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**Mutant HTT seeding activity: a marker of disease progression and
neurotoxicity in models of Huntington's disease**

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SUMMARY

Self-propagating, amyloidogenic mutant huntingtin (mHTT) aggregates may drive progression of Huntington's disease (HD). Here, we report the development of a FRET-based mHTT aggregate seeding (FRASE) biosensor assay that enables the quantification of mHTT seeding activity (HSA) in complex biosamples from HD patients and disease models. Application of the FRASE assay revealed HSA in brain homogenates of presymptomatic HD transgenic and knock-in mice and its progressive increase with phenotypic changes, suggesting that HSA quantitatively tracks disease progression. Biochemical investigations of mouse brain homogenates demonstrated that small rather than large mHTT structures are responsible for the HSA measured in FRASE assays. Finally, we assessed the neurotoxicity of mHTT seeds in an inducible *Drosophila* model transgenic for *HTT^{ex1}*. We found a strong correlation between HSA measured in adult neurons and the increased mortality of transgenic HD flies, indicating that FRASE assays detect disease-relevant, neurotoxic, mHTT structures with severe phenotypic consequences *in vivo*.

KEYWORDS

Huntington's disease, FRASE assay, mutant HTT seeding, huntingtin, *Drosophila*, proteotoxicity

INTRODUCTION

Self-propagating protein aggregates are a pathological hallmark of a large number of neurodegenerative diseases (NDs) including Huntington's disease (HD) (Chiti and Dobson, 2017; Jucker and Walker, 2013). Recent studies indicate that aggregate pathology and associated tissue atrophy do not appear randomly throughout the brain but instead progress along distinct neuronal networks (Brundin et al., 2010). Evidence was provided that amyloidogenic protein assemblies spread from cell to cell, converting free molecules of the same protein into aggregated species. This transcellular propagation may drive pathogenesis in NDs (Guo and Lee, 2014; Pecho-Vrieseling et al., 2014). To understand the mechanisms of disease development and progression, it is of critical importance to specifically monitor the activity of self-propagating protein aggregates in complex biosamples.

A number of assays have been established that allow the quantification of seeding activity of amyloidogenic aggregates in crude protein homogenates (Atarashi et al., 2007; Holmes et al., 2014; Tan et al., 2015). These methods take advantage of the phenomenon that ordered protein aggregates are formed from monomers by a nucleation-dependent process (Jarrett and Lansbury, 1993; Scherzinger et al., 1999), a relatively slow process *in vitro*. However, spontaneous amyloid formation can be accelerated by addition of preformed aggregates that function as seeds for the conversion of monomers from a soluble into an aggregated state (Cohen et al., 2012; Jarrett and Lansbury, 1993). Biosamples that contain seeding-competent protein aggregates might therefore stimulate the polymerization of soluble amyloidogenic proteins with related amino acid sequences in cell-free or cell-based seeding assays.

Based on this premise, the protein misfolding cyclic amplification (PMCA) technology and related methods (Atarashi et al., 2007; Atarashi et al., 2011; Saborio

et al., 2001) have been developed, which allow the detection of minute quantities of seeding-competent PrP^{Sc} aggregates in various biomaterials prepared from patients or rodent models with prion disease (Castilla et al., 2005). Variants of the PMCA technology have also been applied for the amplification of amyloid- β and α -synuclein aggregates from biosamples (Du et al., 2011; Herva et al., 2014). A key feature of PMCA methods is that seed-mediated amyloid polymerization is indirectly monitored through the reporter dye Thioflavin T (ThT), which changes its fluorescence emission upon binding to ordered amyloid fibrils (Biancalana and Koide, 2010). Also cell-based amyloid polymerization assays have been developed (Holmes et al., 2014; Tan et al., 2015). In these assays, ectopically expressed aggregation-prone reporter proteins with fluorescent tags are utilized as biosensors for detecting amyloidogenic aggregates.

Recent studies with brain slices, fly and mouse models provide evidence that mHTT aggregates indeed possess seeding activity and spread from cell to cell (Pecho-Vrieseling et al., 2014; Babcock and Ganetzky, 2015; Pearce et al., 2015), suggesting that proteopathic mHTT seeding in HD patient brains or mouse models drives pathogenesis (Brundin et al., 2010; Jeon et al., 2016). However, it remains unclear whether mHTT seeding is indeed responsible for dysfunction and neurodegeneration. While several mHTT aggregate species, i.e. small oligomers and fibrils, have been described as potentially pathogenic (Nucifora et al., 2012; Pieri et al., 2012; Scherzinger et al., 1997), the mHTT species that exhibit seeding activity *in vivo* still need to be pinpointed.

To be regarded as disease relevant, we propose that seeding-competent mHTT aggregates need to be detectable in affected brain regions in patients and transgenic HD mouse models. To promote disease development, such structures should be present in model systems prior to the appearance of a disease phenotype. Also, their

abundance in affected tissues should increase with the severity of disease symptoms. Finally, perturbation of mHTT seeding activity through genetic manipulation should influence the disease phenotype in model systems. In summary, to elucidate the potential importance of mHTT seeding in disease, it is crucial to detect seeding-competent structures in relevant biosamples, and to investigate their potential impact on biological functions and phenotypes.

Here, we describe the development of a **FRET-based mHTT aggregate seeding (FRASE)** assay, which allows the quantification of **mHTT seeding activity (HSA)** in complex biosamples with high sensitivity and specificity. Application of the method revealed HSA in disease-affected brain tissues of HD patients and mouse models. Furthermore, we observed HSA in brain homogenates of presymptomatic HD mice and its progressive increase with disease development. Biochemical investigations of mouse brain homogenates revealed high HSA predominantly in protein fractions that contain small mHTT fibrils, suggesting that such structures are responsible for this activity *in vivo*. Finally, using an inducible *Drosophila* model of HD, we demonstrated that the formation of seeding-competent HTT_{ex1Q97} structures in adult neurons is associated with a reduced lifespan of transgenic flies, indicating that FRASE assays detect neurotoxic structures.

RESULTS

Establishment of a FRET-based mHTT aggregate seeding assay

To monitor mHTT seeding activity, we first developed a cell-free aggregation assay with recombinant fluorescent reporter proteins (**Figure S1A**). Two soluble glutathione S-transferase HTT exon-1 (HTT_{ex1}) fusion proteins with 48 glutamines C-

terminally fused to CyPet or YPet (GST-Ex1Q48-CyPet or -YPet) were produced in *E. coli* and purified to ~90% homogeneity using glutathione sepharose chromatography (**Figure S1B**).

Recombinant proteins were cleaved with PreScission protease (PSP) to release GST and to initiate the spontaneous aggregation of the fusion proteins Ex1Q48-CyPet and -YPet. The assembly of the tagged Ex1Q48 proteins into insoluble aggregates over time was monitored using an established filter retardation assay (FRA), which specifically detects large SDS-stable mHTT aggregates (Wanker et al., 1999). We found that the proteins Ex1Q48-CyPet and -YPet rapidly self-assemble into SDS-stable aggregates *in vitro* (**Figure 1A**), confirming recently reported results (Wagner et al., 2018). To investigate the morphology of spontaneously formed Ex1Q48-CyPet and -YPet aggregates, we analyzed the aggregation reactions with atomic force microscopy (AFM). We observed that the tagged Ex1Q48 fusion proteins, similar to the untagged Ex1Q48 protein (Wagner et al., 2018), form large fibrillar protein aggregates (**Figure 1B**).

We hypothesized that co-aggregation of CyPet- and YPet-tagged HTT_{ex1} fragments should lead to a time-dependent increase of FRET as the fluorescent tags are brought in close proximity when fibrillar aggregates are formed (**Figure 1C**). We treated mixtures of fusion proteins (1:1 molar ratio; 1 – 3 μ M concentrations) with PSP and quantified the spontaneous formation of Ex1Q48-CyPet/-YPet co-aggregates by repeated FRET measurements. We observed a time- and concentration-dependent increase of FRET efficiency (**Figure 1D**), indicating that FRET measurements are suitable to quantify HTT_{ex1} co-aggregation. In contrast, no time-dependent increase of FRET efficiency was observed in samples that were not treated with PSP, underlining that the removal of the GST tag from CyPet- and YPet-tagged Ex1Q48

fragments is critical for the self-assembly of co-aggregates. Proteolytic cleavage of the GST fusion proteins with PSP was confirmed by SDS-PAGE and immunoblotting (**Figure S1C**). Finally, AFM analysis confirmed that the samples indeed contain typical fibrillar HTT_{ex1} co-aggregates (**Figure S1D**).

In order to assess whether preformed Ex1Q48 fibrils can seed the co-aggregation of Ex1Q48-CyPet/-YPet, we incubated a 1:1 mixture of the GST fusion proteins with PSP and different amounts of preformed Ex1Q48 fibrils as seeds. We observed that addition of fibrils shortens the lag phase of Ex1Q48-CyPet/-YPet polymerization in a concentration-dependent manner (**Figure 1E**), indicating that they possess seeding activity. We termed the established method, which permits the quantification of mHTT seeding activity (**HSA**) in samples of interest, **FRET-based mHTT aggregate seeding (FRASE)** assay.

In independent control experiments, we also investigated whether a mixture of fusion proteins with non-pathogenic polyQ tracts, GST-Ex1Q23-CyPet/-YPet (**Figure S1A**), can be applied as reporter molecules to monitor HSA. We found that preformed, fibrillar Ex1Q48 seeds do not induce FRET when they are added to PSP treated GST-Ex1Q23-CyPet/-YPet fusion proteins (**Figure S1E**). Finally, we confirmed that addition of proteolytically cleaved GST-Ex1Q23 fusion protein as well as of uncleaved GST-Ex1Q23 or GST-Ex1Q48 fusion proteins do not shorten the lag phase of Ex1Q48-CyPet/-YPet polymerization (**Figure S1F**).

Both small and large Ex1Q48 fibrils exhibit HSA in FRASE assays

Our initial experiments indicate that large bundles of Ex1Q48 fibrils (~1-2 μm in length; **Figure 1B**) possess HSA (**Figure 1E**). We next investigated whether such an

activity can also be detected, when small fibrillar Ex1Q48 seeds are added to FRASE assays. We sonicated large preformed Ex1Q48 fibrils for different periods of time and subsequently determined the HSA. We found that seeding activity is high in sonicated Ex1Q48 preparations (**Figure S2A** and **S2B**), indicating that besides large also small Ex1Q48 fibrils possess HSA. To confirm that indeed small fibrils are produced, we analyzed the generated samples by FRA (Wanker et al., 1999). We detected large Ex1Q48 aggregates in non-sonicated samples (**Figure S2C**), while they were not observed in sonicated samples (>30 sec). This indicates that sonication (>30 sec) leads to fibril breakage and the formation of small HTTex1 structures that are no longer retained on filter membranes. Next, the samples were analyzed by dot blot (DB) assays, which allow the identification of protein assemblies on filter membranes independent of their size (Kayed et al., 2003). These experiments revealed Ex1Q48 immunoreactivity in both sonicated and non-sonicated samples (**Figure S2C**), confirming the presence of mHTTex1 protein in all samples. Finally, we analyzed the generated samples with AFM, indicating that small fibrillar Ex1Q48 structures are produced by sonication (**Figure S2D**).

FRASE assays detect HSA with high sensitivity and specificity

To investigate the sensitivity and specificity of FRASE assays, we generated recombinant Ex1Q48 seeds by sonication and analyzed them by blue native PAGE and immunoblotting. We found that sonication for 60 sec leads to the formation of Ex1Q48 structures with an average molecular weight of ~1,250 kDa (~90mers) (**Figure 2A**), while aggregates with a much larger in size were detected in non-sonicated samples.

Next, a large range of concentrations of sonicated Ex1Q48 seeds were analyzed for their activity in FRASE assays. As expected, we observed a dose-dependent shortening of the lag phase when Ex1Q48 structures were added to polymerization reactions (**Figure 2B** and **2C**). We determined a threshold of ~60 fM for detecting Ex1Q48 seeds. Furthermore, FRASE assays responded quantitatively to seeds over a dynamic range of 4 orders of magnitude (**Figure 2C**). At a concentration of ~560 fM the Z' factor (Zhang et al., 1999) was 0.67 (**Figure 2C**).

Finally, we investigated the specificity of the FRASE assay for detecting mHTTex1 aggregates. We produced fibrillar α -synuclein, tau, amyloid- β and IAPP aggregates *in vitro* and subsequently analyzed them in FRASE assays. The unrelated fibrillar aggregates did not significantly influence Ex1Q48-CyPet/-YPet polymerization (**Figure 2D**), indicating that the FRASE assay specifically detects amyloidogenic mHTTex1 aggregates. AFM analysis confirmed that fibrillar α -synuclein, tau, amyloid- β and IAPP aggregates were added to reactions (**Figure 2E**).

HSA is detectable in brains of HD mice and patients

To investigate whether FRASE assays detect HSA in complex biosamples (**Figure 3A**), we first assessed brain homogenates prepared from 12-week-old R6/2Q212 transgenic mice (carrying ~212 CAGs) and age-matched controls. R6/2Q212 mice express low levels of the human HTTex1Q212 protein (Sathasivam et al., 2013), show motor abnormalities from 8 weeks of age (Carter et al., 1999) and typical mHTTex1 inclusion bodies from 3-4 weeks onwards (Li et al., 1999). We detected high levels of HSA in brain homogenates of R6/2Q212 mice but not in those of age-matched littermate controls (**Figures 3B** and **3C**), indicating the presence of

seeding-competent mHTT_{ex1} structures. Independent control experiments with the non-pathogenic reporter molecules Ex1Q23-CyPet/-YPet did not reveal detectable HSA in R6/2Q212 brain homogenates (**Figure S3A**).

