A novel fly model of TDP-43 proteinopathies: N-terminus sequences combined with the Q/N domain induce protein functional loss and locomotion defects.

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Summary statement:
Generation of a novel Drosophila TDP-43 aggregation model for pathogenetic mechanism characterization and drug screening.
Abstract

Transactive response DNA binding protein 43 kDa (TDP-43) is the main protein component of the pathological inclusions observed in neurons of patients affected by different neurodegenerative disorders, including Amyotrophic Lateral Sclerosis (ALS) and Fronto-Temporal Lobar Degeneration (FTLD). The number of studies investigating the molecular mechanisms underlying neurodegeneration are constantly growing however, the role played by TDP-43 in disease onset and progression is still unclear. A fundamental shortcoming that hampers progress is the lack of animal models of aggregation without TDP-43 overexpression. In this manuscript, we have extended our cellular model of aggregation to a transgenic *Drosophila* line. Our fly model is not based on the overexpression of a wild-type TDP-43 transgene. On the contrary, we engineered a construct that includes only the specific TDP-43 amino acid sequences necessary to trigger aggregate formation and trapping of endogenous dTDP-43 into a non-functional insoluble form. Importantly, the resulting recombinant product lacks of functional RNA recognition motifs (RRMs) thus resulting devoid of specific TDP-43-physiological functions (i.e. splicing regulation ability) that might affect the animal phenotype *per se*. This novel *Drosophila* model exhibits an evident degenerative phenotype with reduced life-span and early locomotion defects. Additionally, we show that important proteins involved in neuromuscular junction function, such as syntaxin (SYX), decrease their levels as a consequence of TDP-43/TBPH loss of function implying that the degenerative phenotype is a consequence of TDP-43/TBPH sequestration into the aggregates. Our data lend further support to the role of TDP-43 loss-of-function in the pathogenesis of neurodegenerative disorders. The novel transgenic *Drosophila* model presented in this study will help to gain further insight into the molecular mechanisms underlying neurodegeneration and
will provide a valuable system to test potential therapeutic agents to counteract disease.
Introduction

FTLD and ALS are two neurodegenerative diseases that can exist both as distinct clinical entities and as a clinical continuum with overlapping pathogenic pathways (Van Langenhove et al., 2012). A common feature of these diseases are cytoplasmic aggregates of TDP-43, resulting in nuclear clearance of the protein (Belzil et al., 2013). TDP-43 is a heterogeneous nuclear ribonucleoprotein (hnRNP) with nuclear and cytoplasmic functions (Buratti and Baralle, 2012) that are evolutionarily conserved from invertebrates to rodents and humans (Ayala et al., 2005). By exploiting the functional overlap between the human and fruit fly TDP-43 orthologs (Ayala et al., 2005), different Drosophila models carrying the targeted disruption of the TDP-43 ortholog (TBPH) gene have been generated and virtually all exhibited ALS-like neuromuscular deficits (Romano et al., 2012), indicating a loss of function of TDP-43 being central to the pathogenesis (Buratti and Baralle, 2009; Chen-Plotkin et al., 2010; Da Cruz and Cleveland, 2011; Lee et al., 2012). In accordance with this hypothesis, mutations identified within the TARDBP gene in ALS familial cases are in the C-terminal region and some seem to associate with enhanced TDP-43 aggregation (Arai et al., 2006; Neumann et al., 2006; Sreedharan et al., 2008). The C-terminal region of TDP-43, contains a Q/N rich region involved in protein–protein interaction (D’Ambrogio et al., 2009) and it has been suggested that this sequence resembles a prion-like domain (Gitler and Shorter, 2011; Polymenidou and Cleveland, 2011). The Q/N region is crucial for the aggregation process, as demonstrated by different in-vitro models (Igaz et al., 2009; Fuentealba et al., 2010; Budini et al., 2012b; Budini et al., 2015). In particular, we have shown that expression of 12 repetitions of the Q/N-rich amino acid sequence 331-369 of hTDP-43 (12xQ/N) fused to an EGFP tag (EGFP-12xQ/N) triggers the formation of aggregates that recapitulate the most
relevant properties of the inclusions found in patients (Budini et al., 2012b; Budini et al., 2012a).

The cells expressing EGFP-12xQ/N show co-localization of endogenous TDP-43 with the cytoplasmic aggregates induced by the transgene. However, no significant loss of TDP-43 function was observed. A transgenic *Drosophila melanogaster* line expressing the construct EGFP-12xQ/N in the eye under the control of the GMR-Gal4 driver showed that EGFP-12xQ/N was able to trigger aggregation similarly to that observed in cells and that there was no intrinsic toxicity of the aggregates (Cragnaz et al., 2014). In a follow up of this study the EGFP-12xQ/N construct was expressed as a transgene using the pan-neuronal elav-Gal4 driver. The transgenic fly presents a locomotion defect phenotype in mid-adult life, coinciding with a physiological and age-related four fold reduction of TBPH levels (Cragnaz et al., 2015), while no significant changes in the expression of TBPH-regulated genes were detected in these animals (L. Cragnaz, personal communication). This observation suggests that, although endogenous TDP-43 trapping into EGFP-12xQ/N aggregates occurs, it is not highly efficient using this transgene. Consequently phenotype onset is detected only when endogenous TBPH levels drop.

Further studies in tissue culture cells showed that in addition to the TDP-43 C-terminal region, the 1-75 N-terminal portion of TDP-43 is critical to trigger the formation of aggregates able to efficiently trap endogenous TDP-43 in a non-functional insoluble form (Budini et al., 2015; Romano et al., 2015). These results prompted us to produce a novel transgenic *Drosophila* line based on a construct carrying the N-terminal domain of TDP-43 in addition to the 12xQ/N repetitions. The transgene induced an efficient loss of function of endogenous TBPH in *Drosophila*, in a similar way as that observed in human tissue culture cells. The transgenic fly also
exhibited an evident degenerative phenotype, with reduced lifespan and early locomotion defects.
Results

Generation of a novel construct to model TDP-43 aggregation.

