point of assay.

![Figure 4: TgDNAJB6 mouse show same staining pattern as WT with no aggregates present even at 12 weeks of age](image)
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**Figure 5:** No change in muscle aggregate and muscle wasting in animals from both HTT and HTT/JB6 group

R6/2 mice also develop muscle aggregates, which is not altered in double transgenic mice HTT/JB6 at all the time points. The S829 antibody was used to detect both HTT aggregates (agg) and soluble HTT (sol).
### SUPPLEMENTARY TABLE I

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<td>CTA CGA ATA TGG CAA AGA AGG</td>
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<td>CGG AAT GTG AAG CCA AAC TC</td>
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