Next, we assessed whether HSA is detectable in brain extracts of 12-week-old R6/2Q51 (Larson et al., 2015) mice, which express a HTT_{ex1}Q51 fragment. In comparison to R6/2Q212 mice, these mice do not yet have a disease phenotype at 12 weeks of age, suggesting that HSA should be lower. FRASE analysis revealed that brain homogenates of prodromal 12-week-old R6/2Q51 mice do not possess significant HSA (**Figure S3B**), while activity was detectable in extracts of very old mice (104-105 weeks), which show pathological signs of disease.

We also investigated whether HSA is detectable in the hypothalamus of mouse brains, in which the proteins HTT₈₅₃-Q79 or HTT₈₅₃-Q18 were overexpressed for 8 weeks using viral vectors. Previous studies have demonstrated that hypothalamic expression of HTT₈₅₃-Q79 leads to a gain of body weight and the formation of insoluble mHTT protein aggregates (Hult et al., 2011). We found that HTT₈₅₃-Q79 mice were significantly heavier than control and HTT₈₅₃-Q18 mice (**Figure S3C**). Furthermore, we also observed a significantly higher HSA in brain homogenates of HTT₈₅₃-Q79 compared to HTT₈₅₃-Q18 and controls mice (**Figure S3D**), indicating that seeding activity and alterations in body weight are associated.

We next examined HSA in brain regions of HD patients. Protein extracts prepared from postmortem tissue (cerebral cortex, caudate nucleus and cerebellum) from HD patients and control individuals were systematically analyzed using the FRASE assay. HSA was invariably detected in HD but not in control samples (**Figure 3D**), indicating that the method is suitable to discriminate between patients and healthy individuals. Interestingly, HSA was detectable in the cerebral cortex and the caudate

nucleus, which are severely affected in HD patients (Zuccato et al., 2010), while it was not observed in the cerebellum, which is less affected in disease (DiFiglia et al., 1997). Similarly, no HSA was detectable in postmortem brains of patients with Alzheimer's disease (AD) that do not contain abnormal polyQ aggregates (**Figure S3E**).

Finally, we investigated whether HSA in biosamples indeed originates from mHTT seeds. We produced brain extracts from symptomatic 12-week-old R6/2Q212 mice and littermate controls and immunodepleted potential seeding-competent mHTT_{ex1} seeds using the monoclonal anti-HTT antibody MW8 (Ko et al., 2001). Then, samples were analyzed using FRASE assays. We observed a dramatic decrease of HSA in MW8-immunodepleted R6/2Q212 brain homogenates but not in homogenates treated with an isotype control antibody (**Figures S3F and S3G**), indicating that antibody treatment removes seeding-competent mHTT_{ex1} aggregates from mouse brain extracts. As expected, we did not detect HSA in crude brain extracts of age-matched wild-type control mice. SDS-PAGE and immunoblotting confirmed depletion of mHTT_{ex1} protein aggregates from brain homogenates by MW8 antibody treatment (**Figures S3H and S3I**).

FRASE assay detects HSA in brains of presymptomatic HD mice

To address whether HSA is detectable in brains of presymptomatic HD mice, we first analyzed non-sonicated brain homogenates of young R6/2Q212 mice and age-matched controls using the FRASE assay. We detected significant HSA in brain extracts of 2-week-old R6/2Q212 mice (**Figure 3E**) that progressively increased over time. A similar result was also obtained with sonicated brain extracts (**Figure 3F**). With sonication, significant HSA was already detectable in brains of 1-day-old R6/2Q212

transgenic mice, indicating that seeding-competent mHTTex1 structures are present in brains of R6/2Q212 mice long before inclusion bodies or motor abnormalities can be detected (Davies et al., 1997; Zuccato et al., 2010).

Next, we investigated whether HSA is detectable in presymptomatic *HdhQ150* knock-in mice that express a full-length mHTT protein with a pathogenic polyQ tract of 150 glutamines (Lin et al., 2001). These mice show onset of depressive-like symptoms by 12 months of age (Ciamei et al., 2015) and impairment of motor function at ~18 months of age. Widespread deposition of mHTT aggregates throughout the brain is observed by 8 months of age (Woodman et al., 2007). We systematically analyzed tissue homogenates prepared from cortex, striatum and hippocampus of 2-, 5- and 8-month-old heterozygous *HdhQ150* mice and littermate controls using the FRASE assay. We observed progressively increasing HSA in protein extracts from all three brain regions of *HdhQ150* but not from control mice (**Figure 3G**), confirming that mHTT seeds are detectable in HD mouse brains long before the appearance of inclusion bodies and motor abnormalities (Woodman et al., 2007).

HSA is detectable in protein fractions after depletion of large mHTTex1 aggregates by centrifugation

To investigate whether HSA in HD mouse brains originates predominantly from soluble or insoluble mHTTex1 aggregates, non-sonicated brain homogenates prepared from symptomatic 12-week-old R6/2Q212 mice were centrifuged for 20 min at 2,700 x g (low speed) or 18,000 x g (medium speed), respectively, and the resulting supernatant and pellet fractions (S1_{Low}, P1_{Low} and S1_{Med}, P1_{Med}; **Figure 4A**) were analyzed with FRASE assays. Interestingly, HSA was high in the parental crude lysate

and in the S1_{Low} fraction, while it was relatively low in the P1_{Low} fraction (**Figure 4B**), suggesting that it predominantly originates from soluble rather than insoluble mHTTex1 aggregates. A similar result was obtained when the fractions S1_{Med} and P1_{Med} were analyzed (**Figure 4B**). However, after medium speed centrifugation HSA in the P1_{Med} fraction was higher than in the P1_{Low} fraction, indicating that mHTTex1 seeds can be removed from supernatant fractions using a higher centrifugation speed. This trend was even more pronounced when the generated S1_{Med} fraction was subjected to a high-speed centrifugation (190,000 x g), resulting in the supernatant and pellet fractions S2 and P2 (**Figure 4A**). FRASE analysis revealed a significantly higher HSA in the P2 than in the S2 fraction, indicating that small seeding-competent mHTTex1 aggregates can be removed from the soluble S1_{Med} fraction by high-speed centrifugation (**Figures 4A and B**).

To obtain a first hint about the size of the seeding-competent mHTTex1 aggregates in the brains of R6/2Q212 mice, the supernatant and pellet fractions were analyzed by FRA (Wanker et al., 1999). We found mHTTex1 immunoreactivity predominantly in the P1_{Low} and P1_{Med} fractions. In comparison, weak or no immunoreactivity was detected in the fractions S1_{Low}, S1_{Med}, P2 and S2 (**Figure 4C**), suggesting that HSA in R6/2Q212 mouse brain extracts predominately originates from small rather than large mHTTex1 protein assemblies.

Finally, we used transmission immunoelectron microscopy to assess the size and morphology of mHTTex1 seeds present in P2 fractions. They exhibit high HSA but do not contain large mHTTex1 aggregates. We detected small, immunoreactive mHTTex1 fibrils with diameters of 10.2 ± 3.6 nm and lengths of 157.8 ± 64.1 nm exclusively in P2 fractions of R6/2Q212 mice (**Figure 4D**), suggesting that HSA originates from such structures in P2 fractions.

Establishment of an inducible HD *Drosophila* model

To elucidate whether mHTTex1 seeds are disease-relevant structures that cause dysfunction and toxicity in neurons, it is necessary to investigate their phenotypic consequences *in vivo* in the absence of large insoluble aggregates and without continuous overproduction of mHTT protein. We hypothesized that short-time production of small amounts of mHTTex1 seeds in adult neurons might be sufficient to cause neurotoxicity in HD flies.

Using the bacteriophage Φ C31 integration system, cDNAs encoding HTTex1 proteins with normal and pathogenic polyQ tracts (HTTex1Q17 and HTTex1Q97) were integrated into an intergenic locus (Bischof et al., 2007). Genotypes of newly created strains were confirmed by PCR using genomic DNA (**Figure S4A**). To investigate whether the generated fly strains are comparable regarding their lifespan, we generated the fly lines w1118;HTTex1Q17 and w1118;HTTex1Q97 that carry the transgenes but lack the GAL4 driver, which is required for transgene expression. Measuring fly survival revealed a median lifespan of ~102 days for both strains (**Figure S4B**).

To induce HTTex1 protein expression in adult neurons, we used the inducible, pan-neuronal Elav-GeneSwitch (elavGS) driver (**Figures S4C**). This driver (Osterwalder et al., 2001) was applied previously to create adult-onset *Drosophila* models for spinocerebellar ataxia 7 (Latouche et al., 2007), suggesting that it may also be suitable to temporarily produce polyQ-containing HTTex1 proteins in neurons. Transgene expression in neurons of elavGS flies is induced when they are supplied with food containing the hormonal inducer RU486. Transgene expression can be

switched off again, when flies are transferred back to food lacking the inducer (Rogers et al., 2012).

We first investigated whether RU486 treatment induces transgene expression in *elavGS;HTT_{ex1Q97}* flies. We incubated adult flies for 1, 3, 6 and 12 days on food containing RU486 (400 μ M) and quantified HTT_{ex1Q97} transcript levels in fly heads by qPCR. We measured high amounts of HTT_{ex1Q97} transcripts in brains of RU486 treated but not untreated flies (**Figure S4D** and **S4E**), confirming the functionality of the expression system. Similar results were obtained when *elavGS;HTT_{ex1Q17}* flies were analyzed (data not shown).

Finally, we assessed whether large mHTT_{ex1} aggregates and/or HSA are detectable in brains of RU486 treated *elavGS* HD flies. Starting at an age of 3 days, we treated *elavGS;HTT_{ex1Q97}* and *elavGS;HTT_{ex1Q17}* flies for 24 days with the hormone RU486. Head lysates were prepared and subsequently analyzed by FRA and FRASE assays. We observed the formation of large SDS-stable aggregates by FRA in head extracts of RU486 treated *elavGS;HTT_{ex1Q97}* flies (**Figure S4F**). Such structures, however, were undetectable in untreated *elavGS;HTT_{ex1Q97}* flies. Similarly, no SDS-stable aggregates were detectable in RU486 treated and untreated *elavGS;HTT_{ex1Q17}*, *w1118;HTT_{ex1Q17}* and *w1118;HTT_{ex1Q97}* flies (**Figure S4F**). Strikingly, in head lysates of hormone treated *elavGS;HTT_{ex1Q97}* flies HSA was also detectable by FRASE assays (**Figure S4G**), while it was undetectable in brains of untreated *elavGS;HTT_{ex1Q97}* flies as well as in treated and untreated *elavGS;HTT_{ex1Q17}*, *w1118;HTT_{ex1Q17}* and *-Q97* flies of the same age (**Figure S4G**).

Short-time expression of HTT_{ex1Q97} in adult neurons decreases lifespan and locomotor activity of HD flies

We first confirmed that HTT_{ex1} transcripts decline in neurons when HTT_{ex1Q97} expressing elavGS;HTT_{ex1Q97} flies are placed back on food without the inducer (**Figures S5A** and **S5B**). Next, we investigated whether both long- and short-time expression of HTT_{ex1Q17} or HTT_{ex1Q97} in adult neurons influences survival of HD flies. Starting at an age of 3 days, we treated elavGS;HTT_{ex1Q17} and elavGS;HTT_{ex1Q97} flies either continuously or only for a short time of 3 or 6 days with RU486 (**Figure 5A**); survival was measured by counting dead flies. We found that the lifespan of chronically RU486 treated elavGS;HTT_{ex1Q97} flies was significantly reduced in comparison to untreated flies (**Figure 5B** and **5C**). In strong contrast, chronic treatment with RU486 did not shorten the lifespan of elavGS;HTT_{ex1Q17} flies. We calculated a median lifespan of ~30 and ~85 days for treated and untreated elavGS;HTT_{ex1Q97} flies, respectively. Strikingly, median lifespans (~38 and ~33 days) of short-time and chronically treated elavGS;HTT_{ex1Q97} flies was similar.

As a behavioral measure of neuronal dysfunction, locomotor activity of HD flies was assessed using a negative geotaxis (climbing) assay (Latouche et al., 2007). We observed that RU486-treated elavGS;HTT_{ex1Q97} flies show a significant decline in climbing behavior in comparison to untreated controls (**Figure 5D**), confirming that both short and long-time expression of HTT_{ex1Q97} in adult neurons induces neurotoxicity in HD flies.

Formation of small, seeding-competent HTT_{ex1Q97} structures in adult neurons is associated with reduced survival

We first assessed the correlation between the formation of large, SDS-stable HTT_{ex1} aggregates in neurons and the survival of RU486-treated elavGS;HTT_{ex1}Q97 flies (**Figure 5B and 5C**). Head lysates were prepared from continuously and short-time (3 and 6 days) RU486-treated and untreated flies (**Figure S5C**) and analyzed by FRA using the anti-HTT antibody MW8 (Ko et al., 2001). We found that the abundance of large, SDS-stable HTT_{ex1}Q97 aggregates was high in heads of chronically RU486-treated elavGS;HTT_{ex1}Q97 flies but relatively low in short-time treated flies (**Figure 5E**). A similar result was obtained when the formation of large HTT_{ex1}Q97 aggregates in fly heads was quantified by FRAs using the anti-HTT antibody MAB5492 (**Figure S5D**). These results indicate that large HTT_{ex1}Q97 aggregates detected by FRAs in adult neurons cannot well predict the observed survival phenotypes, which are very similar for short- and long-time RU486-treated flies (**Figure 5B and 5C**).

We next investigated mHTT_{ex1} aggregate formation in brains of hormone-treated elavGS;HTT_{ex1}Q97 flies using an immunohistochemical method. We dissected whole brains of short- and long-time treated elavGS;HTT_{ex1}Q97 flies (**Figure S5C**) and incubated them with the antibody MAB5492. As a control, the brain sections were also immunoassayed with an anti-RBP (RIM-binding protein) antibody, which detects synapses (Liu et al., 2011). As expected, we detected high numbers of HTT_{ex1}Q97 aggregates (green puncta) in long-time and lower numbers in short-time (3 and 6 days) hormone-treated HD flies (**Figure S5E**), confirming the results obtained by FRAs (**Figure 5E**). Interestingly, these investigations also revealed very low numbers of HTT_{ex1}Q97 aggregates in brains of non-induced elavGS;HTT_{ex1}Q97 flies (**Figure S5E**), indicating that the elavGS expression system is leaky and very low levels of HTT_{ex1}Q97 protein are also produced in the absence of hormone treatment. However, such a low expression of HTT_{ex1}Q97 was not sufficient to significantly shorten the lifespan of HD flies (**Figure 5C**).