We have previously shown that expression of the 1-75 N-terminal portion of TDP-43 fused to 12 tandem repeats of its prion like Q/N-rich region (12xQ/N) in a cell line is able to trigger the aggregation of endogenous TDP-43, resulting in its loss of function as determined by POLDIP3 exon 3 alternative splicing (Budini et al., 2015). In this manuscript, we designed a novel construct to make a chimeric protein (Figure 1A) that harbors the entire 1-100 N-terminal domain of the TDP-43 protein (including the Nuclear Localization Sequence, NLS); the linker region between the two RRM; a mutated form of the RRM2 unable to bind RNA, but retaining the Nuclear Export Sequence, NES (RRM2F/L); and, finally, the 12xQ/N repeats. The features of this transgene, Flag-TDP-Δ1-ΔC-RRM2F/L-12xQ/N or Aggregation Inducer (AggIn), provide a reasonable degree of structural integrity to the chimeric protein and includes both the NLS and the NES that ensure the preservation of TDP-43 shuttling abilities between nucleus and cytoplasm. For technical convenience, we also included an N-term Flag tag.

Characterization of a HEK293 AggIn stable cell line.

To test the aggregation efficiency of the transgene, we produced a HEK293 AggIn stable cell line. After tetracycline induction, anti-FLAG staining showed the presence of many, prevalently cytosolic, aggregates (Figure 1B, panel B, anti-FLAG +tet). Interestingly, several cell nuclei appeared to be devoid of endogenous TDP-43 (Figure 1B, Panel B, anti-TDP-43 +tet; empty nuclei marked with asterisks). In order to analyze whether the formation of these aggregates was matched by loss of TDP-43 function, we evaluated the splicing profile of the endogenous gene POLDIP3/SKAR,
whose pre-mRNA processing is determined by TDP-43. In fact, knockdown of TDP-43 causes the exclusion of exon 3 from the mature POLDIP3 mRNA (variant 2) (Fiesel et al., 2012; Shiga et al., 2012). Similarly to what happens with overexpression of the TDP-12xQ/N construct (Figure 2A, Upper panel, lanes 3 and 4) (Budini et al., 2015), tetracycline induction of the AggIn protein expression was associated with a strong increase of POLDIP3 variant 2 at both the mRNA (Figure 2A, Upper panel, lanes 5 and 6) and protein levels (Figure 2A, Lower panel). This effect is specific for constructs able to induce aggregation and efficient trapping of endogenous TDP-43: in fact, no alteration in the POLDIP3 splicing pattern was observed following overexpression of wild type TDP-43 (Figure 2A, Upper panel, lanes 1 and 2) or, as previously reported, of EGFP-12xQ/N (Budini et al., 2012b; Budini et al., 2015).

Furthermore, we also performed the splicing assay on the two additional endogenous transcripts BIM and MADD, whose splicing profile is known to be affected upon TDP-43 depletion (De Conti et al., 2015). For both genes, the splicing profiles of the transcripts in the HEK AggIn stable cell line resembled changes previously observed upon TDP-43 silencing (Figure 2B; increased BIM exon 3 and MADD exon 31 skipping).

Altogether, these experiments provided evidence of the endogenous TDP-43 loss of function upon AggIn expression and indicated AggIn as a promising construct to model TDP-43 aggregation in Drosophila melanogaster.

Pan-neuronal expression of the AggIn construct in Drosophila melanogaster.

To create a novel animal model for TDP-43 aggregation, we cloned the AggIn construct in the pUASTattB vector, under the control of the upstream activating sequence (UAS). After embryo injection, five different fly lines were obtained and
screened for transgene expression by using the GMR-Gal4 driver. While four of these lines expressed the transgene at comparable levels, the fifth demonstrated a higher expression level (data not shown). Therefore, in the subsequent steps of our study, we focused our attention on two of these transgenic lines: the one expressing the transgene at the top expression level (UAS_5A) and one out of the four expressing the transgene at comparable level (UAS_2B) (Figure 3A).

To start studying the effects of transgene expression on the *Drosophila* phenotype, we expressed the construct selectively in neurons, via crossing the UAS_5A and UAS_2B flies with a pan-neuronal elav-Gal4 driver-fly (the flies were grown at 25°C). As expected, elav-Gal4>UAS_5A demonstrated a transgene expression level two-fold higher than the one observed in elav-Gal4>UAS_2B, as calculated from normalized expression values (Figure 3B).

**Flies survival is dramatically affected upon AggIn expression in neurons.**

To study the effects of the AggIn expression in neurons, we first analyzed the life span of elav-Gal4>UAS_5A and elav-Gal4>UAS_2B flies versus two different control flies: one was not expressing any transgene (elav-Gal4>+); the second one was a transgenic fly line expressing the irrelevant protein EGFP (elav-Gal4>UAS_Egfp). As clearly shown in Figure 4A, life span is dramatically reduced in flies expressing AggIn. Indeed, whereas we observed a median survival of 64 days for control flies (both elav-Gal4>+ and elav-Gal4>UAS_Egfp), we found a median survival of only 18 days for elav-Gal4>UAS_5A and 29 days for elav-Gal4<UAS_2B. Such a significant decline in survival during aging suggests that transgene expression has strong phenotypic consequences, whose intensity is related to transgene expression levels.
The intrinsic toxicity of protein aggregates was low. In fact, whereas the expression of a wild type TBPH transgene using the GMR-Gal4 driver resulted in the formation of large necrotic patches in the eye of newly-eclosed flies (Fig. S1A), the expression of the AggIn transgene did not alter significantly the eye anatomy, although there was a modest change in visual ability, particularly in the high-expression-version UAS_5A line (Figure 4B). Furthermore, we also analyzed the external eye phenotype of 15-days-old transgenic flies, and did not observe any appreciable anatomic difference nor increased signs of toxicity, in comparison to the 1-day-old eye (Figure 4B and Fig. S1B). This suggests that AggIn expression does not induce obvious signs of eye-structure degeneration also during aging. Therefore, both fly lines were considered suitable for further studies of the phenotypic effects of aggregation.

**Transgene expression affects climbing ability of flies.**

We then investigated whether a locomotion defect appears at some point of the reduced lifespan of these flies using the climbing ability test. We assayed the flies at five different time-points after eclosion (days: 3, 7, 11, 15, 20). Both elav-Gal4>UAS_5A and elav-Gal4>UAS_2B flies demonstrated a statistically significant impairment of the climbing ability already at the first time-point (day 3). As expected, at each time point analyzed, the elav-Gal4>UAS_5A showed a more severe impairment than the elav-Gal4>UAS_2B (Figure 5). Indeed, whereas about 40% of elav-Gal4>UAS_5A flies were no longer able to reach the top of the cylinder at day 3, only 15% of elav-Gal4>UAS_2B flies demonstrated a similar impairment of climbing at this time point. Similarly, more than 70% of elav-Gal4>UAS_5A flies were no longer able to reach the top of the tube 7 days after
eclosion, whereas a similar percentage of elav-Gal4>UAS_2B flies with impaired climbing ability was observed only 8 days later (day 15 after eclosion).

