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A Genomewide RNA Interference Screen for Modifiers of Aggregates Formation by Mutant Huntingtin in Drosophila

Sheng Zhang, Richard Binari, Rui Zhou and Norbert Perrimon

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FILE S1

Materials and Methods

Plasmid constructs

DNA fragments containing the mutant Httex1-Qn-eGFP variants (Q25, Q46, Q72 and Q103) were cloned from DNA constructs generously provided by the Hereditary Disease Foundation (HDF) (originally from Dr. A. Kazantsev (KAZANTSEV *et al*. 1999)). To clone into the hygromycin-resistance pMK33 vector which contains the copper-inducible *metallothionein* promoter, the Httex1-Q25-eGFP and Httex1-Q103-eGFP fragments were digested with XhoI and SpeI restriction enzymes and inserted into the same sites in the pMK33 vector, while the Httex1-Q46-eGFP as well as Httex1-Q72-eGFP were amplified by PCR and inserted blunt-ended into EcoRV site in the pMK33 vector.

For generating transgenic flies, DNA containing the Httex1-Q25-eGFP and Httex1-Q103-eGFP fragments were digested with XhoI and XbaI and inserted into the same sites in the pUAST vector (BRAND and PERRIMON 1993), and DNA containing the Httex1-Q46-eGFP and Httex1-Q72-eGFP fragments were digested with KpnI and SpeI and inserted into the same sites in the pUAST vector.

A cDNA encoding CG6603 (the fly Hsp110) was obtained from the Berkeley *Drosophila* Genome Project (clone ID LD32979). The EcoRI/XhoI fragment containing full-length CG6603 was cloned into the EcoRI and XhoI sites of pUAST vector.

Cell culture

Drosophila SL2 cells (Schneider's Line S2 cells; http://www.flyrnai.org) were grown at 25°C in Schneider's media (GIBCO) with 5% heat inactivated fetal bovine serum (FBS; JRH Biosciences). Each of the mutant Httex1-Qn-eGFP constructs in pMK33 vector was transfected into SL2 cells using Effectene reagents (QIAGEN) and selected with 0.2mg/ml of Hygromycin consecutively for 5 generations to establish stably-transformed SL2 lines. The resulting stable cell lines are maintained in Hygromycin-containing medium.

Secondary screens

To eliminate genes that could indirectly affect aggregates formation, the 644 candidate genes from the primary screen were further evaluated and tested using the following criteria (Figs. 2E , S2 and their legends for more details): (1) *Remove dsRNAs with significant off-target effects:* we removed candidates with dsRNA amplicons that contain 21-bp overlaps with more than 5 other genes in the genome, as knockdown of the expression of these overlapping genes would be expected to cause a significant non-specific off-target RNAi effects (KULKARNI *et al.* 2006; MA *et al.* 2006). (http://www.flyrnai.org/); (2) *Remove candidates that function in general protein synthesis*: we studied the available information about the known functions of the candidate genes isolated from the primary screen, mainly through checking the references in Flybase (http://flybase.bio.indiana.edu/) and the PubMed in NCBI (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/). From these analyses, we found that a large number of genes are involved in general protein synthesis, including 97 genes encoding cytoplasmic or mitochondrial ribosomal proteins. Knockdown of all these genes in the assay significantly reduced aggregates formation, but since the formation of aggregates depends upon the amount of available mis-folded Htt protein (SCHERZINGER *et al*. 1999), it is highly likely that the observed reduction is not specific to aggregates formation, but simply reflects a general decrease in overall protein synthesis within the cell. Accordingly, most of these ribosomal proteins were not pursued in the following validation steps. (3) *Re-test the dsRNAs from the primary screen*: to ensure the reproducibility of the effects of these candidate dsRNAs on aggregates formation, we performed an intermediate screen by re-synthesizing and re-testing the amplicons specific to genes