Finally, we used the FRASE assay to quantify HSA in head lysates of RU486-treated *elavGS;HTT_{ex1Q97}* flies. Strikingly, we measured high HSA in protein lysates of both short- and long-time hormone-treated flies (**Figure 5F** and **5G**), demonstrating that FRASE assays provide information that is fundamentally different from that obtained by FRAs. As the abundance of large fibrillar aggregates is very low in protein extracts of short-time treated flies (**Figure 5E** and **S5D**), HSA in these fractions must predominantly result from small structures that are not retained by the filter membrane. In contrast to the FRA results (**Figure 5E** and **S5D**), HSA levels measured with the FRASE assay (**Figure 5F** and **5G**) correlate significantly better with the increased mortality of RU486-treated *elavGS;HTT_{ex1Q97}* flies (**Figure 5H**). As expected, HSA was undetectable in head lysates of 27-day-old *elav;HTT_{ex1Q17}* control flies, which constitutively express the protein HTT_{ex1Q17} in neurons (**Figure 5F**).

Short-time expression of Hsp70 extends the lifespan of HD flies and decreases HSA in neurons

We hypothesized that co-expression of the molecular chaperone Hsp70 (*HSPA1L*) (Chan et al., 2000) might influence HSA and neurotoxicity in HD transgenic flies. To address this question, we generated *elavGS;HSPA1L;HTT_{ex1Q97}* flies, which upon hormone treatment co-produce both Hsp70 and HTT_{ex1Q97} in adult neurons. We first assessed whether in brains of RU486-treated *elavGS;HSPA1L;HTT_{ex1Q97}* and *elavGS;HTT_{ex1Q97}* flies similar levels of HTT_{ex1Q97} transcripts are expressed. We treated 3-day-old flies for 6 days with RU486 (400 μ M) and subsequently quantified mHTT_{ex1} transcript levels in fly heads by qPCR. Similar HTT_{ex1Q97} transcript levels were observed in both strains, indicating that co-expression of *HSPA1L* does not significantly influence mHTT_{ex1} expression in *elavGS;HSPA1L;HTT_{ex1Q97}* flies

(Figure S6A). To confirm the expression of Hsp70 in elavGS;HSPA1L;HTT_{ex1Q97} flies, we also analyzed protein extracts of hormone-treated animals by SDS-PAGE and immunoblotting. As expected, similar Hsp70 protein levels were detectable in head lysates of hormone-treated elavGS;HSPA1L;HTT_{ex1Q97} and elavGS;HSPA1L control flies **(Figure S6B).**

Next, we assessed whether short-time co-expression of Hsp70 (for 6 days) in adult neurons influences the survival of elavGS;HSPA1L;HTT_{ex1Q97} HD flies. In control experiments, the survival of short-time RU486-treated elavGS;HTT_{ex1Q97} flies was analyzed. We determined median lifespans of ~39 and ~33 days for RU486 treated elavGS;HSPA1L;HTT_{ex1Q97} and elavGS;HTT_{ex1Q97} flies, respectively, **(Figure 6A-6C)**, indicating that short-time co-expression of Hsp70 in adult neurons improves the survival of elavGS;HSPA1L;HTT_{ex1Q97} flies. As expected, a median lifespan of ~89 days was observed for non-treated elavGS;HTT_{ex1Q97} flies, confirming our initial results **(Figure 5C).**

To examine whether short-time co-expression (6 days) of Hsp70 in adult neurons influences mHTT_{ex1} aggregation, head lysates of 13-day-old RU486-treated elavGS;HSPA1L;HTT_{ex1Q97} and elavGS;HTT_{ex1Q97} flies were analyzed by FRAs and FRASE assays. We found that the abundance of large HTT_{ex1Q97} aggregates and HSA both were significantly decreased in brains of hormone treated elavGS;HSPA1L;HTT_{ex1Q97} flies in comparison to elavGS;HTT_{ex1Q97} flies **(Figure 6D-6F)**, substantiating our hypotheses that quantification of HSA predicts survival of HD transgenic flies.

Finally, we addressed the question whether the molecular chaperone Hsp70 associates directly with mHTT_{ex1} aggregates in fly neurons. Immunohistochemical investigations of brains prepared from 9-day-old elavGS;HSPA1L;HTT_{ex1Q97} flies

treated for 6 days with RU486 revealed partial co-localization of Hsp70 and HTT_{ex1Q97} aggregates (**Figure 6G**), supporting previous observations that Hsp70 directly targets aggregation-prone polyQ-containing HTT_{ex1} fragments (Warrick JM, 1999).

Depletion of Hsc70 increases Q35-YFP seeding activity in a *C.elegans* model

Our studies in HD flies indicate that short-time overproduction of Hsp70 decreases HSA in neurons (**Figure 6F**), suggesting that a decrease of chaperone expression might have the opposite effects. To address this question, we performed RNAi knockdown experiments in transgenic worms that overproduce the aggregation-prone protein Q35-YFP in body wall muscle cells. Previous studies have demonstrated that Q35-YFP aggregation in these cells leads to motor impairment and that this phenotype increases in severity upon knock-down of *hsp-1* (Hsc70) gene expression by RNAi (Brehme et al., 2014). We treated Q35-YFP expressing worms with *hsp-1* RNAi and assessed their motility at day five. We observed a significant reduction of motility in RNAi-treated in comparison to untreated worms (**Figure S6C**), confirming previously published results (Brehme et al., 2014). Furthermore, this phenotypic change was associated with a significant increase in Q35-YFP seeding activity measured by FRASE assays (**Figure S6D and S6E**), supporting our hypothesis that HSA is a marker of dysfunction and toxicity in model systems.

DISCUSSION

There is increasing experimental evidence that self-propagating mHTT aggregates, or seeds, play an important role in HD model organisms (Babcock and

Ganetzky, 2015; Pecho-Vrieseling et al., 2014). Whether they are indeed critical or even responsible for the appearance and progression of disease, however, is still unclear. To address this question, we developed a sensitive FRET-based biosensor (FRASE) assay that enables the quantification HSA in complex biosamples. With this assay in hand, we assessed the potential correlation between HSA in affected tissues and the appearance of disease phenotypes in various HD models (**Figures 3-6**). We e.g., detected HSA in crude brain extracts of mice weeks before manifestation of disease (**Figures 3E-3G**). Furthermore, we observed an increase of mutant HSA in mouse brain extracts concomitantly with the appearance of symptoms, suggesting that it quantitatively tracks disease progression. Finally, mechanistic studies with a newly established inducible *Drosophila* model of HD indicate a correlation between HSA in adult neurons and reduced survival of HD flies, supporting our hypothesis that mHTT seeding is a disease-relevant process. Taken together, these studies indicate that HSA is a valuable early disease marker that can predict severe downstream phenotypic changes in various HD models.

The fluorescent dye Thioflavin T (ThT) is currently utilized in a large number of cell-free assays as a reporter molecule to monitor the seeding activity of amyloidogenic protein aggregates (Gupta et al., 2012). ThT exhibits enhanced fluorescence when it is bound to β -sheet-rich amyloid structures (LeVine, 1993). However, its binding to such structures is significantly decreased, when competing proteins are present in complex amyloid polymerization reactions (Biancalana and Koide, 2010). Therefore, previously established ThT-based seeding assays are relatively insensitive when biosamples such as brain homogenates are analyzed (Gupta et al., 2012). To overcome these limitations, we have established the FRASE assay, which does not require ThT reporter molecules for the quantification of HSA in biosamples. Two

fluorescently tagged aggregation-prone HTT exon-1 fusion proteins are used as reporter molecules to monitor HSA (**Figure 3A**). This assay is highly robust and affected by contaminating proteins in complex biosamples only to a very small extent. Therefore, the FRASE assay can be employed without the need for upstream purification of mHTT seeds, which would complicate the protocol and decrease accuracy of quantification.

We observed high HSA in soluble fractions of HD mouse brain extracts (**Figure 4B** and **4C**), suggesting that seeding activity in transgenic animals predominately originates from small rather than large mHTT_{ex1} aggregates. However, inclusions with insoluble fibrillar HTT_{ex1} aggregates (Bäuerlein et al., 2017) may also possess seeding activity. Further studies will be necessary to purify fibrillar HTT_{ex1} structures of different sizes from mouse and fly brains and to compare their specific seeding activity (i.e., seeding activity per unit of protein). Our results are in agreement with previous investigations indicating that small, fibrillar polyQ-containing HTT assemblies are detectable in the cytoplasm of cells besides large inclusions with fibrillar mHTT aggregates (Sahl et al., 2012). They are also consistent with studies demonstrating that proteotoxicity in mammalian cells is associated with small, diffusible HTT oligomers rather than large inclusions (Arrasate et al., 2004; Leitman et al., 2013). However, our present study advances beyond the state-of-the-art. For the first time, we provide experimental evidence that the abundance of small seeding-competent polyQ structures correlates with dysfunction and toxicity in HD transgenic flies and worms (**Figures 5, 6** and **S6**).

Together, our investigations suggest that HSA in HD mouse and fly brain extracts is a biological marker of disease long before its onset. Previous studies argue that large inclusions with insoluble mHTT aggregates in brains of HD mice and patients

are not predictive for the development of symptoms (Kuemmerle et al., 1999). However, neuronal inclusions are commonly detected with immunohistological methods, which fail to identify small, seeding-competent mHTT assemblies. The application of the FRASE assay overcomes this important limitation associated with standard histology and is likely to yield new mechanistic insights into the progressive development of HD. We propose that in future drug trials with HD mice HSA could be utilized as an outcome marker to monitor the efficacy of therapeutic molecules *in vivo*, before and independent of changes in phenotype. As we detected robust HSA in the striatum of 2-month-old *Hdh*Q150 knock-in mice (**Figure 3G**), drug treatment could start before that point in time and animals could be assessed for HSA at any later age. Furthermore, we propose that the FRASE method can be applied as a drug screening assay to identify therapeutic molecules that directly target mHTT seeding *in vitro*. As the assay can monitor HSA in protein extracts prepared from postmortem patient brains and transgenic animals (**Figure 3D-3G**), it seems now feasible to investigate aggregate-targeting therapeutic candidate molecules in assays which contain disease-relevant seeds.

Through the application of FRASE assays, we have demonstrated that HSA is a robust, early disease biomarker in HD transgenic mice and flies. We propose that it also might be of high value for monitoring disease onset and progression in HD patients if HSA could be quantified in biosamples whose collection is technically and ethically possible, like cerebrospinal fluid, blood or muscle tissue. Through the quantification of HSA in patient samples, the optimal time point for the initiation of clinical trials could be determined and the efficacy of therapeutic interventions could be monitored. In this way, our findings may help to develop novel disease-modifying therapeutic strategies for HD and other polyQ diseases.

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AUTHOR CONTRIBUTIONS

A.B., A.A., F.S. and E.E.W. conceived the study and designed the experiments; A.B., A.A. and F.S. analyzed the data. A.A., R.H., I.J. and K.K. performed experiments with *in vitro* seeds. A.A. and R.H. performed mouse and human tissue experiments. F.S., A.A, L.B. and J.E. performed all studies with the HD transgenic flies. L.D. and A.Boe. performed aggregation experiments with non-polyQ proteins. R.H. performed immunodepletion experiments and FRAs. A.S. and A.G. performed toxicity assays with primary neurons. S.K. and B.P. labeled and analyzed EM samples. J.K. performed all experiments with nematodes. L.B. analyzed protease cleavage reactions with SDS-PAGE. B.M. performed experiments with AAV5 HTT853 injected mice. H.N. performed cell culture studies. H.K. provided cell lines. B.B. and Å.P. performed the injections of AAV vectors in FVBN/ mice and provided samples. J.M. coordinated breeding of R6/2Q51 mice and provided samples. S.F. and G.B. coordinated breeding of R6/2Q212 and *Hdh*Q150 mice and provided samples. L.B. and G.G. analyzed AFM samples. E.E.W., A.B., A.A. and S.S. conceived and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURES LEGENDS

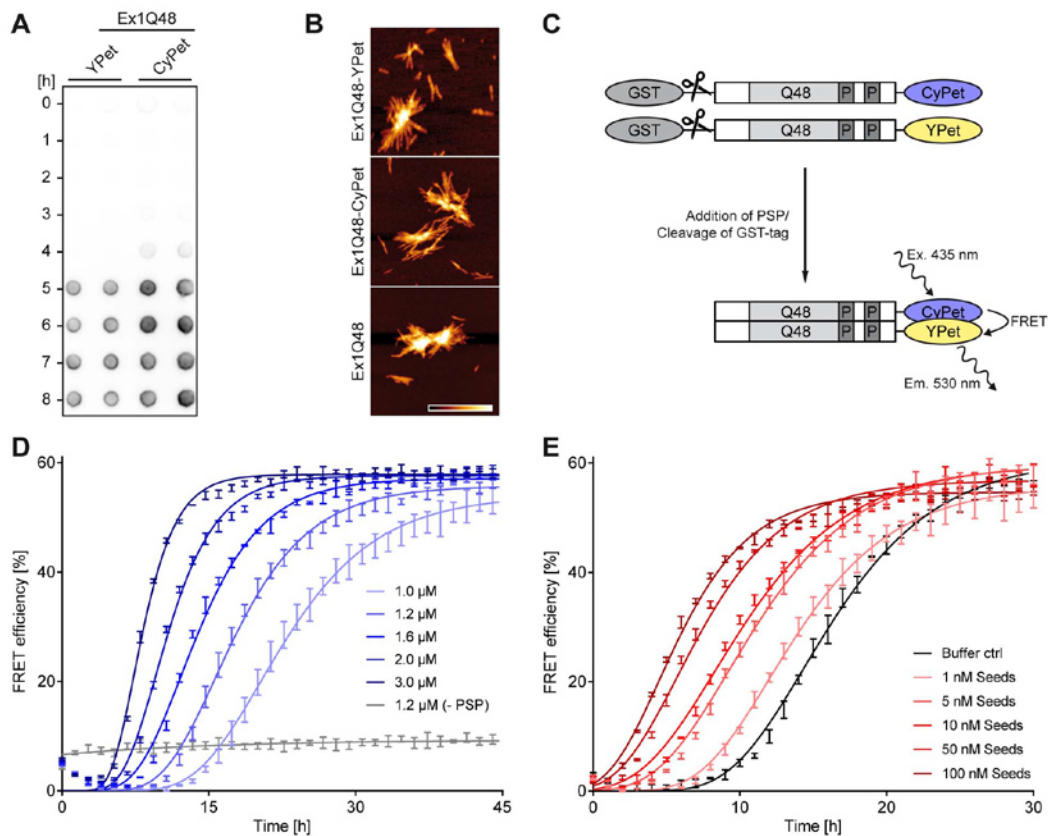


Figure 1. Establishment of a FRET-based mutant HTT aggregate seeding assay

(A) Time-dependent aggregation of Ex1Q48-CyPet and -YPet fusion proteins (3 μ M) monitored by FRA (500 ng protein per dot). Immunoblot, anti-GFP antibody.