Taken together, these results suggest that the AggIn product affects the motility of flies in an expression-dependent manner.

**Pan-neuronal expression of the transgene in elav-Gal4>UAS_5A results in early locomotion impairment, detectable in the larval stage.**

The possibility of an early lethality of the AggIn flies was checked: we selected third instar larvae and transferred them to fresh food tubes. 6 days later we calculated the percent ratio between eclosed flies:pupal lethality:larval lethality (Fig. S2). Whereas the elav-Gal4>UAS_2B line did not show differences in larval lethality compared to controls (elav-Gal4>+, elav-Gal4>UAS_Egfp) and revealed only a slight increase in pupal lethality, elav-Gal4>UAS_5A animals demonstrated an higher larval and pupal lethality compared to both the controls and to the elav-Gal4>UAS_2B line. Nonetheless, we were able to analyze the phenotype of the animals during the larval stage. To this aim, we assayed third instar larvae movement by counting the number of their peristaltic waves in two minutes on a suitable solid substrate (see Materials and Methods for details). In addition, as a negative control of the experiment, we used a transgenic line expressing the irrelevant protein EGFP in neurons (elav-Gal4>UAS_Egfp) and the wild type control line w1118. On the other hand, as a positive control, we analyzed the movement of TBPHΔ23 larvae, the first TBPH-null allele fly line that shows a severe neurodegenerative phenotype with locomotion defect in larval stages and dramatic locomotive defects after eclosion (Feiguin et al., 2009). We did not observe any significant difference in larval motility of the elav-Gal4>UAS_2B larvae with respect to the negative controls (Figure 6). The elav-
Gal4>UAS_5A larvae instead showed a significant motility impairment, as compared to the negative controls, with a reduced number of peristaltic waves, quantitatively comparable to those counted with TBPH-null larvae (TBPH\(^{\Delta 23}\)) (Figure 6). Therefore, these results show that the locomotion impairment of the elav-Gal4>UAS_5A fly line is comparable with the one of the TBPH-null model.

**Biochemical and functional assays support the notion of endogenous TBPH loss-of-function in transgenic AggIn fly models.**

The creation of the HEK293 AggIn stable cell line has shown that transgene expression efficiently triggers the formation of aggregates able to recruit and trap the endogenous TDP-43 protein and give rise to a TDP-43 loss of function effect (as demonstrated by the alteration of the splicing profile of the endogenous genes POLDIP3, BIM and MADD).

To show that AggIn expression induces the formation of insoluble aggregates able to trap endogenous TBPH also in flies, we performed solubility experiments on transgenic fly heads co-expressing AggIn and a Flag-tagged form of TBPH under the control of the GMR-Gal4 driver. As a control experiment, Flag-TBPH was co-expressed with the unrelated protein EGFP. This experiment clearly shows that AggIn expression results in the formation of insoluble aggregates (Flag-AggIn, *; Fig. 6B) and induces a very strong shift of TBPH from the soluble to the insoluble fraction (Flag-TBPH, #; Fig. 6B). As expected, the expression of control EGFP does not result in the formation of insoluble aggregates nor alter the solubility pattern of TBPH, that remains mainly soluble.
Western blot analysis of SYX and CSP proteins expression.

In order to investigate if biochemical evidence of TBPH loss of function could also be found in the transgenic flies, we analyzed the expression levels of genes known to be altered in TBPH-null fly models and previously characterized as molecular targets of TBPH potentially related to neurodegeneration pathogenesis.

In particular, we focused our attention on the elav-Gal4>UAS_5A line, which showed a striking degenerative phenotype during both adulthood and larval stage.

SYX and Cysteine-string protein (CSP) are two presynaptic vesicular proteins. It has been recently reported that their down-regulation is an early event of TBPH dysfunction in-vivo. In fact, the expression of these proteins was found to be significantly altered in the heads of our TBPH-null fly model TBPH Δ23 and in neuromuscular junctions (NMJs) presynaptic boutons in muscle 6/7 of third instar larvae (Romano et al., 2014). Starting from these observations, we verified the endogenous TBPH function in Drosophila melanogaster expressing the AggIn transgene. In particular, we compared by western blot the expression of SYX and CSP proteins in the heads of elav-Gal4>UAS_5A versus elav-Gal4>UAS_Egfp control flies. These proteins appeared to be significantly downregulated in our transgenic model (Figure 7A). Interestingly, the drop in expression was found at all three time points assayed (day 3, 7, 11), in agreement with the observation that this fly line has a severe phenotype already 3 days after eclosion.

Confocal analysis of SYX protein expression in larval NMJs presynaptic boutons

In order to support the hypothesis that endogenous TBPH loss of function was also responsible for the phenotype observed during the larval stage in elav-Gal4>UAS_5A line, we analyzed SYX protein expression in the NMJ presynaptic boutons in muscle
6/7 of these transgenic larvae via immunohistochemistry. As expected, SYX levels were strongly reduced in the synaptic terminals of elav-Gal4>UAS_5A third instar larvae compared to elav-Gal4>UAS_Egfp controls (Figure 7B, both anti-SYX and merge). On the contrary, the presynaptic marker positive for horseradish peroxidase (HRP) antibody did not exhibit any alteration (Figure 7B, anti-HRP), confirming the specificity of the SYX downregulation in elav-Gal4>UAS_5A flies. Furthermore, the shape of motoneuron terminals at the NMJ did not appear altered in the transgenic larvae versus control.

To provide evidence that the N-terminal portion of TDP-43 is critical to enhance the trapping of endogenous TBPH in an insoluble non-functional form, we included in the analysis also elav-Gal4>UAS_Egfp-12xQ/N larvae, which do not exhibit any locomotion impairment during the larval stage (Cragnaz et al., 2015). This allowed to perform a side by side phenotypic comparison of EGFP-12xQ/N and AggIn at the NMJ: as expected, in contrast to what observed in elav-Gal4>UAS_5A larvae, the EGFP-12xQ/N line did not show any reduction of the SYX levels in the synaptic terminals (Figure 7B), thus supporting the role ascribed to the N-terminal region of TDP-43.