retained from the primary screen up to this point. More specifically, DNA templates for the dsRNA amplicons used in the primary screen were re-amplified, and the corresponding dsRNAs were re-synthesized and re-tested for 8 additional rounds. Candidates that failed to repeat their effect on modulating aggregation in these additional rounds of testing were removed; (4) *Luciferase assay*: since the expression of the Httex1-Q46-eGFP reporter in the screen was controlled by the inducible *metallothionein* promoter, genes that regulate the activity of this promoter would also be identified in the primary and intermediate screens. To eliminate such promoter-related false positives, we established stable cell lines in which the expression of luciferase was controlled by the same *metallothionein* promoter, and performed luciferase-based assays to examine the effect of candidate dsRNAs on the activity of the *metallothionein* promoter (see below). Using this luciferase-based secondary assay, we eliminated a number of candidates that affect cellular copper uptake or the activity of the *metallothionein* promoter in the pMK33 vector, which controls the expression of the Httex1-Qp46 reporter (*e.g.,* COP complex components). (5) *Validation with 2nd set of dsRNAs*: to further ensure that the modulating effect observed in the primary screen was specific only for the candidate genes, for each candidate that passed the above selections, one or two more sets of dsRNAs targeting different regions of this candidate gene were synthesized and re-tested. Genes that failed to repeat their effect on modulating aggregation in these additional rounds of testing were removed from consideration.

As with the primary screen, 384-well plates were used in all the secondary assays, with 5ul of 50ng/ul dsRNA samples or water controls aliquoted into each well in the plate. As different to the primary screen, in each secondary assay plate, more than 100 evenly-positioned wells were aliquoted with 5ul of water as controls. For all the secondary assays, the effect of dsRNA treatment on aggregates formation was evaluated on the same three evaluation parameters (*i.e.,* the average number, size and intensity of the aggregates), but instead of using the values from the whole plate as an evaluation standards, average values and standard deviation (SD) from these more than 100 water control wells were used as evaluation standards for each plate. Accordingly, in the secondary assays, those dsRNAs that decreased or increased aggregates formation by more than 2xSD of the water controls on the plate were considered to have a significant effect on aggregates formation and were selected as hits.

126 hits passed all the above selection steps. Table S2 provides details regarding the amplicons used in this study, and additional information is available at the DRSC website (http://www.flyrnai.org).

Luciferase-based assay on the *metallothionein* promoter

Two stable cell lines (RZ-1 and RZ-14) were generated, each carrying three transgenes encoding the Firefly luciferase, Renilla luciferase and a hairpin (a *Renilla luciferase* hairpin in RZ-14, a *firefly luciferase* hairpin in RZ-1), all under the control of the *metallothionein* promoter. About 20,000 cells were treated with ~200 ng dsRNA in 384-well plates and induced with 25 uM $CuSO_4$ 72 hours after dsRNA treatment. Luciferase assay was performed after another 48 hours following the manufacture's recommendation (Promega). The firefly luciferase activity (in RZ-14) and the *Renilla* luciferase activity (in RZ-1) were employed to access the effect of dsRNA treatment on the *metallothionein* promoter activity.

***Drosophila* stocks and genetic crosses**

pUAST-dHsp110 (CG6603) DNA and pUAST-Httex1-Qn-eGFP DNA were injected into *w1118* embryos and transformants were selected following standard procedures. Around 20 independent transgenic lines for each of the constructs were established and tested. Targeted expression of Httex1-Qn-eGFP (Q25, Q46, Q72 and Q103) or dHsp110 (CG6603) was achieved using the binary UAS-Gal4 expression system (BRAND and PERRIMON 1993). A *gmr-Gal4* driver was used for all eye-specific expression (HAY *et al*. 1994).

Although CG6603 encodes the only Hsp110 ortholog in *Drosophila*, alleles of CG6603 refer to it as Hsc70Cb, solely due to its cytological location at polytene band 70C. To avoid confusion with the general Hsp70 proteins, we renamed it it as dHsp110. The following mutant alleles for *Drosophila dhsp110* (CG6603) were tested: *l(3)70Ca*^{1} (From the Bloomington Stock Center, stock # BL-4911), *l(3)00082* (BL-11485), *l(3)S148513* (from the Szeged *Drosophila* Stock Centre at University of Szeged, stock