(B) Analysis of spontaneously formed Ex1Q48-CyPet, Ex1Q48-YPet and Ex1Q48 aggregates by AFM (3 μ M) after 24 h. Scale bars: 1 μ m; color gradient represents 0-20 nm height.

(C) Schematic model of FRET-inducing co-aggregating Ex1Q48-CyPet and -YPet upon cleavage of GST fusion proteins with PSP.

(D) Spontaneous time-dependent co-aggregation of Ex1Q48-CyPet and -YPet sensor proteins (1:1 mixture) upon incubation of GST fusion proteins with PSP at 25 $^{\circ}$ C. FRET efficiency is displayed as mean \pm SD of technical triplicates.

(E) Fibrillar Ex1Q48 aggregates (seeds) induce a concentration-dependent shortening of the lag phase in Ex1Q48-CyPet and -YPet (1:1 mixture, 1.2 μ M) co-polymerization reactions. Seed concentrations are equivalent to monomer concentrations. FRET efficiency is displayed as mean \pm SD of technical triplicates.

See also Figure S1.

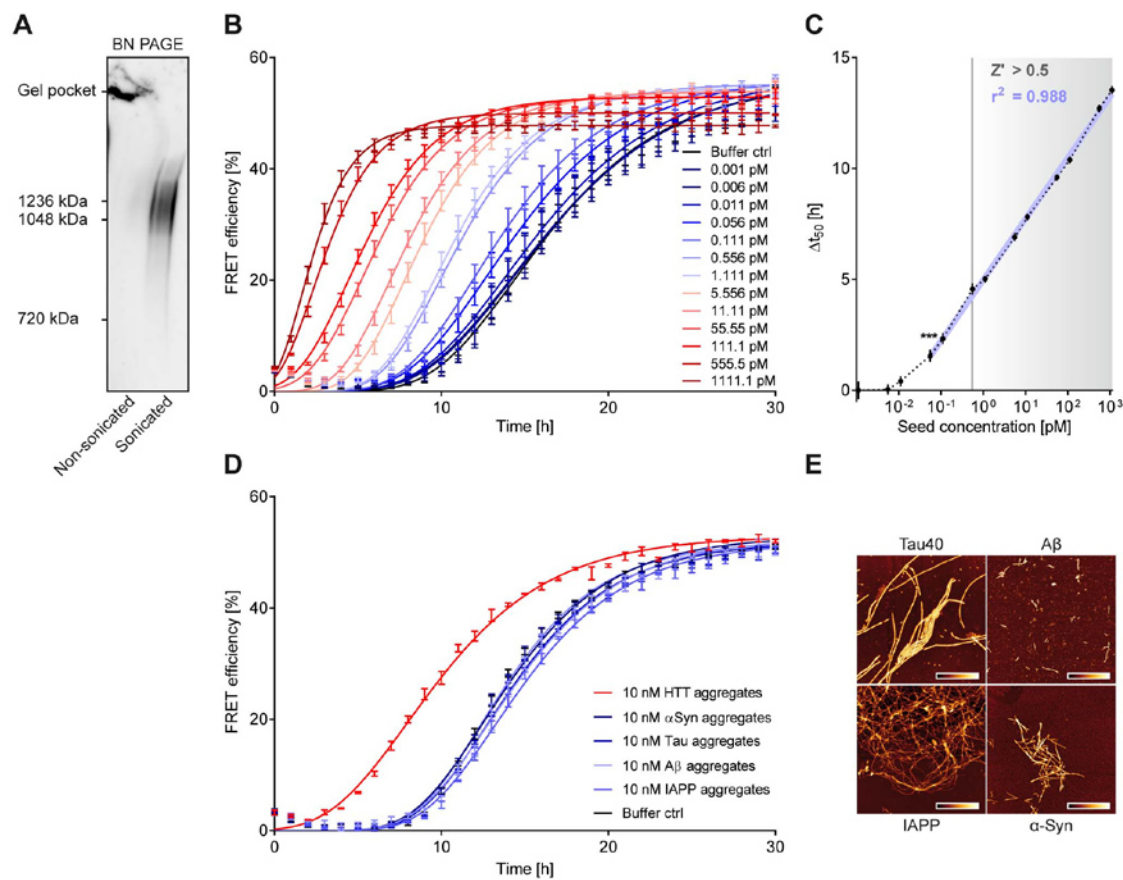


Figure 2. FRASE assays facilitate detection of HSA with high specificity and sensitivity

(A) Analysis of sonicated (1 min) and non-sonicated fibrillar Ex1Q48 aggregates by blue native (BN) PAGE and immunoblotting using HD1 antibody.

(B) Effects of small, preformed Ex1Q48 seeds (1250 kDa) on Ex1Q48-CyPet and -YPet (1:1 mixture, 1.2 μ M) co-aggregation. Data are mean \pm SEM (n = 5).

(C) Calculation of HSAs (Δt_{50} values) from aggregation profiles in **B**. Data are mean \pm SEM (n = 5).

(D) Effects of non-polyQ fibrils on Ex1Q48-CyPet and -YPet (1:1 mixture, 1.2 μ M) co-aggregation. Data are mean \pm SD of triplicates.

(E) Analysis of α -synuclein (α -Syn), amyloid- β 42 ($A\beta$), islet amyloid polypeptide (IAPP) and Tau (Tau40) fibrils by AFM. Scale bars: 1 μ m; Height of color gradients: 0-10 nm (α -Syn), 0-5 nm ($A\beta$), 0-30 nm (IAPP) and 0-10 nm (Tau40).

See also Figure S2.

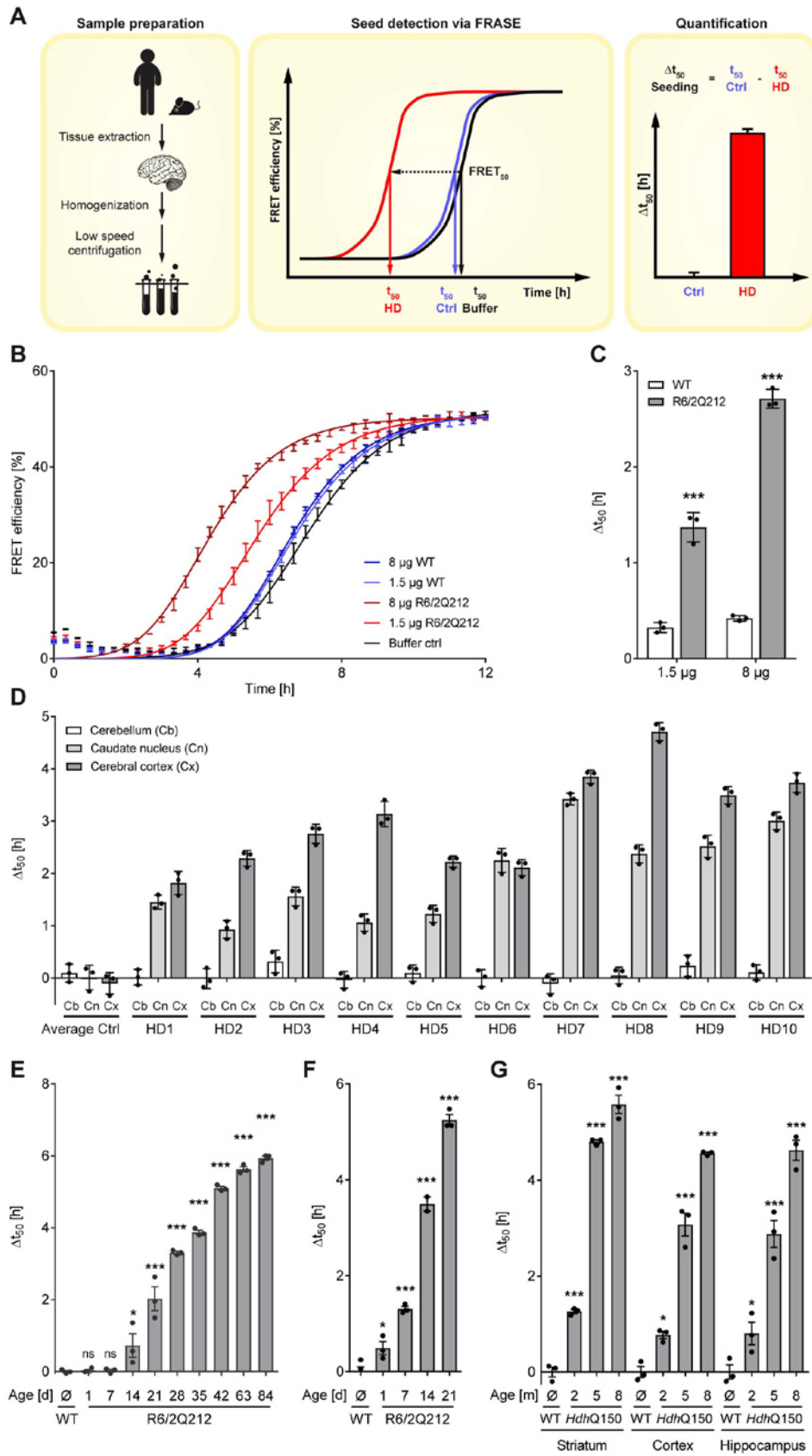


Figure 3. Quantification of HSA in brain extracts of patients and HD mice

(A) Schematic workflow for quantifying HSA in brain tissue homogenates.

(B) Effects of mouse brain homogenates on Ex1Q48-CyPet and -YPet (1:1 mixture, 3 μ M) co-aggregation. Data are mean \pm SD of technical triplicates.

(C) HSA (Δt_{50} values) of mouse brain extracts investigated in **B**. Statistical analysis: two-way ANOVA followed by Bonferroni's multiple comparison post hoc test against the respective WT controls. Δt_{50} is displayed as individual values (\bullet) and as mean \pm SD of technical triplicates.

(D) Quantification of HSA in brain homogenates prepared from HD patients and controls. For clarity, the average Δt_{50} values obtained from 3 healthy control samples are depicted (Average Ctrl). Individual values of Δt_{50} (\bullet) and mean \pm SD of triplicates are displayed.

(E) Quantification of HSA in brain extracts of R6/2Q212 and wild-type (WT) control mice (3 mice per age). Results from WT mice are shown as an average Δt_{50} value.

(F) Quantification of HSA in brain extracts of presymptomatic R6/2Q212 and wild-type (WT) control mice after sonication. Results from WT mice are shown as an average Δt_{50} value.

(G) Quantification of HSA in brain tissue extracts of *Hdh*Q150 heterozygous knock-in and WT mice. Data are mean \pm SEM ($n = 3$). One-Way ANOVA followed by Dunnett's multiple comparisons test.

See also Figure S3.

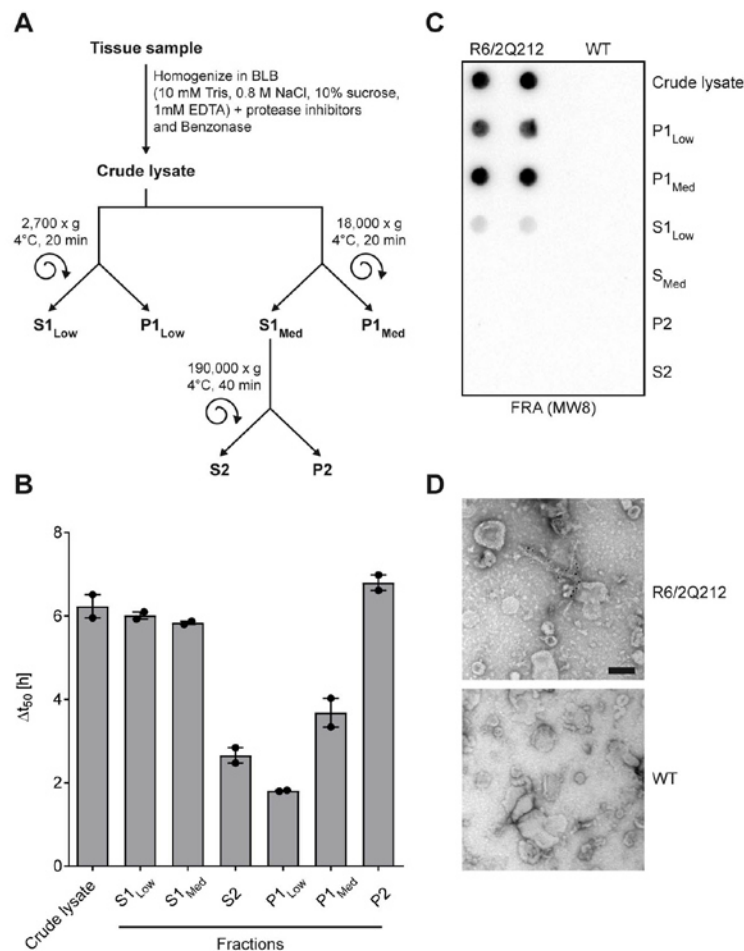


Figure 4. Detection of small HTTEx1 fibrils in soluble brain fractions with high HSA

(A) Schematic workflow for preparing soluble and insoluble protein fractions from crude tissue homogenates by centrifugation.

