

A *Drosophila* model for TDP-43 proteinopathy

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Neuropathology involving TAR DNA binding protein-43 (TDP-43) has been identified in a wide spectrum of neurodegenerative diseases collectively named as TDP-43 proteinopathy, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD). To test whether increased expression of wide-type human TDP-43 (hTDP-43) may cause neurotoxicity in vivo, we generated transgenic flies expressing hTDP-43 in various neuronal subpopulations. Expression in the fly eyes of the full-length hTDP-43, but not a mutant lacking its amino-terminal domain, led to progressive loss of ommatidia with remarkable signs of neurodegeneration. Expressing hTDP-43 in mushroom bodies (MBs) resulted in dramatic axon losses and neuronal death. Furthermore, hTDP-43 expression in motor neurons led to axon swelling, reduction in axon branches and bouton numbers, and motor neuron loss together with functional deficits. Thus, our transgenic flies expressing hTDP-43 recapitulate important neuropathological and clinical features of human TDP-43 proteinopathy, providing a powerful animal model for this group of devastating diseases. Our study indicates that simply increasing hTDP-43 expression is sufficient to cause neurotoxicity in vivo, suggesting that aberrant regulation of TDP-43 expression or decreased clearance of hTDP-43 may contribute to the pathogenesis of TDP-43 proteinopathy.

amyotrophic lateral sclerosis | animal model | RNA binding protein

TDP-43 (TAR DNA binding protein of 43 kDa) is encoded by *TARDBP*, a highly conserved gene on chromosome 1 of the human genome (1–3). Initially identified as a transcriptional repressor of HIV-1 gene expression (4), TDP-43 is a multifunction protein involved in transcription, splicing, and mRNA stabilization (reviewed in ref. 5). Recent studies show that TDP-43 is a major protein component of neuronal inclusion bodies in the affected tissues in a range of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), frontotemporal lobar dementia (FTLD) (6, 7), Alzheimer's disease (AD) (8–10), and other types of dementia (10–13). Decreased protein solubility, hyperphosphorylation, abnormal cleavage, and cytoplasmic mislocalization of TDP-43 have been associated with TDP-43 proteinopathy (14–16). It is not clear whether TDP-43 proteinopathy is caused by loss-of-function of TDP-43 or gain-of-function neurotoxicity. Here, we report the generation and characterization of transgenic flies expressing human TDP-43. In different types of neurons, including photoreceptors, mushroom bodies, or motor neurons, simply overexpressing hTDP-43 by itself is sufficient to cause protein aggregate formation and neuronal loss in an age-dependent manner, suggesting that increased hTDP-43 expression or aberrant accumulation of hTDP-43 may lead to TDP-43 proteinopathy. Our transgenic flies recapitulate important pathological and clinical features of ALS, representing a powerful animal model for TDP-43 proteinopathy.

Results

Generation of Transgenic Flies Expressing Human TDP-43. To study human TDP-43 (hTDP-43) in vivo, we used *Drosophila*, a powerful genetic model widely used to study neurodegeneration (17, 18). We generated transgenic flies expressing monomeric red fluorescent protein (RFP) as a control or hTDP-43 fused to RFP in different populations of neurons using UAS/Gal4 system (19) (Fig. S1C).

We also generated transgenic flies expressing a mutant hTDP-43, T202, containing the carboxyl-terminal glycine-rich domain but lacking the amino-terminal RNA recognition motif, because expression of this mutant led to neuronal death in vitro, consistent with a recently published study (16). Both RT-PCR and Western analyses confirmed hTDP-43 expression, consistent with RFP signals detected in the eye discs of hTDP-43-RFP larvae (Fig. S1).

To test whether TDP-43 proteinopathy can be caused by insufficient TDP-43 expression, we also generated flies in which the expression of the endogenous fly homolog, Tar DNA binding protein homolog (TBPH), was reduced by RNA interference (RNAi) with two fly lines (numbers 38377 and 38379). Using TBPH-specific primers, we demonstrated that TBPH was significantly reduced in TBPH-RNAi-expressing flies as compared to the control flies (Fig. S1).

Expression of hTDP-43 in the *Drosophila* Eye Leads to Progressive Retinal Degeneration.

We used a GMR-Gal4 driver to express hTDP-43 specifically in *Drosophila* eyes beginning at the third instar larval stage. RFP control flies showed normal eye morphology (Fig. 1A), whereas flies expressing hTDP-43 began to show obvious ommatidia loss at eclosion with different levels of severity (Fig. 1B). Although there were significant variations in the eye defects among individual flies of the same age and the same genetic background, hTDP-43-expressing flies showed progressive degeneration with aging (Fig. 1C). Such retinal degeneration phenotype was not detectable in flies expressing the hTDP-43 mutant T202. We also investigated effects of decreasing expression of the endogenous fly TBPH on eye morphology using TBPH-RNAi flies. No obvious eye defect was found in these TBPH RNAi flies, consistent with a recent study in TBPH knockout flies (20).