010975), *l(3)S004112* (stock # 0100040), *l(3)S031820* (stock # 0100228), *l(3)S064906* (stock # 0100467), and *l(3)S0134802* (stock # 0100866). The following *dhsp110* alleles showed dosage-dependent genetic interaction with the HD93 flies: *l(3)00082*, *l(3)S031820, l(3)S064906, l(3)S004112* and *l(3)S0134802*. To test for genetic interactions, HD93 flies (Httex1p-Q93, genotype *gmr-Gal4/+; UAS-Httex1p-Q93*. from Drs. L. Thompson and J.L. Marsh (STEFFAN *et al*. 2001)) were crossed to the above *dhsp110* mutant alleles or the *UAS*-dHsp110 transformants, and *w1118* or *UAS*-LacZ transgenic flies were used as cross controls. The resulting trans-heterozygous progeny were collected and aged for the same time as the progeny from the *w1118* and *UAS*-LacZ control crosses (genotype for the mutant *dhsp110* crosses: *gmr-Gal4/+; UAS-Httex1p-Q93/+; dhsp110*$^{-}$/+; genotype for the *UAS*-dHsp110 cross: *gmr-Gal4/+; UAS-Httex1p-Q93 /+; UAS-dHsp110*/+; genotype for the *w1118* control: *w1118; gmr-Gal4/+; UAS-Httex1p-Q93*/+. genotype for the *UAS*-LacZ control: *gmr-Gal4/+; UAS-Httex1p-Q93/+; UAS-LacZ*/+). Eye imaging was done using a Zeiss Stemi SV11 microscope. To generate mosaic mutant clones, three *dhsp110* alleles, *l(3)00082, l(3)S031820* and *l(3)S064906,* were recombined onto an FRT80B chromosome, and mosaic mutant clones in adults were generated according to standard procedures using the *eyeless*-Flipase and *hs*-Flipase drivers *(XU and RUBIN 1993)*. In the eye, mosaic clones homozygous for either of the three *dhsp110* alleles were not viable.

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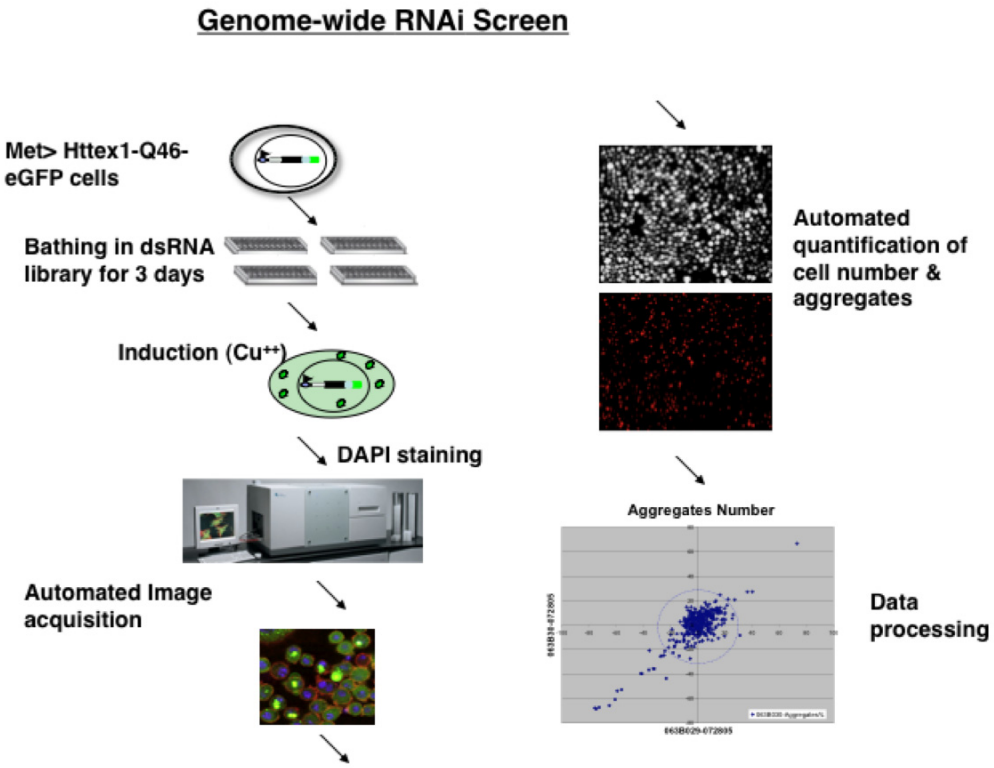