(B) Quantification of HSA in soluble and insoluble fractions prepared from brains of R6/2Q212 transgenic mice. In all cases, data were normalized to average Δt_{50} values of age-matched WT control mice. Bars are mean \pm SEM ($n = 2$). HSAs measured for individual mice are displayed as dots (\bullet).

(C) Detection of large, SDS-stable fibrillar mHTTEx1 aggregates in protein fractions by FRA.

(D) Detection of small, fibrillar HTTEx1 aggregates in P2 fractions of R6/2Q212 mice by immunoelectron microscopy using the anti-HTT antibody Agg53. Scale bar: 100 nm.

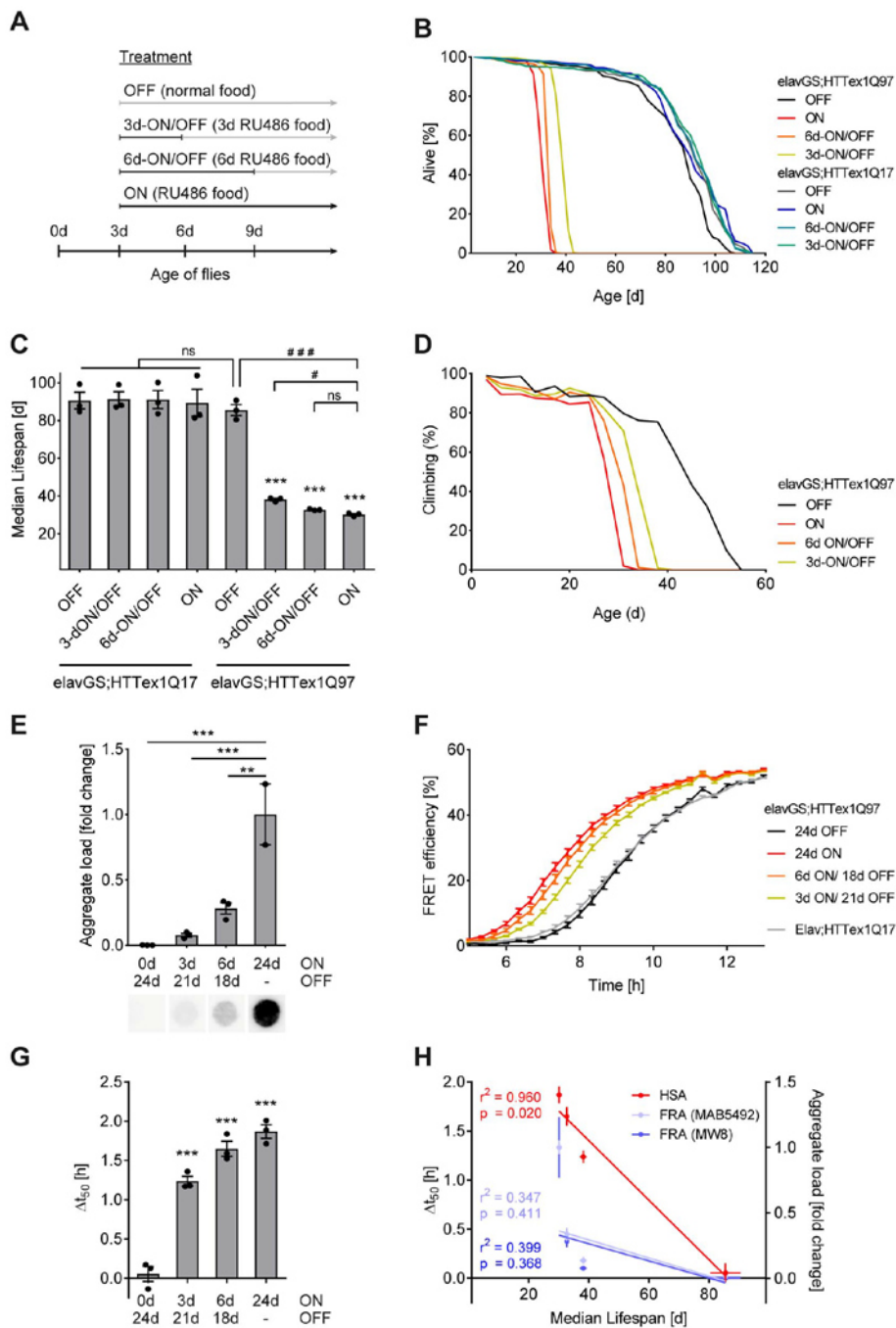


Figure 5. Short-term HTTEx1Q97 expression-induced decrease in *Drosophila* lifespan correlates with HSA but not with aggregate load in general

(A) Scheme for short- and long-time RU486 treatment of adult HD transgenic flies.

(B) Survival of RU486 treated and untreated elavGS;HTTEx1Q97 and elavGS;HTTEx1Q17 flies ($n = \sim 100$ flies/group). Survival was plotted as the percentage of surviving flies of 3 biological replicates.

(C) Median life span calculated from survival curves in **B**. Average survival of each experiment (n = ~100 flies/group) is presented as dots (●). Bars are mean ± SEM from 3 independent experiments; One-way ANOVA Dunnett's post-hoc test; ns, not statistically significant; */# p < 0.05; **/## p < 0.01; ***/### p < 0.001.

(D) Analysis of motor performance of RU486 treated and untreated elavGS;HTTex1Q97 flies (n = ~100 flies/group; three independent experiments).

(E) Quantification of large, SDS-stable fibrillar HTTex1 aggregates in heads of RU486 treated and untreated elavGS;HTTex1Q97 flies by FRA using the MW8 antibody. Representative images for each condition are shown. Data are mean ± SEM of individual measurements (●); One-way ANOVA Dunnett's post hoc test.

(F) FRASE analysis of head lysates analyzed in **E**. Values are means ± SEM of three biological replicates each performed in triplicates.

(G) Quantification of HSA (Δt_{50} values) from FRET-based aggregation profiles depicted in **F**. Results are displayed as mean ± SEM; individual measurements (●); One-way ANOVA Dunnett's post hoc test.

(H) Pearson correlation analysis. Data are mean ± SEM of the three independent experiments.

See also Figure S4 and S5.

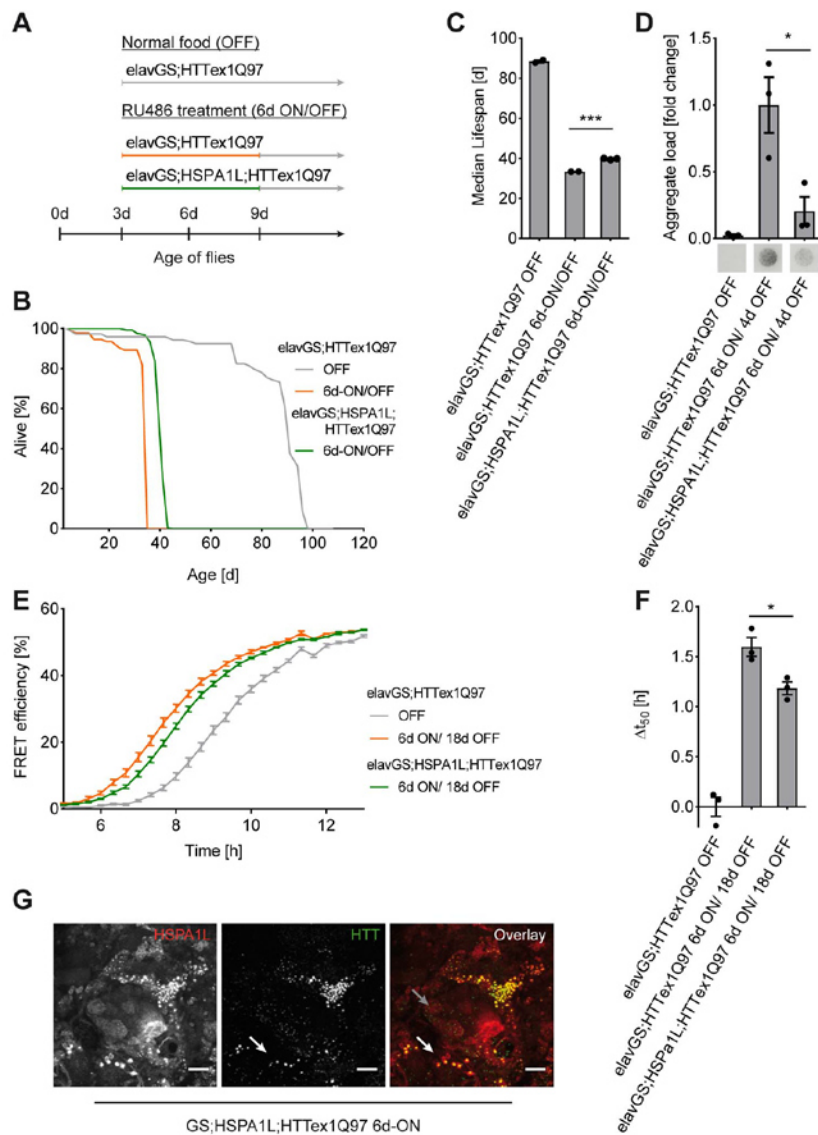


Figure 6. Co-production of Hsp70 extends survival of HD flies

(A) Scheme depicting the workflow for the temporary treatment of HD transgenic flies with RU486.

(B) Life span analysis of short-time RU486 treated elavGS;HSPA1L;HTTex1Q97 flies ($n = \sim 90$ flies/group). Hormone-treated and untreated elavGS;HTTex1Q97 ($n = \sim 40$ flies/group) were also analyzed. Three biological replicates are shown.

(C) Median life span calculated from survival curves in **B**. Bars are mean \pm SEM from three independent replicates; Unpaired t test.

(D) Quantification of large, SDS-stable HTTex1Q97 aggregates in fly heads by FRAs using the MW8 antibody. Representative images for each condition are shown. Data are mean \pm SEM; Individual measurements are presented as dots (●); Unpaired t-test.

(E) FRASE analysis of fly head lysates. Values are plotted as mean \pm SEM of 3 biological replicates each performed in triplicates.

(F) Quantification of HSA from FRET-based aggregation profiles in **E**. Data are mean \pm SEM; Individual measurements (●); Unpaired t test.

(G) Representative confocal images of the right central brain region of elavGS;HSPA1L;HTTex1Q97 flies treated for 6 days with RU486 and immunostained for HTT (Mab5492, green) and Hsp70 (anti-HSP70/HSP72, red). White arrows indicate co-localization of Hsp70 with HTTex1 aggregates; grey arrow indicates areas with no co-localization. Scale bars: 20 μ m.

See also Figure S6.

STAR * METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the corresponding author Erich E. Wanker (ewanker@mdc-berlin.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

HD mouse models

Hemizygous R6/2Q212 mice (Mangiarini et al., 1996) were bred by backcrossing R6/2Q212 males to (CBA/Ca x C57Bl/6J) F1 females (B6CBAF1/OlaHsd, Harlan Olac, Bicester, UK). *HdhQ150* heterozygous knock-in mice (Lin et al., 2001; Woodman et al., 2007) on a (CBA/Ca x C57Bl/6J) F1 background were generated by intercrossing *HdhQ150* heterozygous CBA/Ca and C57BL/6J congenic lines (inbred lines from Harlan Olac, Bicester, UK). All animals were subject to a 12 h light/dark cycle with unlimited access to drinking water and breeding chow (Special Diet Services, Witham, UK). Housing conditions and environmental enrichment were described previously (Hockly et al., 2003). R6/2 mice were always housed with wild-type mice. The CAG repeat size of the R6/2Q212 mice used in this study was 212 ± 5.27 (s.d.) and that of the *HdhQ150* heterozygotes was 160 ± 2.86 (s.d.). Hemizygous R6/2Q51 mice were derived from R6/2 parent lines by selective breeding (Larson et al., 2015) and bred by backcrossing R6/2Q51 males to (CBA x C57Bl/6) F1 females (Charles Rivers, UK). R6/2Q51 mice were maintained and bred as described previously (Larson et al., 2015). Female mice from the FVB/N strain (Charles River Laboratories, Germany) were injected at eight to ten weeks of age with recombinant adeno-associated viral (AAV) vectors of serotype 5 encoding the first 853 amino acids of either the WT form of HTT

with 18Q (HTT853- Q18) or the mutant form of the protein with 79Q (HTT853-Q97) (Baldo et al., 2013). All mice were housed in groups at a 12 h light/dark cycle. At 8 weeks post-injection, FVB/N mice were sacrificed. Overall, mice were sacrificed at different ages from 1 day up to 2 years. Tissues were strictly stored at -80 °C until use.

All animal work with R6/2Q212 and *Hdh*Q150 mice was approved by the King's College London Ethical Review panel and performed under a Home Office project license in accordance with the United Kingdom 1986 Animals (Scientific procedures) Act. All animal work with R6/2Q51 mice was approved by the University of Cambridge Ethical Review panel and performed under a Home Office project license in accordance with the United Kingdom 1986 Animals (Scientific procedures) Act. All experimental procedures with FVB/N mice were approved by the Regional Ethical Committee in Lund, Sweden.