Consistently, the observation that TBPH functional loss is detected in elav-Gal4>UAS_5A but not in elav-Gal4>UAS_Egfp-12xQ/N larvae correlates with the ability of aggregates to sequester endogenous TBPH. Indeed, we recently demonstrated that the climbing impairment in elav-Gal4>UAS_Egfp-12xQ/N flies overlaps with an age-related physiological drop of TBPH (Cragnaz et al., 2015). In order to demonstrate that a more efficient TBPH-trapping ability of the AggIn construct mediates the earlier onset of degenerative effects observed in this novel animal model, we analyzed the correlation between phenotype onset and endogenous
TBPH levels in elav-Gal4>UAS_5A flies. In this transgenic line, western blot experiments confirmed the same trend of endogenous TBPH physiological drop observed during aging in wild-type flies (Fig. S3). Interestingly, whereas the phenotype onset in elav-Gal4>UAS_Egfp-12xQ/N flies matches with a strong decrease of endogenous TBPH at day 10 (Cragnaz et al., 2015), the elav-Gal4>UAS_5A flies show an evident climbing impairment already at day 3 after eclosion (Figure 5), a time-point in which TBPH expression has already started to drop, as the decrease in its levels appears to occur in at least two main steps (see day 3 and day 10 in Fig. S3; compare with TBPH expression at day 1), but is significantly higher than at day 10.
Discussion

In the last decade several lines of evidence have shown that TDP-43 plays a key role in the pathogenesis of several neurodegenerative disorders, including ALS and FTLD (Neumann et al., 2006; Van Langenhove et al., 2012; Ling et al., 2013). Notwithstanding the large number of studies devoted to characterization of the molecular mechanisms linking TDP-43 aggregation to neurodegeneration, it is still unclear what the role of TDP-43 and TDP-43 positive aggregates is in disease onset and progression.

However, previous studies identified the structural determinants of the TDP-43 protein that mediate its self-aggregation and trapping into a non-functional insoluble form: they demonstrated that the C-terminal Q/N prion-like domain is important in the protein aggregation process and that the N-terminal region 1-75 is essential to enhance the trapping of endogenous TDP-43 in the aggregates in a non-functional insoluble form (Budini et al., 2012b; Budini et al., 2015). The sequestered TDP-43 loses its functional capacity but the aggregates did not show intrinsic significant toxicity in the HEK293 cells (Budini et al., 2012b) and in the *Drosophila* eye, a tissue that does not need TDP-43 for its development (Cragnaz et al., 2014).

Furthermore, while the role of the TDP-43 C-terminal part in the aggregation process is well established (D'Ambrogio et al., 2009; Igaz et al., 2009; Yang et al., 2010; Jiang et al., 2013; Wang et al., 2013), the implication of the N-terminal region in a growing number of physiological and pathological functions of the protein has been highlighted only by more recent studies, where the TDP-43 N-terminus, in particular the first 10 residues, appear to play a role not only in RNA recognition (Buratti and Baralle, 2001) and cellular localization (Winton et al., 2008), but also in regulating TDP-43 folding, homotypic interaction, splicing functionality and cytoplasmic
sequestration (Zhang et al., 2013). Other studies have also suggested that the TDP-43 N-terminus encodes a novel ubiquitin-like fold involved in binding of nucleic acids, that is normally in equilibrium with an unfolded form: the formation of irreversible inclusions relevant for both physiological and pathological processes might occur when this equilibrium is altered (Qin et al., 2014). Finally we have recently reported that the elimination of the first 75 residues of TDP-43 N-terminus reduces the efficiency of intracellular aggregates to interact with and sequester endogenous TDP-43 (Budini et al., 2015).

In fact, the insertion of the N-terminal sequence upstream of an artificial repetition of the Q/N rich region of TDP-43 (12xQ/N) results in a chimeric protein that induce aggregation and alters the splicing pattern of POLDIP3, commonly used as reporter of TDP-43 dysfunction. This experiment demonstrated that TDP-43 N-terminal is crucial for efficient sequestration of the endogenous TDP-43 within the inclusions (Budini et al., 2015).

Taking all the above points into consideration, we have now optimized an aggregation inducer with minimal TDP-43 sequences and tested it both in HEK293 cells and in *Drosophila melanogaster*. This novel construct Flag-TDP-Δ1-ΔC-RRM2F/L-12xQ/N was named, for simplicity, AggIn. We show here that AggIn is able to trigger aggregation, to deplete nuclei of endogenous TDP-43 and to induce loss of the splicing function of endogenous TDP-43 in HEK293 cells (Figures 1B and 2). Significantly, it is also able to induce evident survival and behavioral impairments when expressed as a transgene in *Drosophila melanogaster* neurons. We have studied two transgenic fly lines (UAS_2B and UAS_5A) with different levels of the AggIn transgene expression. This characteristic was useful to model differential levels of the TDP-43 aggregation process. In fact, only the elav-Gal4>UAS_5A line (AggIn
expression double vs. elav-Gal4>UAS_2B) showed motility impairment at the larval stage, while both the elav-Gal4>UAS_5A and elav-Gal4>UAS_2B lines resulted in a reduced lifespan and impaired climbing ability during adulthood. These latter effects were more severe when the aggregation was more efficient. In fact, lifespan was reduced to about 1/4 (elav-Gal4>UAS_5A line) and 1/2 (elav-Gal4>UAS_2B line) with respect to the median survival of control elav-Gal4>UAS_Egfp flies. Importantly, these effects were observed at physiological growth temperature (25°C).

These results suggest that the stronger effects observed in the UAS_5A line are due to the higher levels of AggIn expression and support the hypothesis of a direct correlation between efficiency of endogenous TBPH-trapping and onset, as well as severity of the phenotype. Interestingly, the higher transgene expression in elav-Gal4>UAS_5A line was able to trigger a very early locomotion impairment, quantitatively comparable to that observed in the null TBPHΔ23 larvae (Feiguin et al., 2009). In addition, the genotype-phenotype studies were complemented by the observation that TBPH function is lost. This supports the hypothesis that the effect of aggregation is a depletion of functional endogenous TBPH. In fact, the levels of CSP and SYX dropped significantly in the heads of elav-Gal4>UAS_5A flies and the latter was strongly reduced also in the larval NMJ compared to elav-Gal4>UAS_Egfp controls. These results suggested that the expression of the AggIn construct in Drosophila causes biochemical outcomes associated with endogenous TBPH loss of function. These observations are consistent with the results derived from the stable AggIn transgenic cell line HEK293, demonstrating that the transgene expression causes the formation of aggregates, able to trap endogenous TDP-43 in a non-functional form. In conclusion, we generated a novel transgenic Drosophila line to
model TDP-43 aggregation *in-vivo* and demonstrated that aggregation contributes to the onset of neurological impairments through a TBPH loss-of-function mechanism. We cannot exclude a minor contribution of AggIn aggregates to neurotoxicity (as suggested by a modest reduction of vision, as shown in Figure 4B). However, the observation that protein aggregation caused a decrease of presynaptic markers, whose expression requires presence of functional TBPH (Romano et al., 2014), strengthens the role of TDP-43 loss-of-function in the pathogenesis of neurodegenerative disorders.