FIGURE S1.—Procedure for genome-wide RNAi screen on aggregation modulators. Httex1-Qp46 cells were mixed with the dsRNAs in 384-well plates for 3 days to knock down target gene expression. Copper ($CuSO_4$) was then added to induce reporter expression and aggregates formation, and after two days, the cells were fixed and stained with DAPI and Tritc-labeled phalloidin to reveal the cell nuclei and overall cell morphology, respectively. Images, from four sites in each well (equal to about 4,000 cells), were then collected to identify the eGFP aggregates. Information on both the aggregates and cell number in the imaged fields were automatically quantified using the Metamorph analytic software (Fig. 2C, also see Materials and Methods for details). This method allowed us to accurately quantify the effect of dsRNA treatment on the average number, size and intensity of aggregates, which were normalized with cell numbers. For each plate, the average value and standard deviation (SD) for these three parameters from the whole plate samples were also calculated. In the primary screen, for a dsRNA-treated sample, if the value of any of the three parameters was beyond 2xSD of the whole plate average, it was considered to have a significant effect on aggregates formation and was selected as a potential candidate.

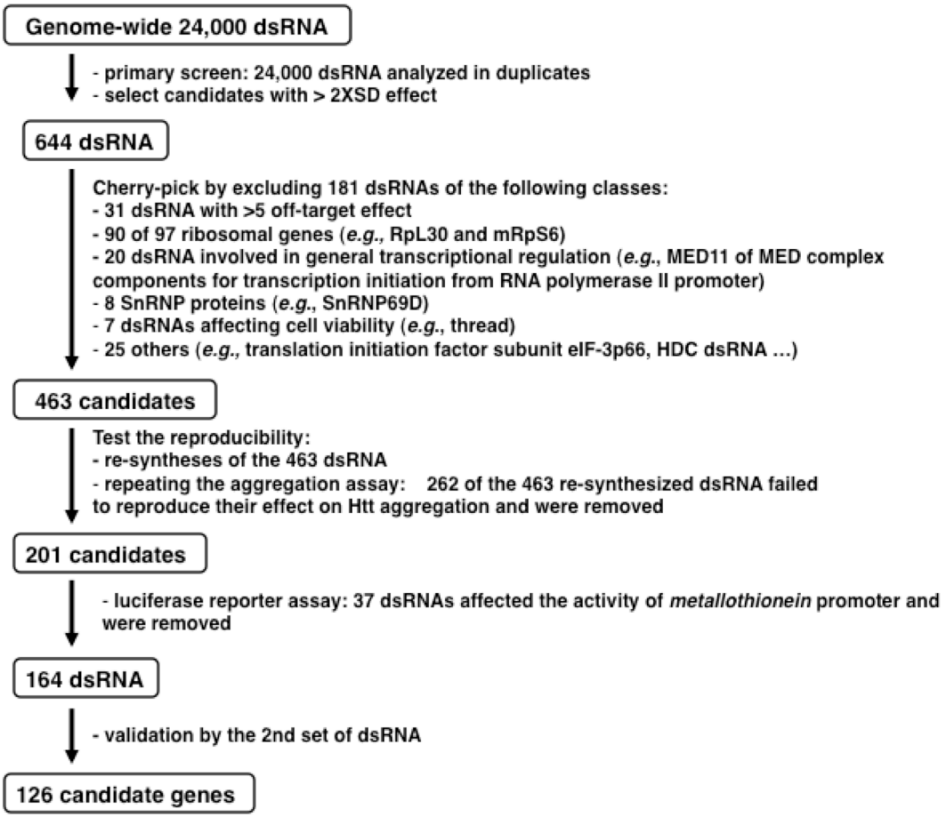

FIGURE S2.—Flow chart of the RNAi screening and validation steps for aggregation modulators of mutant Htt. In primary screen, genome-wide *Drosophila* RNAi libraries containing >24,000 dsRNA were tested in duplicates and 644 dsRNA with significant effect (over 2XSD of a plate average) on aggregates formation were isolated (see Fig. S1 and "Secondary screens" below for details).