Human brain tissue

Post mortem brain tissues from human HD and AD patients and unaffected control individuals (both male and female) were obtained from the Newcastle Brain Tissue Resource (NBTR, Newcastle University, UK). Experiments were performed in accordance with the approval of the joint Ethics Committee of Newcastle and North Tyneside Health Authority and following NBTR brain banking procedures. Tissues were collected at 34.5 ± 21.0 h post-mortem from HD patients and controls with an average age of 57.8 ± 10.7 years.

Generation and maintenance of *Drosophila* strains

ElavGS-GAL4, Elav-GAL4 and HSPA1L lines were obtained from the Bloomington *Drosophila* Stock Center. Transgenic flies were generated through cloning of cDNAs encoding HTTEx1Q17 and HTTEx1Q97 into pUAST-attB-rfA (provided by Prof. S. Sigrist, Freie Universität, Berlin) and subsequent site-directed insertion on the third chromosome (68E) using the PhiC31 integrase [Rainbow Transgenic Flies Inc. (Camarillo, CA, USA)]. All *Drosophila* strains were cultured on standard medium at 25°C and 65% humidity with a 12 h light-dark cycle. Expression of transgenes was induced by culturing flies on standard medium containing 400 µM RU486.

***C. elegans* strains and maintenance**

C. elegans Q35 AM140 (rmls132 (*unc-54p::Q35::YFP*)) were grown on NGM plates seeded with the *E. coli* OP50 strain at 20 °C. Nematodes were transferred to fresh wells or plates every day in the course of the experiment to separate them from their progeny.

METHOD DETAILS

Cloning of expression vectors

For the construction of plasmids encoding CyPet- and YPet-tagged HTTEx1Q48 fusion proteins, the coding sequence of HTTEx1Q48 was PCR-amplified from pGEX-6P1-HTTEx1Q48 using the primers 5'-gacgacgaattcatggcgaccctg-3' and 5'-gacgacctcgagtggcggtgcagcgg-3'. The resulting PCR product was digested with the restriction enzymes EcoRI and NotI. Additionally, CyPet cDNA was PCR amplified from pBAD33-CyPet-His (Addgene plasmid #14030) (Nguyen and Daugherty, 2005) with the primers 5'-acgacctcgagggtggcggtggcggtatgtctaaaggtgaagaattattcgg-3' and 5'-

gacgacgcgccgcttatttgtacaattcatccataccatg-3'. YPet cDNA was amplified from pBAD33-YPet-His (Addgene plasmid #14031) (Nguyen and Daugherty, 2005) with the primers 5'-gacgacctcgagggtggcggtggcggtatgtctaaaggtgaagaattattcactgg-3' and 5'-gacgacgcgccgcttatttgtacaattcattcataccctcg-3'. The resulting PCR fragments were cloned into the plasmids pGEX-6P1 using the EcoRI/XhoI/NotI restriction sites to obtain plasmids pGEX-6P1-HTTEx1Q48-CyPet and -YPet, respectively. To generate the plasmids encoding GST-Ex1Q23-CyPet and -YPet or GST-Ex1Q23 the coding sequence of HTTEx1Q23 was PCR-amplified from pDONR221-HTTEx1Q23 using the primers 5'-gacgacgaattcatggcgaccctg-3' and 5'-gacgacgcgccgctcgcctcgagtggcggtgcagcgg-3' (GST-Ex1Q23-CyPet and -YPet) or 5'-gacgacgaattcatggcgaccctg-3' and 5'-gacgacgcgccgctcgcctcgagtgtatggcggtgcagcgg-3'. The resulting PCR products were digested using EcoRI and XhoI endonucleases and cloned into the plasmids pGEX-6P1-HTTEx1Q48-CyPet, -YPet or pGEX-6P1-HTTEx1Q48 after excision of HTTEx1Q48 fragments by EcoRI/XhoI endonucleases.

Recombinant protein expression

The proteins GST-Ex1Q23, -Ex1Q48, -Ex1Q23-CyPet, -Ex1Q23-YPet, -Ex1Q48-CyPet and -Ex1Q48-YPet were produced in *E. coli* BL21-CodonPlus-RP and affinity-purified on glutathione-sepharose beads. Purified proteins were dialyzed over night at 4 °C against 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 5% glycerol, snap-frozen in liquid N₂ and stored at -80 °C. Protein concentrations were determined with a NanoDrop spectrophotometer. Prior to use, protein solutions were ultra-centrifuged at 190,000 x g for 40 min to remove aggregated material. α -Synuclein (α -Syn) was produced in *E. coli* BL21 (DE3) and monomeric α -Syn was purified as described elsewhere (Theillet et al., 2016). Expression of Tau40 protein was performed

in *E. coli* BL21 using a 50 l bioreactor. After cell disruption using a French press, Tau40 protein was purified via cation exchange chromatography and gel filtration. Expression and purification of Tau were performed by InVivo BioTech Services (Hennigsdorf, Germany) using proprietary company protocols.

Preparation of *in vitro* seeds

Spontaneous Ex1Q48 aggregation was initiated by addition of 14 U PreScission protease (GE Healthcare) per nmol purified GST-Ex1Q48 fusion protein (2 μ M). The aggregation reaction was performed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 mM DTT at 25 °C and constant agitation (450 rpm) for 24 h. Ex1Q23 protein for seeding experiments was prepared from GST-Ex1Q23 fusion protein using the same protocol. Synthetic human IAPP was aggregated as described previously (Gao et al., 2015). Lyophilized α -Syn was dissolved in PBS at 500 μ M and centrifuged (4 °C, 265.000 x g) after a 5 min sonication step to remove aggregated material. The supernatant was incubated for 7 d at 37 °C under constant shaking in the presence of a single glass bead. Tau40 was aggregated for 6 d at 37 °C under constant shaking in 100 mM sodium acetate, pH 7.4, and 1 mM DTT in the presence of heparin. Synthetic human A β ₁₋₄₂ was dissolved in 100 mM NaOH and diluted to 200 μ M in low salt buffer (10 mM K₃PO₄, pH 7.4, 10 mM NaCl). Aggregation was performed for 6 h at 37 °C under constant agitation.

Atomic force microscopy

Aliquots of 15 μ l aggregation reactions (24 h) were spotted onto freshly cleaved mica glued to a microscope slide. After incubation for 30 min to allow adsorption, samples

were rinsed 4 times with 40 µl distilled water and dried over night at RT. Samples were imaged with a digital multimode Nanowizard II (JPK, Germany) atomic force microscope operating in intermittent-contact mode.

Filter retardation assays

FRA were essentially performed as described previously (Wanker et al., 1999). Briefly, equal volumes of 500 ng of Ex1Q48 aggregates and 4 % SDS solution with 100 mM DTT were mixed and boiled at 95 °C for 5 min. By applying vacuum, samples were filtered through a cellulose acetate membrane with 0.2 µm pores (Schleicher and Schuell, Germany) and washed twice with 100 µl 0.1 % SDS. For analysis of tissue homogenates, 60 µg of total protein for mouse brain and 75 µg of total protein for *Drosophila* heads were filtered per dot. Membranes were blocked with 5% skim milk in PBS/0.05 % Tween20 (PBS-T) for at least 30 min. Aggregates retained on the membrane were detected using GFP, N18, MW8, Mab5492 or HD1 antibody followed by an appropriate peroxidase-coupled secondary antibody. Signals were quantified using the AIDA image analysis software (Raytest, Germany).

Dot blot assays

To estimate total HTT protein, native dot blot (DB) assays were performed as described previously (Kayed et al., 2003). Briefly, 250 ng of Ex1Q48 protein were filtered onto a nitrocellulose membrane and blocked with 5% skim milk in PBS-T. For detection, the membrane was incubated with HD1 antibody followed by an appropriate peroxidase-coupled secondary antibody. Signals were quantified using the AIDA image analysis software (Raytest, Germany).

Native gels

Protein solutions were mixed with sample buffer and loaded onto a Novex NativePAGE 3-12% Bis-Tris gradient gel (Life Technologies). NativePAGE and immunoblotting were performed according to manufacturer recommendations. Ex1Q48 aggregates were visualized as for SDS-PAGE.

SDS-PAGE and Western blotting

Samples of aggregation reactions were mixed with loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 0.1% bromophenol blue) and boiled at 95 °C for 5 min. Samples were loaded onto Novex NuPAGE 4-12% Bis-Tris gradient gels (Life Technologies). SDS-PAGE and immunoblotting were performed according to manufacturer recommendations. Ex1Q48 distribution was visualized by N18 antibody (Santa Cruz) or Ex1Q48-CyPet/-YPet with a GFP antibody (Abcam) followed by appropriate peroxidase labeled secondary antibodies.

Genotyping of *Drosophila* strains

Total genomic DNA from transgenic flies was extracted using the DNeasy® Blood & Tissue Kit (Qiagen). cDNAs encoding HTT_{ex1Q17} and HTT_{ex1Q97} were PCR amplified using the Pwo DNA polymerase (Roche) and the primers 5'-aaccgccgtaaactgc-3' and 5'-atctctgtaggtagttgtc-3'). The sizes of the resulting PCR products were analyzed by agarose gel electrophoresis.

Quantitative polymerase chain reaction (qPCR)

RNA was extracted from *Drosophila* heads using TRIzol™ Reagent (Invitrogen). cDNA was synthesized using M-MLV Reverse Transcriptase (Thermo Scientific) and qPCR was performed using the SYBR Green PCR Master Mix (Thermo Scientific). Primer pairs for HTT (sense, 5'-gacctggaaaagctgatga-3' and antisense 5'-tcatggtcggtgcagcggct-3'), and control primers for rp49 (sense 5'-tacaggccaagatcgtgaa-3', and antisense 5'-acgttgtgcaccaggaact-3') were utilized. SYBR Green analysis was performed using the ViiA7 Real-time PCR system (Thermo Scientific). The amount of mRNA detected was normalized to control rp49 mRNA values.

Viability analysis of adult *Drosophila melanogaster*

Viability assays were performed with elavGS;HTT_{ex1Q17}, elavGS;HTT_{ex1Q97} and elavGS;HSPA1L;HTT_{ex1Q97} transgenic flies by quantification of lethality of at least 100 females of each genotype and expression condition in three independent biological replicates. Flies were aged at 25°C, with 10 flies per vial and were transferred every 3-4 days. Median lifespan (age at which half of the tested population has died) was calculated by fitting survival curves to the log(inhibitor) vs. normalized response (variable slope) equation using GraphPad Prism. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparison post hoc test. *, p≤0.05; **, p≤0.01; ***, p≤0.001.

Analysis of motor performance (climbing assay)

Ten female flies were placed in a closed empty vial and gently tapped to the bottom of the vial. The percentage of flies that climbed 8 cm within 15 s was recorded. Flies were aged at 25°C (10 flies per vial) and were monitored and transferred twice a week. Motor performance was assessed for *elavGS;HTT^{ex1Q17}* and *elavGS;HTT^{ex1Q97}* flies expressing the HTT transgenes for the indicated times. 100 females of each genotype and expression condition in each of the three biological replicates were investigated.

Preparation of *Drosophila* head lysates for FRAs

Drosophila head lysates were produced by homogenizing fly heads in 2 % SDS and complete protease inhibitor cocktail using a micro pestle. Lysates were centrifuged for 10 min at 8,000 rpm (4°C). The supernatant was transferred to a new tube and total protein concentration was determined with a Pierce™ BCA assay using BSA as a standard.

Dissection and immunostaining of *Drosophila* adult brain

The whole brains of adult flies were dissected in ice-cold haemolymph-like saline (HL3) solution (Stewart et al., 1994), fixed for 20 min in 4 % paraformaldehyde (PFA) in PBS and permeabilized in PBS-T (1 % Triton™ X-100) for 20 min at RT. Samples were blocked in 10 % normal goat serum (NGS) in PBS-T (0.3 % Triton™ X-100) for at least two hours. Brains were incubated with the indicated primary antibody (1:500) in brain staining buffer (5 % NGS, 0.1 % NaN₃ in PBS-T (0.3 % Triton™ X-100)) for 48 hours at 4 °C. Subsequently, brains were washed in PBS-T (0.3 % Triton™ X-100) for 24 hours at 4°C with multiple buffer exchanges. Next, samples were incubated with appropriate secondary antibody in brain staining buffer for 24 hours at 4 °C, washed

six times for 30 min in PBS-T (0.3 % Triton™ X-100) at RT and stored in VectaShield H-100 (Vector Laboratories) at least for one day at -20 °C. Brains were mounted and imaged using the Leica TCS SP8 Confocal Microscope. Images were analyzed using Fiji.

RNA interference

For synchronization, gravid adults from one 10 cm NGM plate were collected in a canonical tube and treated with 20% alkaline hypochlorite solution under vigorous agitation for 4 min. The eggs were then washed three times with cold 0.1 M NaCl solution. The eggs were allowed to hatch in M9 medium at 20 °C for 22 h. Animals were then placed as L1 larvae onto RNAi plates that were seeded with *E. coli* expressing dsRNAi against *hsp-1* or the empty vector L4440 (control).

Fluorescence microscopy

The aggregation propensities of Q35-YFP were analyzed throughout adulthood. Animals were subjected to RNAi treatment from the first larval stage on and maintained on RNAi plates throughout the experiment. For imaging, nematodes were mounted onto 2 % agarose (Sigma) pads on glass slides and immobilized with 2 mM Levamisole (Sigma). Images were taken on a Zeiss LSM780 confocal microscope at 20x magnification. The Q35-YFP expressing nematodes were analyzed as whole nematode for quantification of the aggregates and an image was taken of the head region of every animal. 20 animals were analyzed for each condition.