In comparison with other models based on the overexpression of fully functional TDP-43 variants to trigger neurodegeneration (Walker et al., 2015), our aggregation-model is based on the expression of a minimal construct containing specific TDP-43 subdomains, which lacks functional RRMs thus mimicking a splicing-defective version of TDP-43. This greatly limits the risk of possible intrinsic effects of the transgene activity. These novel transgenic *Drosophila* models could help to gain more insight into the molecular mechanisms underlying neurodegeneration and provide a valuable system to test potential therapeutic agents able to prevent, counteract or slow down disease progression by increasing aggregate clearance or by preventing the capture of endogenous TDP-43/TBPH.
Materials and methods

Expression plasmid and stable cell line generation

The AggIn (Flag-TDP-Δ1-ΔC-RRM2F/L-12xQ/N) plasmid was generated by site-directed mutagenesis using the pcDNA5/FRT/TO-Flag-TDP-12x-Q/N plasmid (Budini et al., 2015) as a template. The final construct included: an N-term Flag-tag; the amino acid stretches 2-100, 173-190, 191-264 (with phenylalanine in positions 229 and 231 mutated to leucine) of the hTDP-43 protein; 12 repetitions of the hTDP-43 amino acids stretch 331-369 (referred to as 12xQN). Figure 1A recapitulates the main features of the construct.

For stable cell lines generation, HEK293 flip-in cells were grown in DMEM-Glutamax-I (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) and Antibiotic/Antimycotic stabilized suspension (SigmaAldrich, St. Louis, MO, USA). Cells were transfected using Effectene Transfection reagent (QIAGEN Inc, Gaithersburg, Maryland) following manufacturer’s instructions. Co-transfection of 0.5 μg of plasmid together with 0.5 μg of pOG44 (Thermo Fisher. Scientific, Waltham, MA, USA) vector allowed recombination in the genome of the cells. After co-transfection, cells were grown in DMEM-Glutamax-I supplemented with 10% fetal bovine serum and Antibiotic/Antimycotic until they reached 80% of confluence. The stable integration of the plasmid was then gradually selected using 100 μg/ml Hygromicin B (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) and 10 μg/ml of Blasticidin (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA). Once cells were selected, expression of the protein was achieved by adding 1 μg/ml of tetracycline (SigmaAldrich, St. Louis, MO, USA) to the culture medium.
Splicing Assay

For the splicing assay, 5x10^5 Flag-TDP-43-WT, Flag-TDP-43-12xQ/N and AggIn cells were seeded in 6-well-plate and induced with tetracycline for 72 hours. Uninduced cells were used as a control. After induction, cells were collected and RNA was extracted using Trifast reagent (Euroclone, Milan, Italy) according to the manufacturer’s instruction. Reverse transcription was performed using M-MLV Reverse Transcriptase (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) following manufacturer’s protocol. A PCR with TAQ DNA Polymerase (Roche Diagnostics, Mannheim, Germany) was performed for 35 amplification cycles (95°C for 30s, 55°C for 30s, 72°C for 30s) to amplify POLDIP3, BIM and MADD cDNAs. The primers used to test the splicing pattern of POLDIP3, BIM and MADD endogenous genes were: POLDIP3 Forward (5’-gcttaaatgcagaccggagttgga-3’); POLDIP3 Reverse (5’-tcattcttctcagcctggtataatt-3’); BIM Forward (5’-ctgagtgtgaccgagaagg-3’); BIM Reverse (5’-ctcttgggcgatccatatctc-3’); MADD Forward (5’-gacctgaattgggtggcagtccct-3’); MADD Reverse (5’-cattggtgtctttgaactgtgctc-3’).

Protein expression and immunoblot of HEK293 stable cell lines

Protein expression of the AggIn construct was analyzed in 5x10^5 cells seeded in 6-well-plate and induced for 72 hours. Uninduced cells were also seeded as control. After induction, cells were collected and lysed with 100 μl of RIPA lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA pH 8, 1 mM PMSF, 0.5% Sodium Deoxycholate) supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Cell lysates were incubated at +4°C for 30 minutes, then lysed by sonication and centrifuged at 500xg at +4°C for 5 minutes. Total protein amount in cell lysates was then quantified by
Bradford and 20 μg were loaded in a 10% SDS-PAGE. An anti-Flag (SigmaAldrich, St. Louis, MO, USA) primary antibody and an anti-Mouse (DAKO, Glostrup, Denmark) secondary antibody were used for protein detection. Western blot using a primary anti-POLDIP3 (Cell Signaling Technology, Beverly MA) antibody and a secondary anti-Rabbit (DAKO, Glostrup, Denmark) antibody was also performed to detect POLDIP3 isoforms.

**Immunofluorescence microscopy.**

For indirect immunofluorescence, 5x10⁵ HEK-AggIn cells were seeded on slides and induced with tetracycline for 72 hours. Not induced cells were also seeded as control. Immunofluorescence was performed as previously described (Ayala et al., 2008). As primary antibodies an anti-Flag (SigmaAldrich, St. Louis, MO, USA) and an anti-TDP-43 (ProteinTech, Chicago, IL, USA) were used. The secondary antibodies were anti-mouse-AlexaFluor 594, anti-rabbit-AlexaFluor 488 and TO-PRO3 dye for nuclei staining, all purchased from Life Technologies. Cells were then analyzed on a Zeiss LSM 510 Meta confocal microscope.

**Fly stocks**

AggIn and EGFP constructs were cloned in the pUASTattB vector and subsequently sequenced. The constructs were used to create transgenic flies by standard embryo injections (BestGene Inc., Chino Hills, CA, USA). Transgenes were subsequently balanced on the required chromosome to obtain fly stocks. w¹¹¹⁸ and elav-Gal4 flies were supplied by Bloomington Drosophila Stock Center at Indiana University. Flies were fed on standard fly food (agar 6 gr/l; sugar 41.6 gr/l; yeast 62.5 gr/l; cornmeal 29 gr/l; propionic acid 4.1 ml/l), maintained and crossed in a humidified incubator at 25°C with a 12 hours-12 hours light–dark cycle.
Life span

Adult flies were collected for 2 days from eclosion and transferred to fresh food tubes in a 1:1 male:female ratio (20 total flies/tube). At the third day, death events were scored and viable flies were transferred to fresh tubes. The same was done every three days. Survival proportions were plotted as percentage of alive flies against days. More than 120 flies were tested for each genotype.