Out of these 644 dsRNA, sequences of 31 dsRNA turned out to have high off-target effect (targeting over five different genes) and were removed from the ensuing studies. Curation of the remaining corresponding genes' known functions revealed that many are involved in general protein synthesis, including 97 ribosomal proteins, 8 SnRNP proteins, components of transcription initiation complexes and translation initiation factors. 131 of such dsRNA were also excluded from further analyses.

Amplicons for the remaining 463 dsRNA were cherry-picked and their dsRNA were re-synthesized and re-tested in the same aggregation assay. 262 of the re-synthesized dsRNA failed in the repeating experiments while the other 201 dsRNA showed reproducible effect.

In a luciferase-based assay to identify false positives that act by regulating the activity of the *metallothionein* promoter employed in the aggregation assay, 37 of the above 201 dsRNA showed significant effect, including those involved in general transcriptional regulation or cellular endocytosis (*e.g.*, Cop complex components such as alpha-COP, beta-Cop and zeta-Cop). These 37 dsRNA were excluded from further consideration.

Lastly, to confirm the specificity of the dsRNA with their corresponding genes, one or two more set of dsRNA targeting different regions of the remaining 164 candidates were synthesized and re-tested. In total, 126 genes passed all these validation steps.

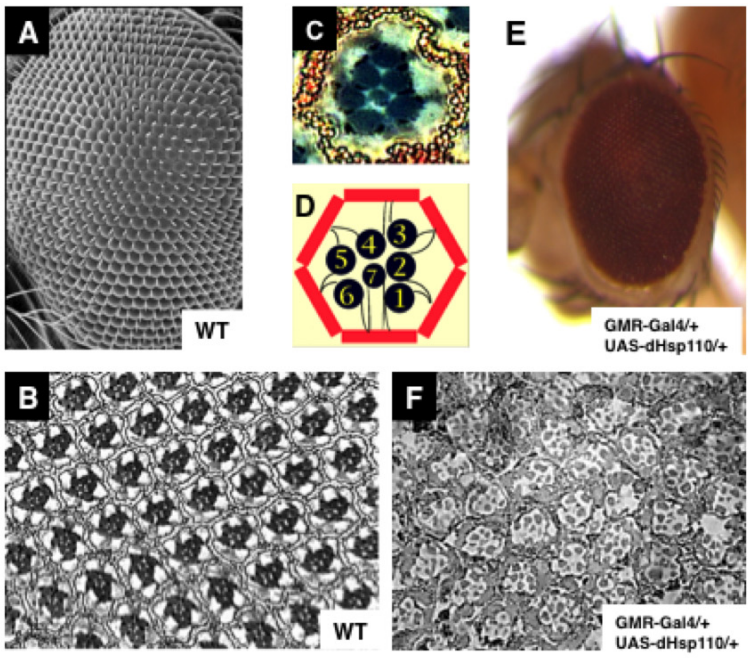

FIGURE S3.—High level of *dhsp110* (*CG6603*) expression disrupts the proper formation of adult *Drosophila* eye.

(A-D) Wild-type (wt) adult flies have well-patterned eye structure. (A) A wt adult eye imaged by scanning electronic microscopy. Each eye is composed of about 800 ommatidia. (B) Well-organized internal structure of adult eye, which is composed of lattice-like ommatidium units as revealed by tangential section. (C and D) (C) High magnification view of a single ommatidium unit and (D) its cartoon representation. Each ommatidium is composed of 8 photoreceptor cells (PR) surrounded by pigment cells. Only 7 PR cells are visible in each sectioned layer. (E and F) Images of adult fly eyes with high-level *dhsp110* expression. Genotype: *GMR-Gal4/+; UAS-dhsp110/+*. Although these flies show normal external eye morphology (E, bright-field imaging), their internal eye structure are severely disrupted (F, tangential section image), including a thickening of pigment cells, loss of PR cells and abnormally formed rhabdomeres.