Motility assay

Nematodes were transferred from liquid culture onto a blank (unseeded) NGM plate and allowed to acclimate for 15 min. The movement of the animals was digitally recorded at 20 °C using a Leica M165FC microscope with a DFC3000G digital camera and the Leica LASX Software. Movies of 10 s were captured at 10 frames/s. Animals that crossed each other or those that escaped from the field of view were excluded from analysis. 20 animals were analyzed for each condition. Captured frames were merged into *.avi format, imported into Fiji (Schindelin et al., 2012) and analyzed using the wrMTrck plugin (<http://www.phage.dk/plugins>). The average speed of each animal was calculated by dividing its body length by the duration of each track (body length per second).

Tissue homogenization

Frozen brain tissue was cut on dry ice, weighed and homogenized in a 10-fold excess (w/v) of ice-cold 10 mM Tris-HCl pH 7.4, 0.8 M NaCl, 1 mM EDTA, 10% sucrose, 0.25 U/μl benzonase and complete protease inhibitor cocktail with a dounce homogenizer. The homogenate was incubated for 1 h at 4 °C on a rotating wheel and centrifuged for 20 min at 2,700 x g (4 °C) to remove cell debris. *Drosophila* heads were processed comparably using 10 μl of ice-cold 10 mM Tris-HCl pH 7.4, 0.8 M NaCl, 1 mM EDTA, 10% sucrose and a complete protease inhibitor cocktail per fly head. Homogenates were centrifuged for 10 min at 8,000 rpm (4 °C). After centrifugation, supernatants were transferred to a new tube and total protein concentration was determined with a Pierce™ BCA assay using BSA as a standard. For FRASE analysis, 0.8-5 μg total protein per replicate were applied.

Electron microscopy

Total brain homogenate was centrifuged at 18,000 x g at 4 °C for 20 min; the resulting supernatant was pelleted by ultra-centrifugation at 190,000 x g for 40 min and resuspended in 10 mM Tris-HCl (pH 7.4). Immunolabeling was performed with minor modifications as described (Laue, 2010). Briefly, samples were incubated on formvar-coated copper grids (Plano) for 10 min before immunolabeling. Grids were blocked and washed in PBS supplemented with 1% BSA and 0.1% glycine. Labeling was performed with the anti-HTT aggregate antibody Agg53 and an appropriate 12 nm colloidal gold-labeled secondary antibody (Jackson ImmunoResearch). Samples were stained with 2% uranyl acetate and imaged with a Zeiss EM 910 transmission electron microscope at 80 kV. Acquisition was performed with a CDD camera (Quemesa, Olympus Viewing System).

Immunodepletion of HTTex1 aggregates from mouse brain extracts

Protein G-coupled magnetic beads (Life Technologies) were incubated with 4 µg MW8 (Developmental Studies Hybridoma Bank, DSHB) or IgG isotype control (Invitrogen) antibody, respectively, for 10 min at RT to allow antibody binding. Free binding sites were saturated with Pierce protein-free blocking solution according to manufacturer recommendations. 500 µg brain homogenate in brain lysis buffer were incubated with antibody coupled beads for 3 h at 4 °C under constant overhead rotation. Subsequently, aliquots from the supernatants were taken and analyzed with the FRASE assay.

FRASE assay

Purified GST-Ex1Q48-CyPet and GST-Ex1Q48-YPet were diluted in aggregation buffer at an equimolar ratio to a final concentration of 1.2 μM (0.6 μM each) with 14 U PSP per nmol sensor proteins if not stated otherwise. The solution was then mixed with preformed aggregates of Ex1Q48 (seeds) at varying concentrations with or without prior sonication and transferred to a black 384-well plate (with a final reaction volume of 30 μl per well and a sensor protein concentration of 1.2 μM). For quantification of seeding-competent HTT species in tissue samples, the sensor-protein mixture was supplemented with up to 10% (v/v) tissue homogenate. Fluorescence signals were measured every 20 min following a 5 s pulse of vertical shaking with a Tecan M200 fluorescence plate reader at 25 °C for up to 48 h. CyPet donor fluorescence was measured at excitation (Ex): 435 nm/emission (Em): 475 nm; YPet acceptor fluorescence at Ex: 500 nm/Em: 530 nm; the FRET channel (DA) was recorded at Ex: 435 nm/Em: 530 nm. Raw signals were processed by subtracting the background fluorescence of unlabeled Ex1Q48 in all channels. Signals in the FRET channel were corrected for donor bleed-through (c_D) and acceptor cross excitation (c_A) using donor- and acceptor-only samples to obtain sensitized emission. Finally, sensitized emission was normalized to the acceptor signals (Jiang and Sorkin, 2002). In brief, the FRET efficiency E (in %) was calculated as follows: $E = (DA - c_D \times DD - c_A \times AA) / AA$ with DD = donor channel signal and AA = acceptor channel signal.

Quantification of mutant HTT seeding activity (HSA)

Seeding effects (Δt_{50} [h]) were quantified by subtracting the t_{50} values (time at half-maximal FRET efficiency) of the respective sample from the negative control. To obtain

the t_{50} values, the aggregation kinetics were curve fitted by Richard's five-parameter dose-response curve using GraphPad Prism.

$$y = y_0 + \left(\frac{y_\infty - y_0}{(1 + 10^{((\text{Log}xb - x) \times \text{HillSlope}))^s}} \right)$$

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters including the exact value of n , the definition of center, dispersion and precision measures (mean \pm SEM or mean \pm SD) as well as the statistical analysis chosen and statistical significance are reported in the figures and figure legends. Data is judged to be statistically significant when $p < 0.05$ by the indicated statistical test. In figures, asterisks denote statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Statistical analysis was performed in GraphPad PRISM 7.

Figure 1

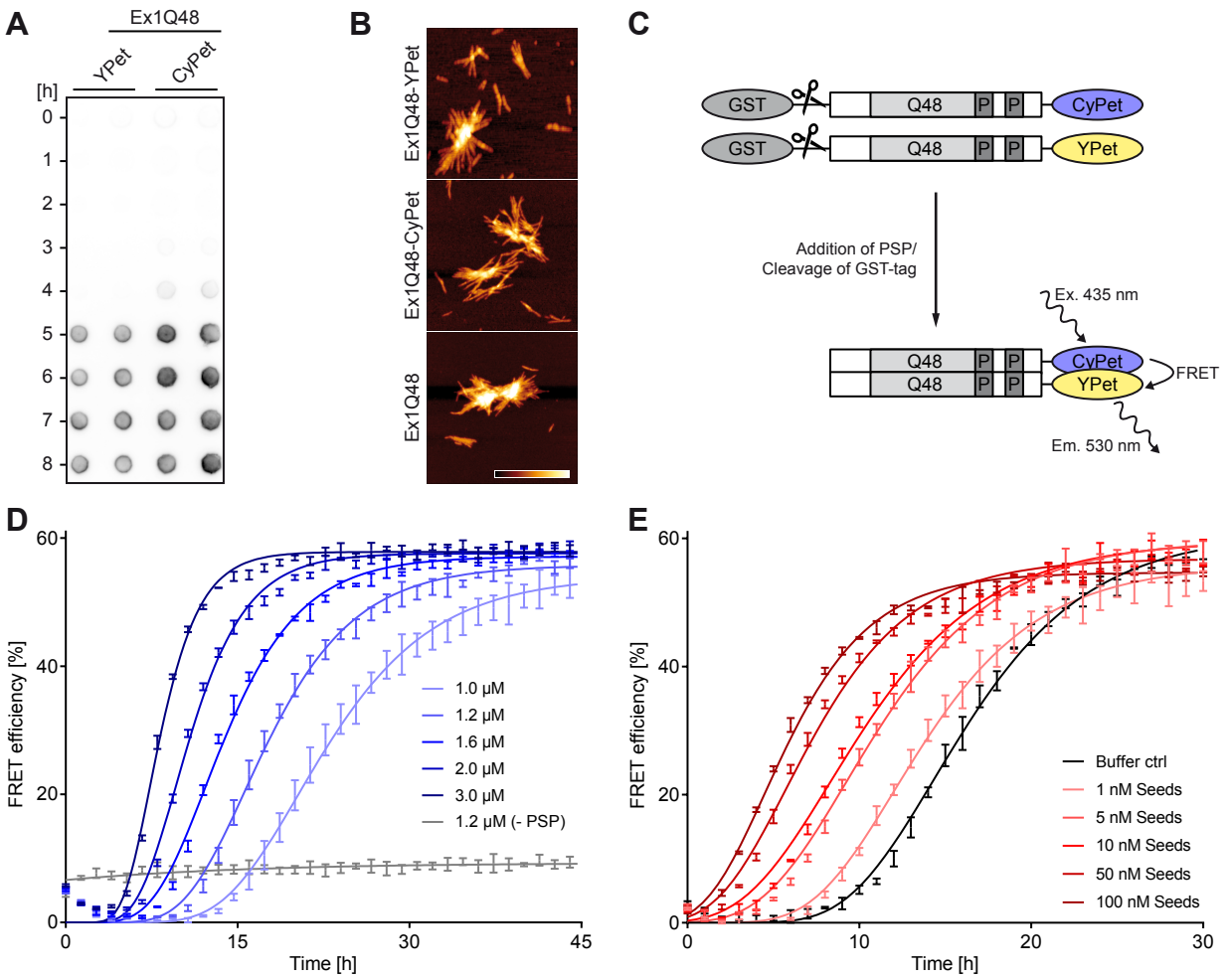
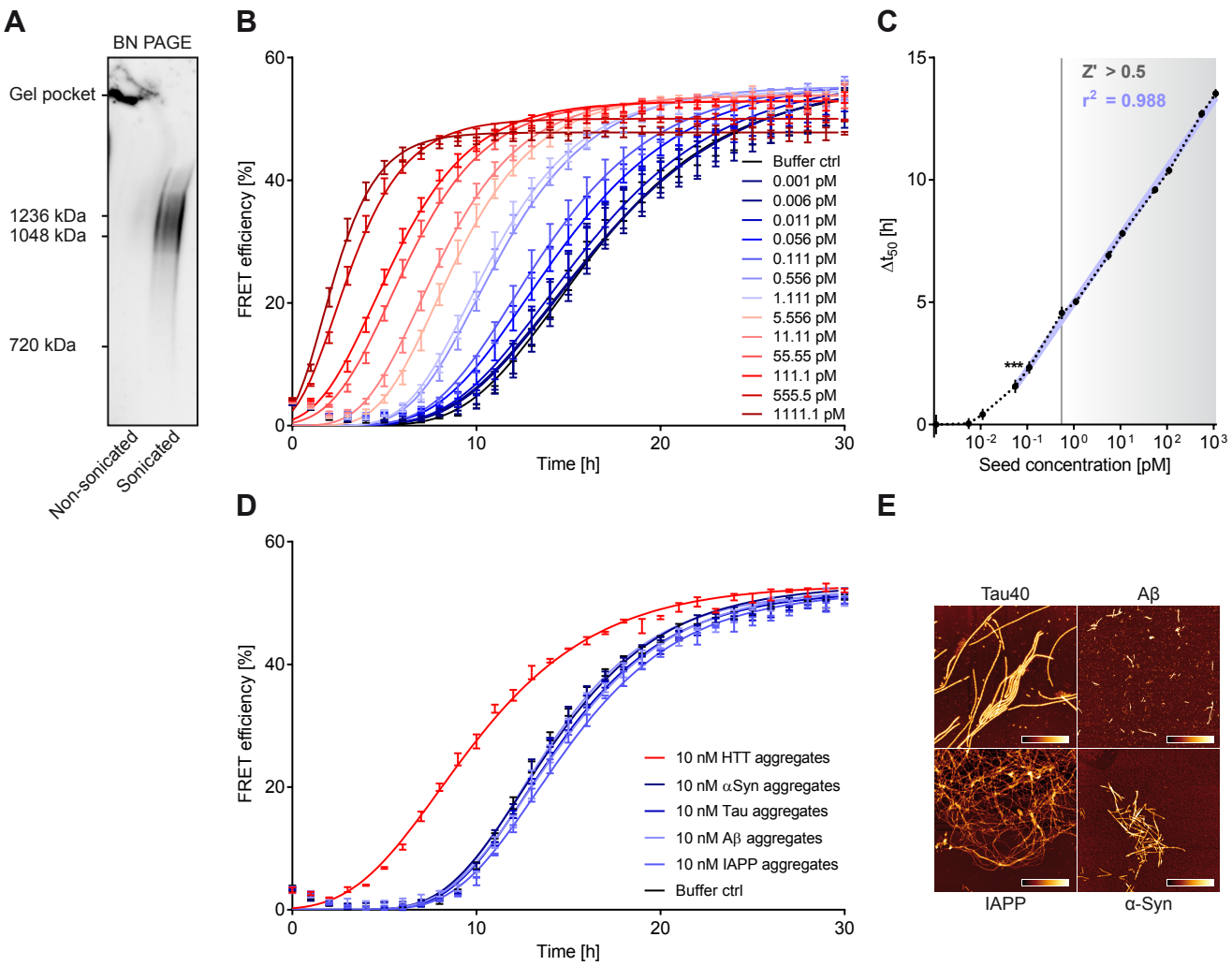