Phototaxis assay

The assay was performed as previously described (Cragnaz et al., 2014). Briefly, individual flies from each genotype were introduced into the stem of an Y-maze with one arm exposed to violet light (400 nm) and the second arm completely in the dark. The number of flies that moved versus the illuminated chamber within one minute was determined. At least 50 flies/experiment for each genotype were tested.

Climbing assay

Age-synchronized cohorts of flies were transferred without anesthesia to a 50 ml glass-cylinder, tapped to the bottom with cotton. After a period of adaptation of 30 seconds, the climbing ability of flies was quantified as number of animals that reached the top of the cylinder (10 cm) in 15 seconds. Flies were assayed in batches of 20 (1:1 male:female ratio) and the test was repeated three times for each batch of animals. More than 120 flies were tested for each genotype. The number of top climbing flies was converted into % value, and the mean % value (±SEM) was calculated for at least 6 experiments.
Larval movement

Wandering third instar larvae were selected, gently washed and transferred to a Petri dish (0.7% agarose in distilled water). After a period of adaptation (30 seconds), the peristaltic waves were counted within 2 minutes. At least 20 larvae were assayed for each genotype. The median number of peristaltic waves performed in 2 minutes by each genotype was plotted on a graph (±SEM).

Immunoblotting of fly head samples.

*Drosophila* heads were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% Glycerol, 50 mM NaF, 5 mM DTT, 4M Urea, and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany)). Proteins were separated by 8 % SDS-PAGE, transferred to nitrocellulose membranes (Whatman, Clifton, NJ, USA), blocked overnight in a 5% non-fat dried milk solution and probed with the following primary antibodies: rabbit anti-TBPH (1:1500, home-made), mouse anti-SYX 8C3s (1:2500, Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, USA), anti-CSP2c (1:9000, Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, USA), mouse anti-tubulin CP06 (1:4000, Calbiochem, San Diego, CA, USA) and mouse anti-FLAG M2 (1:1000, SigmaAldrich, St. Louis, MO, USA). The membranes were incubated with the secondary antibodies: HRP-labeled anti-mouse (1:1000, Thermo Scientific, Rockford, IL, USA) or HRP-labeled anti-rabbit (1:1000, Thermo Scientific, Rockford, IL, USA). Finally, protein detection was assessed with Femto Super Signal substrate (Thermo Scientific/Pierce, Rockford, IL, USA) for anti-TBPH and anti-CSP2c.
immunoblotting and with ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA) for anti-syntaxin and anti-tubulin antibodies.

Protein expression was quantified using the NIH ImageJ software (Schneider et al., 2012) and normalized versus tubulin. Histograms are representative of 3 independent experiments.

**Solubility test**

The solubility assay was performed as previously described (Cragnaz et al., 2014). Briefly, 24 adult fly heads/genotype were homogenized in RIPA buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% NonidetP40 (v/v), 0.1% SDS, 1% Na-deoxycholate and a cocktail of Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany)). Following incubation on a rotating wheel for 1 h at 4 °C, samples were centrifuged at 1000xg for 10 minutes at 4 °C. An aliquot was taken at this point as the input, and after a further centrifugation step at 100000xg for 30 minutes at 4 °C, the supernatant was collected as the soluble fraction. The resulting pellet was re-suspended in urea buffer (9 M urea, 50 mM Tris–HCl, pH 8, 1% CHAPS and Complete Protease Inhibitor Cocktail) and collected as the insoluble fraction. Proteins were separated by 10% SDS-PAGE. The different samples were loaded in a 1:1:1 ratio for the input, soluble and insoluble fractions. Proteins were immunoblotted as already described for fly head samples and probed with the following reagents. Primary antibodies: mouse anti-FLAG M2 (1:1000, SigmaAldrich, St. Louis, MO, USA), rabbit anti-GFP sc-8334 (1:2000, Santa Cruz Biotechnology Inc., Dallas, Texas, USA) and mouse anti-tubulin CP06 (1:4000, Calbiochem, San Diego, CA, USA). Secondary antibodies: HRP-labeled anti-mouse
(1:1000, Thermo Scientific, Rockford, IL, USA) or HRP-labeled anti-rabbit (1:1000, Thermo Scientific, Rockford, IL, USA).

**NMJ immunohistochemistry and images quantification**

Third instar larvae were selected, briefly washed in water and dissected in saline solution (0.1 mM CaCl$_2$, MgCl$_2$ 4 mM, KCl 2 mM, NaCl 128 mM, sucrose 35.5 mM and Hepes 5 mM pH 7.2), fixed for 20 minutes in 4% paraformaldehyde, washed in PBS 0.1% Tween 20, blocked with 5% normal Goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS 0.1% Tween. Primary antibodies, anti-HRP 1:150, (Jackson ImmunoResearch Lab, West Grove, PA, USA) and anti-SYX 8C3s 1:15 (DSHB, Iowa City, IA, USA) were incubated overnight at 4°C and then the secondary antibodies, Alexa-Fluor® 488 rabbit 1:500 and Alexa-Fluor® 555 mouse 1:500 (Thermo Fisher Scientific, Waltham, MA, USA), were incubated for 2 hours at room temperature. SlowFade Gold (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) has been used for the mounting. Images were acquired on a Zeiss LSM 510 Meta Confocal Microscope with a 63x oil lens and 40x lens, then analyzed using NIH ImageJ software (Schneider et al., 2012).

The larvae analyzed for these experiments were processed simultaneously and the same microscope settings were employed to acquire all images. The presynaptic terminals of second abdominal segment on muscle 6 and 7 were analyzed. The samples were double labeled with anti-HRP and anti-SYX: the mean intensity of both was quantified and a ratio calculated, (adapted from (Thomas et al., 1997)). The statistical analyses were performed using Prism6 (GraphPad, San Diego, CA, USA).
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Competing interests

None of the authors have any competing interests in the manuscript.