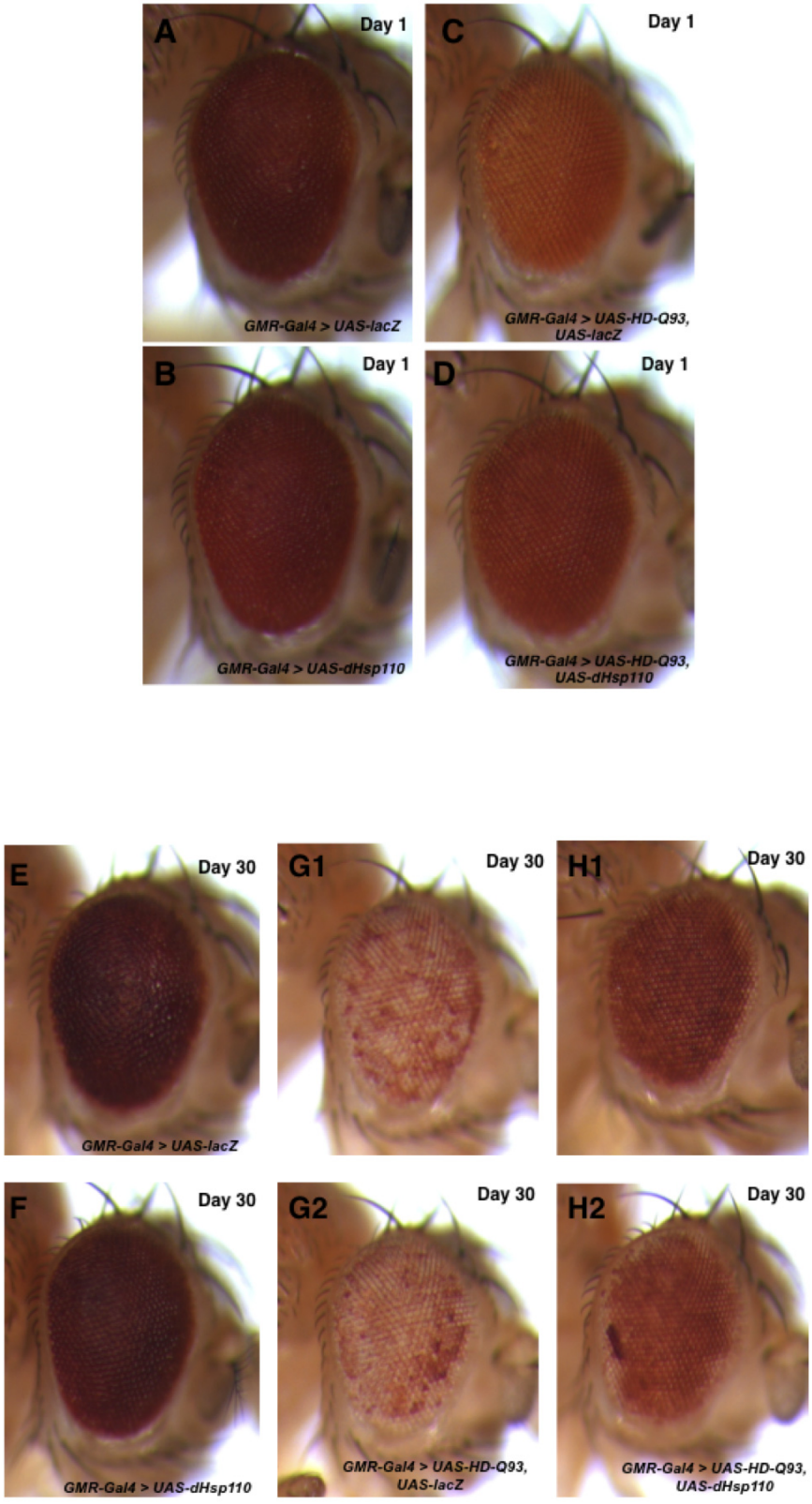

FIGURE S4.—Modification of HD93 toxicity by *dhsp110*. Bright-filed images of adult fly eyes at age (A-D) day 1 or (E-H) day 30. Control flies that express (A and E) lacZ gene or (B and F) dhsp110 alone did not show obvious loss of pigmentation as