Figure 2



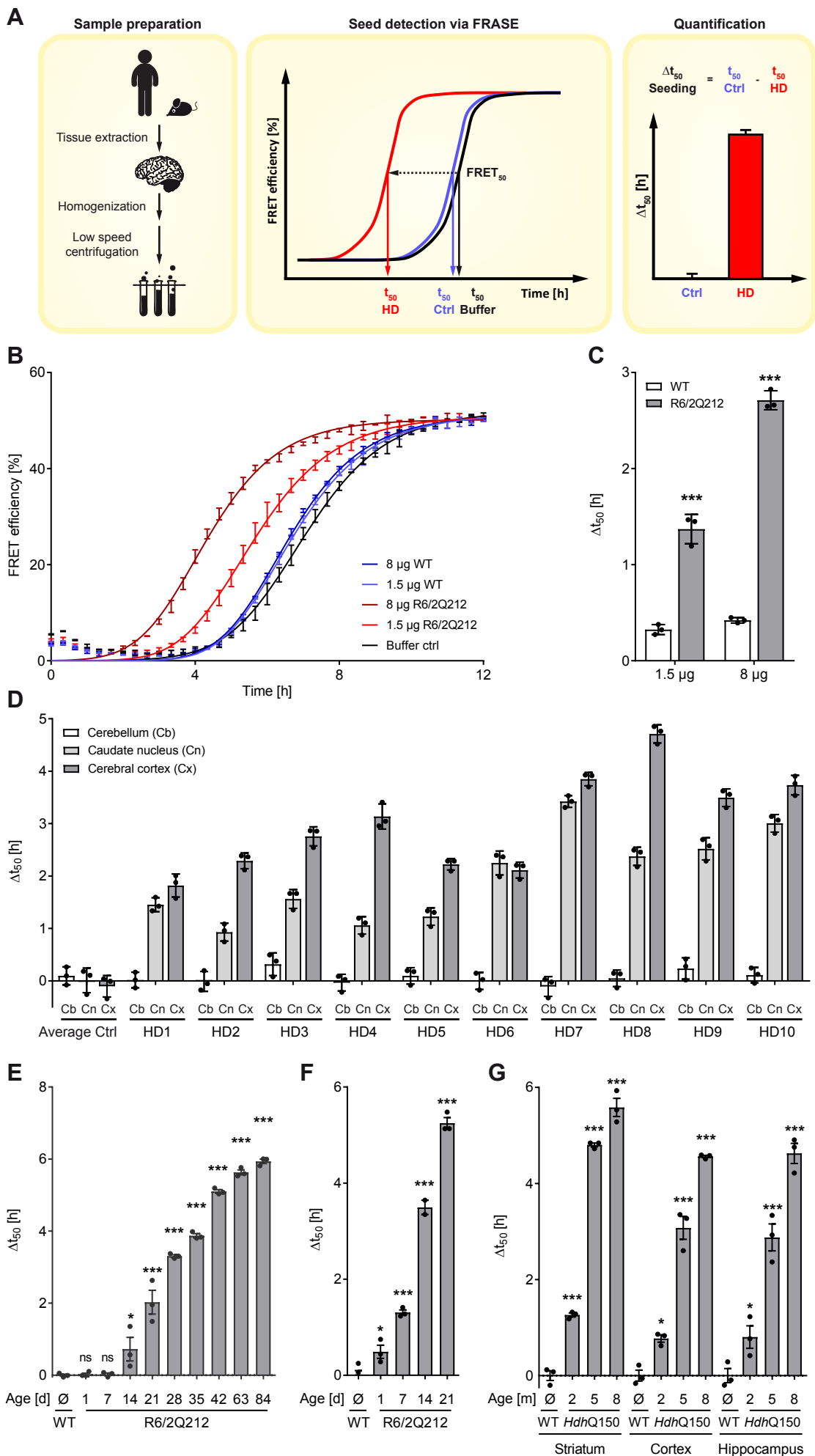
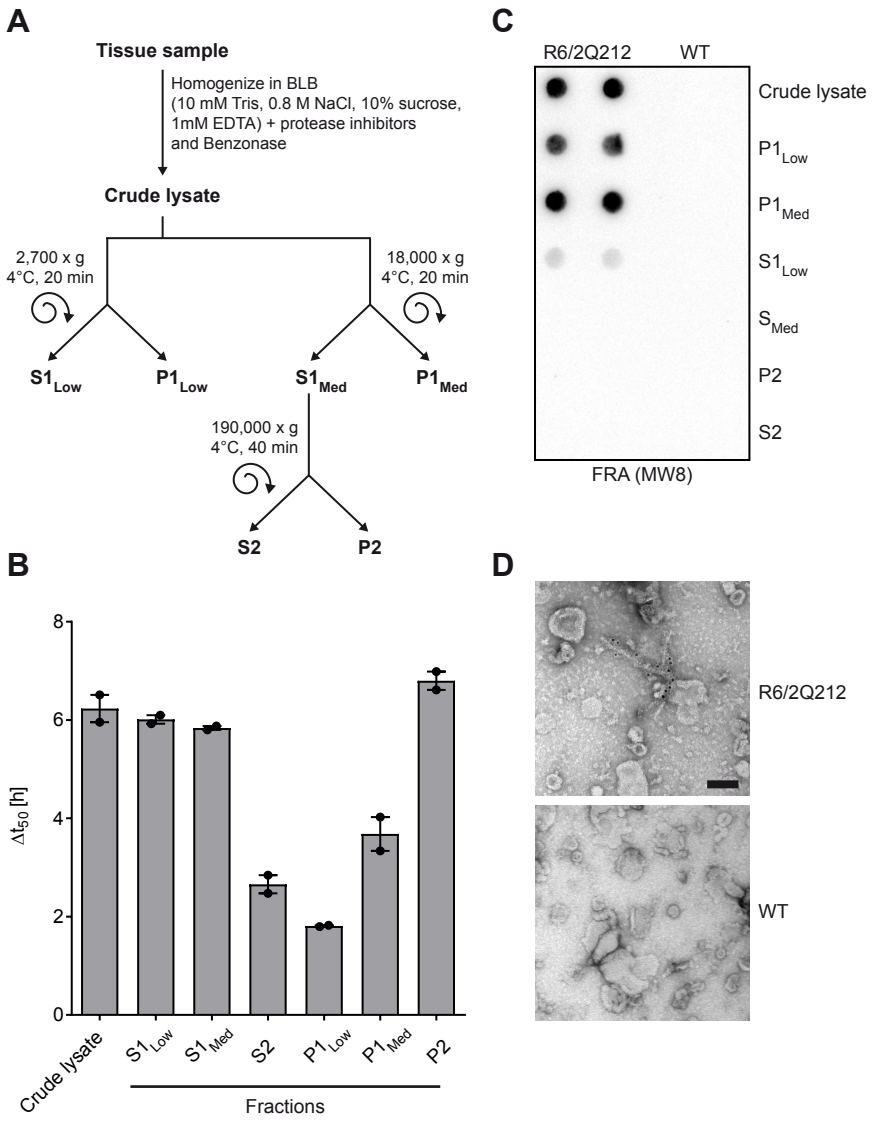


Figure 3

Figure 4



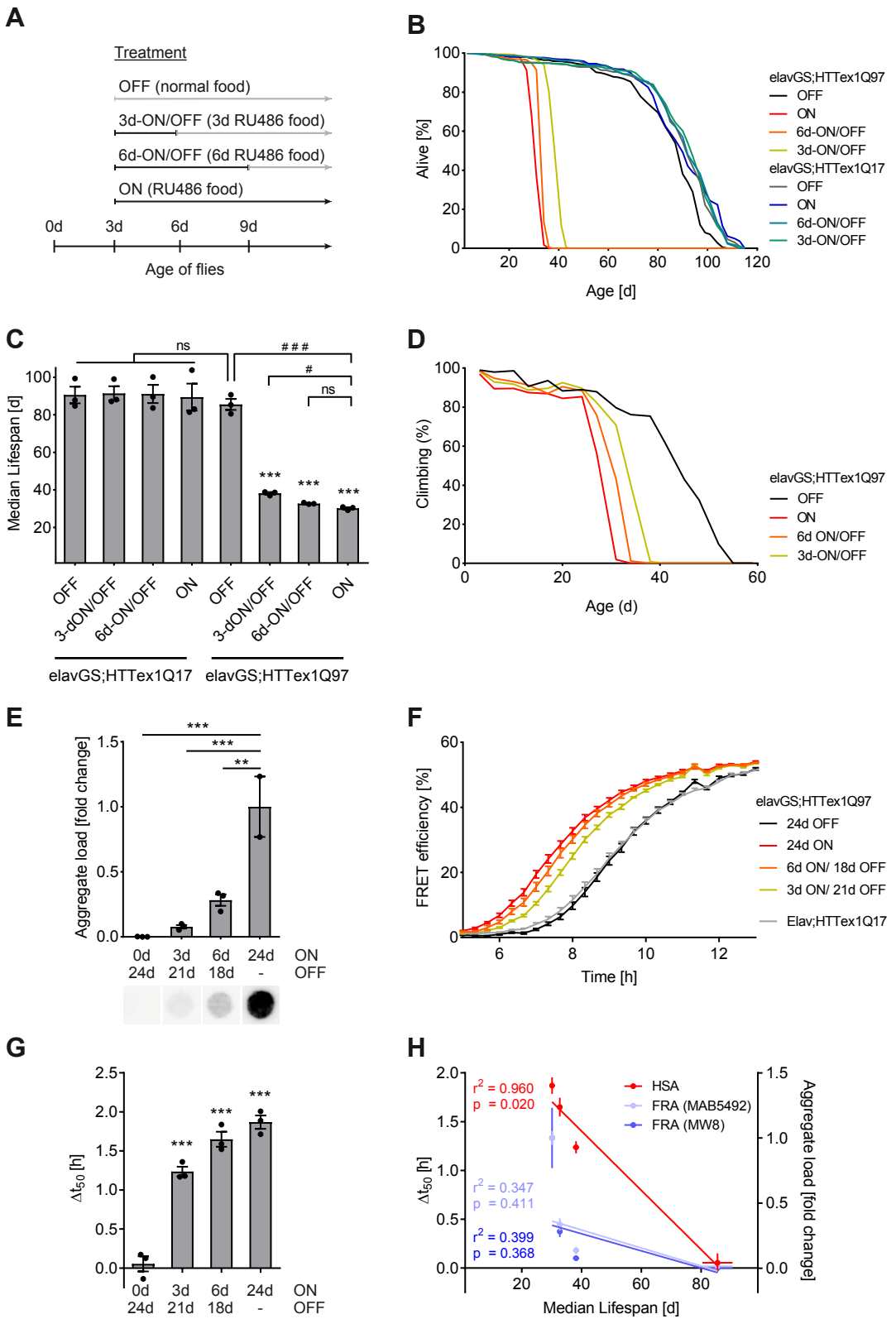


Figure 5

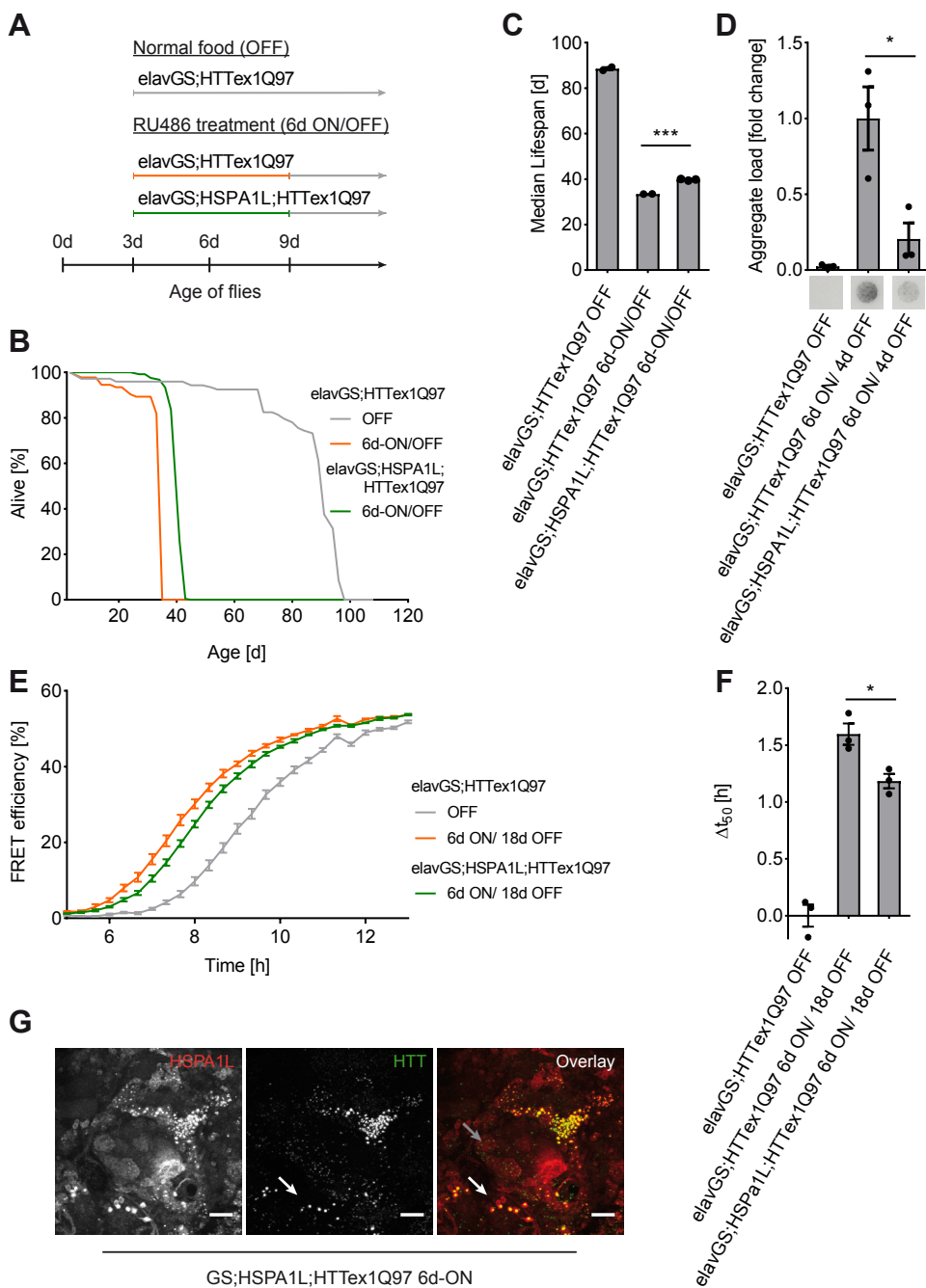


Figure 6