Author contributions:

S.L. carried out molecular, genetic and behavioral studies with flies, and drafted the manuscript. V.R. carried out molecular studies with cell lines and tested the characteristics of the construct. G.R. performed NMJ immunohistochemistry experiments, R.K. and F.F. participated in the phenotypic characterization of flies. L.C. carried out molecular studies on TBPH age-related variations. M.R. participated in the design of the study, interpretation of the results, performed the statistical analysis and helped to draft the manuscript. F.E.B. conceived the study, participated in its design, in the interpretation of the results, coordination of the project and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figure 1:
Schematic representation of the AggIn construct and immunofluorescence of the HEK293 AggIn stable cell line.

(A) All relevant elements within the AggIn (Flag-TDP-Δ1-ΔC-RRM2F/L-12xQ/N) construct are identified along with their relative position to the human TDP-43 wild-type protein (TDP-43). (B) Panel A shows HEK293 AggIn cells without tetracycline induction and Panel B shows HEK293 AggIn cells after tetracycline induction. Anti-
Flag immunofluorescence (IF) is visualized as red fluorescence, while anti-TDP-43 IF as green fluorescence. Cell nuclei were stained with the reagent TOPRO-3 (blue). Empty nuclei in AggIn-expressing cells were marked with asterisks. A merge between anti-Flag/anti-TDP-43/TOPRO-3 is shown. Higher magnification of the + tet merge panel is also reported.
Figure 2:

Effect of transgene expression on TDP-43 target genes in HEK293 Aggln stable cell line.

(A) Upper panel: RT-PCR: splicing pattern of endogenous POLDIP3/SKAR gene (exon 3) after tetracycline induction (+) or not (-) of flag-TDP wt (lanes 1,2), flag-TDP-12xQ/N (lane 3,4), Aggln (lane 5,6) HEK293 stable cell lines. Lower panel: Western blot analysis, using anti-POLDIP3/SKAR antibody, of total protein lysates extracted from the same samples used in lanes 5 and 6 of panel A. (B) RT-PCR: splicing pattern of endogenous BIM (exon 3) and MADD (exon 31) genes after tetracycline induction (+) or not (-) of Aggln HEK293 stable cell line.
Figure 3:

Expression levels of transgene in two *Drosophila* lines.

(A) Western blot analysis of total protein extracts from fly heads of GMR-Gal4>UAS_5A and GMR-Gal4>UAS_2B and (B) elav-Gal4>UAS_5A and elav-Gal4>UAS_2B lines. Eye-specific and pan-neuronal expression of the AggIn construct was achieved using the GMR- and elav-Gal4 drivers, respectively. Western blot densitometry was performed using the ImageJ software and the normalized expression of the transgenic protein is reported in the graph (n=3).
Figure 4:

Effect of transgene expression on *Drosophila* lifespan and on external eye structure/function.

(A) Lifespan is dramatically reduced in flies expressing the AggIn transgene in neurons (elav-Gal4>UAS_5A and elav-Gal4>UAS_2B) versus a control fly not expressing any transgene (elav-Gal4>+) or a transgenic fly line expressing the control protein EGFP (elav-Gal4>UAS_Egfp). Median survival is: 18 days for elav-Gal4>UAS_5A; 29 days for elav-Gal4>UAS_2B; 64 days for controls (both elav-Gal4>+ and elav-Gal4>UAS_Egfp). n>120 animals for each genotype; p-values p<0.001, calculated by long-rank test, for all the following genotype pairs: elav-Gal4>+ versus elav-Gal4>UAS_2B; elav-Gal4>+ versus elav-Gal4>UAS_5A; elav-Gal4>UAS_Egfp versus elav-Gal4>UAS_2B; elav-Gal4>UAS_Egfp versus elav-
Gal4>UAS_5A; elav-Gal4>UAS_2B versus elav-Gal4>UAS_5A; time points (days) corresponding to 25%, 50% and 75% survival are also shown in the graph for each genotype and reported in the flanking summary table (B) External eye phenotype and phototaxis assay of 1-day-old flies. a/a’) Oregon; b/b’) GMR-Gal4>UAS_Egfp; c/c’) GMR-Gal4>UAS_2B; d/d’) GMR-Gal4>UAS_5A. The AggIn expression in the eye, using the GMR-Gal4 driver, did not result in any significant alteration of the external eye phenotype (upper panel, compare pictures c/d versus a/b controls). However the vision assay (lower panel) revealed that a minor fraction of the AggIn expressing population of flies (8.5% of GMR-Gal4>UAS_2B, and 20.4% of GMR-Gal4>UAS_5A) exhibited vision defects, since they did not reach the light within 1 minute of time (compare c’ and d’ white fractions in the lower panel graphs versus a’/b’ negative controls). Error bars indicate SEM (n=3).
Figure 5:

Effect of transgene expression on *Drosophila* climbing ability.

Climbing assay performed in flies expressing the AggIn transgene (elav-Gal4>UAS_5Aand elav-Gal4>UAS_2B) versus a control fly not expressing any transgene (elav-Gal4+) and a transgenic fly line expressing the control protein EGFP (elav-Gal4>UAS_Egfp).

A statically significant impairment of climbing is observed already at day 3 in both the AggIn expressing flies. The climbing defect gets worse with age, from day 3 to 20. n>120 animals for each genotype, ***p<0.001, *p<0.05 calculated by one-way ANOVA. Error bars indicate SEM.
Figure 6:

Effect of transgene expression on *Drosophila* larval motility and solubility assay on adult fly heads.

(A) The larval motility assay was performed on third instar larvae. Strong reduction in larval motility of elav-Gal4>UAS_5A (5A) larvae is observed, as compared to a transgenic line expressing the control protein EGFP (elav-Gal4>UAS_Egfp) and to the wild-type line (w^{1118}). No impairment in larval motility is observed in elav-Gal4>UAS_2B larvae (2B). A TBPH null-allele line (TBPH^{Δ23}) was used as a positive reference control. x axis: genotype; y axis: peristaltic waves counted in two minutes. n=20 animals for each genotype, ***p<0.001 calculated by one-way ANOVA. Error bars indicate SEM.

(B) Solubility assay. Western blot of fractionated proteins obtained from adult fly heads of the following genotypes: GMR-Gal4>UAS_TBPH;UAS_5A - GMR-Gal4>UAS_TBPH;UAS_Egfp.
Gal4>UAS_TBPH; UAS_2B - GMR-Gal4>UAS_TBPH; UAS_Egfp. Upper panel: Input, soluble and insoluble fractions of each genotype were loaded in a 1:1:1 ratio and probed by immunoblotting. AggIn and TBPH were detected with anti-FLAG antibody. EGFP was detected using anti-GFP antibody. Anti-tubulin served as protein loading control. Lower panel: to improve the separation of Flag-AggIn (see * ) and Flag-TBPH (see #) protein bands, that have a close molecular weight, the three sample fractions from each genotype were also loaded on additional gels and were run for a longer time, before anti-FLAG immunoblotting. TBPH is prevalently insoluble when it is co-expressed with AggIn. On the contrary, it remains mainly soluble when it is co-expressed with the unrelated protein EGFP.
Figure 7:

Transgene effects on TBPH-target genes expression.