flies age. Flies that co-express *Httex1p-Q93* with (C, G1 and G2) lacZ gene show a clear de-pigmentation of adult eyes as they age, (D, H1 and H2) while such degeneration phenotype was significantly suppressed by the presence of *dhsp110* gene. Genotypes: (A and E) *GMR-Gal4/+; UAS-LacZ/+*. (B and F) *GMR-Gal4/+; UAS-dhsp110/+*. (C, G1 and G2) *GMR-Gal4/ UAS-LacZ; UAS-Httex1p Q93/+*. (D, H1 and H2) *GMR-Gal4/ UAS-dhsp110; UAS-Httex1p Q93* /+ (STEFFAN *et al*. 2001). In all eye images, the anterior side is up and the ventral side is to the left.

Tables S1-S7 are available for download as Excel files at http://www.genetics.org/cgi/content/full/genetics.109.112516/DC1

TABLE S1

List of hits identified from the RNAi screen as regulators of aggregates formation

Please note that in this study, "**Suppressor**" is defined genetically as the candidates that cause an increased formation of aggregates after dsRNA-mediated knockdown of the corresponding genes, whereas "**Enhancer**" is similarly defined as those that cause a decreased formation of aggregates in the assay.

The columns in the Table S1 are as follows: (1) Gene symbol; (2) Modifier class "Enhancer" and "Suppressor".; (3) Functional categorization (based on the "GeneOntology (GO)" index biological function or protein domains.); (4) IDs of DRSC amplicons (http://www.flyrnai.org/); (5) FBGN: ID of FlyBase Genome annotations; (6) Protein domain (from the Flybase: http://flybase.bio.indiana.edu/); (7) Molecular function (curated from the Flybase)

TABLE S2

List of hits identified from the RNAi screen as regulators of aggregates formation and their human homologues

The columns in the Table S2 are as follows: (1) Gene symbol; (2) Gene full name; (3) Functional categorization (based on the "GeneOntology (GO)" index biological function or protein domains); (4) Modifier class (see Table S1 for definition of "Enhancer" and "Suppressor"); (5) Gene ID by CG number (http://flybase.bio.indiana.edu/) (6) Human homologues by "Database of Pairwise Orthologs" (http://inparanoid.cgb.ki.se/); (7) Human homologues (curated from the Homophila website http://superfly.ucsd.edu/homophila); (8) Disease-related human orthologs (curated from the Homophila website http://superfly.ucsd.edu/homophila).

TABLES S3-S7

Notes

1. Please note that for consistence, the effects of the *C. elegans* modifiers and their *Drosophila* homologues on aggregates formation are described according to Nollen et. al., (2004) as "*Enhance*" or "*Suppress*", respectively. "Enhance" indicates that the cognate dsRNA treatment increases aggregation formation, and *vise versa*, "Suppress" suggests that the cognate dsRNA treatment reduces aggregation formation.

2. **Importantly**, in our study and in Table S1 and S2, the identified modifiers are listed as "***Suppressor***" and "***Enhancer***". "Suppressor" is defined *genetically* as the genes for which their cognate dsRNA treatment enhances aggregation formation, that is, causing an increased formation of aggregates after dsRNA-mediated knockdown of the corresponding genes, whereas "Enhancer" is similarly defined as those that lead to a decreased formation of aggregates in the assay. Accordingly, genes that cause "Enhance" and "Suppress" effect in Tables S3-S6 correspond to the "Suppressor" and "Enhancer" in our study as listed in Tables S1 and S2, respectively.

3. In Tables S3-S6, information on the *C. elegans* modifiers are directly from the corresponding studies. *Drosophila* homologues (column E) of the *C. elegans* genes were identified manually by first downloading the protein sequences of the worm modifiers from the NCBI website with the "cosmid nr." or other information listed in respective studies, which were then used to search the *Drosophila* database (**http://flybase.bio.indiana.edu/**) using the BLASTp program from the NCBI site. In most cases, only the closet homologues were listed and compared with the hits from our study.