(A) Western blot for TBPH-target genes performed in adult transgenic flies. Western blot analysis with anti-syntaxin (SYX) and anti-Cysteine-string protein (CSP) demonstrates the specific drop in expression of these presynaptic vesicular markers in elav-Gal4>UAS_5A fly heads, as compared to a transgenic line expressing the control protein EGFP (elav-Gal4>UAS_Egfp). Three different time-points were assayed (days 3, 7, 11). Total protein samples were extracted from adult heads; anti-alpha tubulin (tub) was used as a loading control. The relative expression of target proteins in transgenic lines was calculated by optical densitometry with ImageJ software (Schneider et al., 2012). For each time-point, the percent expression of SYX and CSP in 5A line versus control EGFP was calculated and reported in the western blot panel. SYX: at day 3, the expression of SYX in 5A line was 43% + 15% SEM compared to Egfp line; at day 7, it was 53% +18% SEM; at day 11, it was 40% + 15% SEM. CSP:
at day 3, the expression of CSP in 5A line was 38% ± 15% SEM compared to Egfp line; at day 7, it was 83% ± 8% SEM; at day 11, it was 73% ± 10% SEM. All results shown are representative of at least three independent experiments. (B) Anti-syntaxin immunofluorescence performed in third instar larvae NMJs. Confocal images of NMJs presynaptic boutons in muscle 6/7, II segment. SYX expression (anti-SYX, red) appears strongly reduced in the synaptic terminals of elav-Gal4>UAS_5A larvae compared to elav-Gal4>UAS_Egfp control larvae. On the contrary, anti-HRP-positive presynaptic marker expression is not affected (anti-HRP, green). No significant alteration of the SYX protein expression was detected in elav-Gal4>UAS_Egfp-12xQ/N synaptic terminals. The SYX-normalized expression is quantified in the graph.
Translational Impact

Clinical issue

Amyotrophic Lateral Sclerosis (ALS) and Fronto-Temporal Lobar Degeneration (FTLD) are two distinct severe neurodegenerative diseases. ALS is characterized by progressive weakness and loss of motor neurons, with a life expectancy of 2-5 years from diagnosis. FTLD is the second leading cause of dementia under 65 years of age, after Alzheimer’s disease (AD). Frontotemporal dementia (FTD) comprises a large subgroup within the broad spectrum of neurological disorders that constitute FTLD. It is characterized by significant cognitive and behavioural involvement. Both FTD and ALS are phenotypically and genetically heterogeneous. However, a significant percent of ALS patients (up to 50%) also show FTD signs (including behavioral, cognitive or language dysfunctions), implying that these two disorders can share clinical, genetic and neuropathological features. Indeed, ubiquitin-positive inclusions are nowadays considered the pathological hallmark of both FTD and ALS. Moreover, the TAR DNA-binding protein 43 (TDP-43) has been identified as the main component of these common pathological inclusions in ALS and ubiquitine-positive FTLD (FTLD-U) patients. Notwithstanding the numerous studies aimed at investigating the molecular mechanisms underlying neurodegeneration, they are still unclear as it is the role played by TDP-43 in disease onset and progression. No really effective FDA-approved drugs are yet available to treat FTD and ALS.

Results

In the present work it has been generated a novel transgenic *Drosophila* line suitable to model and study TDP-43 aggregation *in-vivo*. The transgene, namely “AggIn”, was engineered to include molecular determinants able to induce its self-aggregation with
simultaneous trapping of endogenous dTDP-43. AggIn expression results in an evident degenerative phenotype characterized by reduced life-span and early locomotion defects. Moreover, biochemical and immunofluorescence experiments provide evidences supporting the notion that such a severe phenotype in these animals is linked to dTDP-43 loss of function, resulting from the sequestration of this protein into the aggregates.

Implications and future directions

The lack of animal models mimicking features of the disease (i.e. aggregates formation) without overexpression of TDP-43 itself hampers progress in elucidating the underlying pathogenic mechanisms as well as the discovery of effective drugs. The novel transgenic fly model presented in this study allowed to obtain evidences strengthening the role of TDP-43 loss-of-function in the pathogenesis of neurodegeneration. Moreover it will help to further characterize the molecular mechanisms underlying neurodegeneration, to keep investigating the role of TDP-43 aggregation in the pathogenesis of these disorders and will also provide a valuable system to test potential therapeutic agents to counteract disease.
**Fig. S1. Effect of transgenes expression on external eye structure.**

(A) External eye phenotype of 1-day old GMR-Gal4>UAS_TBP flies: overexpression of wild-type TBPH results in the formation of large necrotic patches.

(B) External eye phenotype of 15-day old transgenic flies of the following genotypes: GMR-Gal4>UAS_Egfp; GMR-Gal4>UAS_2B; GMR-Gal4>UAS_5A. No appreciable anatomic differences nor increased signs of toxicity are detectable in Aggl transgenic flies in comparison to the 1-day-old eye photos shown in the Figure 4B.
Fig. S2. Assessment of early lethality in AgglIn flies.

Third instar larvae (at least 100 animals, for each of the following genotypes: elav-Gal4>+, elav-Gal4>UAS_Egfp, elav-Gal4>UAS_2B, elav-Gal4>UAS_SA) were selected and transferred to fresh food tubes. 6 day after it was estimated: the number of eclosed flies; the number of pupae unable to eclose and of larvae unable to reach the pupal stage, that were present in the food or on the tube walls. The percent ratio between eclosed flies:pupal lethality:larval lethality was determined and reported in a graph.
**Fig. S3.** TBPH levels in fly heads physiologically drop during aging.

Upper panel: Western blot analysis of endogenous TBPH in the heads of adult wild-type (w^{1118}, left panel) and elav-Gal4>UAS_5A (right panel) flies aged at 25 °C. Lower panel: the relative TBPH expression (normalized with tubulin) is shown in the graph. The arrows indicate the time-points when the climbing ability of the transgenic flies start to be significantly reduced (thin arrow for elav-Gal4>UAS_EGFP-12xQ/N; thick arrow for elav-Gal4>UAS_5A). Error bars indicate SD. D stands for day.