4. *Drosophila* homologues that were also identified in the primary screens in our study are labeled as "1st", those identified as final candidates after passing all the secondary assays are marked as "F".

TABLE S3

Comparison of modifiers from this study in Drosophila and the Nollen *et. al.,* study (2004) in *C. elegans*

Drosophila homologues that were also identified in our primary screens are labeled in column G as "1st", those identified as final candidates after passing all the secondary assays are marked in column H as "F. The effects of the *C. elegans* modifiers and their *Drosophila* homologues on aggregates formation are listed in column C and F, respectively (See the above **Notes** for more details).

The protein sequences for the worm modifiers were retrieved using the "cosmid nr." listed in Nollen et. al., (2004) study. "**-**" in column E indicates that no *Drosophila* homologue of the corresponding worm gene was identified from the search. Protein sequences for a few worm modifiers could not be retrieved from the NCBI website using the "cosmid nr." provided and were indicated as "none" in column E.

TABLE S4

Comparison of modifiers for mutant Htt aggregation from this study in Drosophila and for mutant *a-Synuclein* by the Hamamichi *et al.* study in *C. elegans* (2008)

The effects of the genes on aggregates formation in corresponding assays are listed in columns C and G, respectively. Please see "**Notes**" in front of the Table S3 for more details.

* For *dnj-19*, a DnaJ domain co-chaperone, its closest homologue in *Drosophila* is *droj2* (FBGN0038145) with E value at 4.79546e-47. *dnaJ-1* (FBgn0015657), another homolog of this gene (E value of 1.63364e-20), marked in column H as "1st" and in column I as "F", was the only overlapping hit from these two studies

TABLE S5

Comparison of modifiers for mutant Htt aggregation from this study in *Drosophila* and for mutant *α-Synuclein* by the *van Ham et al.* study in *C. elegans* (2008)

Information on the *C. elegans* modifiers are directly from the Table 1 and Table S1 in the *van Ham et al*. (2008) study**.** Please see "**Notes**" in front of the Table S3 for more details.

The protein sequences of the worm modifiers were downloaded from the NCBI website with the "*Cosmid no*." or "Gene" provided in Table 1 and Table S1 in *van Ham et al*. (2008) study. **"-"** in column E indicates that no *Drosophila* homologues was identified from the search. Protein sequences for a few worm modifiers could not be retrieved from the NCBI website using the "*Cosmid no*." or "Gene" provided in Table 1 and Table S1 in *van Ham et al*. (2009) study and were indicated with "**?**".

The five *Drosophila* homologues that were isolated in the primary screens from our study are labeled in column H as "1st".

* For chaperone *R151.7*, its closest *Drosophila* homolog is *trap1* (FBgn0026761) with E value at 2.73574e-150. *hsp83* (FBgn0001233), another homolog of this gene (E value of 1.06342e-42), was also isolated as a final candidate in our study and is marked as "F" in column I.

TABLE S6

Comparison of modifiers for mutant Htt aggregation from this study in *Drosophila* and for mutant SOD from the Wang *et. al.,* study (2009) in *C. elegans*

Drosophila homologues that were identified in the primary screens from our study are labeled in column H as "1st", those isolated as final candidates are marked as "F" in column I. Please see "**Notes**" in front of the Table S3 for more details.

"-" in column E indicates that no *Drosophila* homologue of the corresponding worm gene was identified.

"*" For *dnj-19*, a DnaJ domain co-chaperone, its closest homolog in *Drosophila* is *droj2* (FBgn0038145) with E value at 4.79546e-47. Its homology with *dnaJ-1* (FBgn0015657) is at a E value of 1.63364e-20.

TABLE S7

Comparison of modifiers from Doumanis *et. al.,* study (2009) with the candidates from this study

Please see "**Notes**" in front of the Table S3 for more details. The same modifiers that were also identified in our primary screens are labeled in column D as "1st", those identified as final candidates after passing all the secondary assays are marked in column F as "F".