We examined eye structures of hTDP-43 transgenic flies in frontal sections after toluidine blue staining. Both the RFP-expressing control flies and flies expressing hTDP-43 mutant T202 showed intact rhabdomere structure throughout their lifespan (Fig. 1D and F). In contrast, the ommatidia organization was completely lost in hTDP-43 transgenic flies, with large vacuoles frequently detected (arrow in Fig. 1E), although their cone cells remained largely normal. Using electron microscopy, we compared the ultrastructure of fly eyes expressing hTDP-43 with controls (Fig. 2). Well organized rhabdomeres (Rh) were seen in the control group of both young (1 day) and old (30 day) flies (Fig. 2A and B). However, the normal rhabdomere arrangement was disrupted in hTDP-43 flies as early as day 1, with very few rhabdomeres detected (Fig. 2C). By day 30, the rhabdomere structure was completely lost in hTDP-43 flies with morphological features

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The authors declare no conflict of interest.

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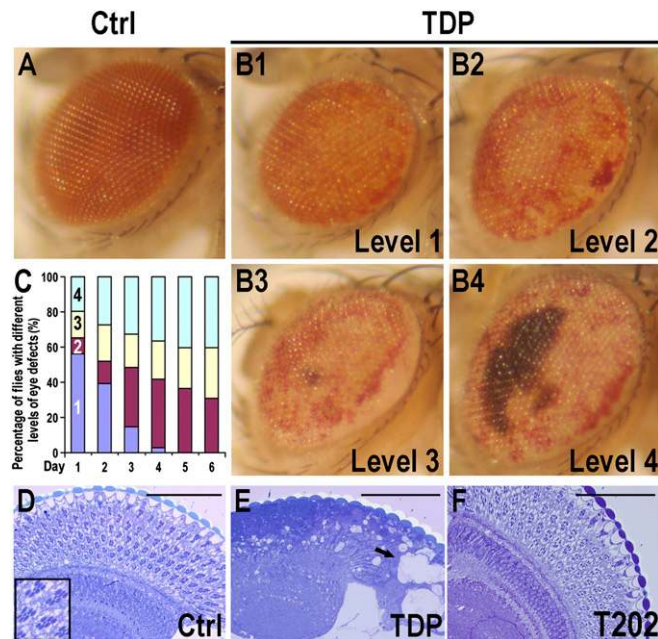


Fig. 1. Expression of hTDP-43 in fly eyes leads to progressive eye defects in an age-dependent manner. (A and B) Eye morphology of one control fly (A, GMR-Gal4/UAS-RFP) and hTDP-43 expressing flies (B, GMR-Gal4/UAS-hTDP-43-RFP). GMR-Gal4/UAS-hTDP-43-RFP eyes exhibit ommatidia loss and necrotic lesions. B1–B4 show hTDP-43-expressing fly eyes with ommatidia loss at different levels. (C) Quantification of the eye defects in hTDP-43-expressing flies during aging, showing significant difference from that in the control RFP-expressing flies ($P < 0.0001$ in χ^2 test). Eye defects were classified into four levels: 1, <25% ommatidia loss; 2, 25–50% ommatidia loss; 3, 50–75% ommatidia loss with small regions of necrosis (appearing as black dots); 4, >75% ommatidia loss with massive regions of necrosis. The percentages of flies with each level of eye defects are shown in different colors (1, blue; 2, red; 3, yellow; 4, cyan). By day 6, most flies expressing hTDP-43 showed moderate to severe eye defects. (D–F) Retinal sections stained with toluidine blue showed intact ommatidial and rhabdomere structure. (D) GMR-Gal4/UAS-RFP control fly eye (high magnification shown in *Inset*). Rhabdomere structures were disintegrated, and ommatidia arrangement was disrupted in GMR-Gal4/UAS-hTDP-43-RFP fly eyes (E) but not in GMR-Gal4/UAS-hTDP-43T202-RFP fly eyes (F). The arrow in E marks a large vacuole in the eye. (Scale bars: 100 μm .)

of neurodegeneration including degenerating mitochondria (as marked by the asterisk), multilamellar bodies (MLB), multivesicular bodies (MVB), and autophagic vacuoles (AV) (Fig. 2 D–F). However, flies expressing mutant hTDP-43, T202, show the normal ultrastructure (Fig. 2 G and H). These results indicate that expressing hTDP-43 in *Drosophila* eyes leads to age-dependent progressive degeneration of photoreceptors. The expression of the carboxyl-terminal fragment of hTDP-43 alone is not sufficient to cause neurodegeneration in flies.

Expression of hTDP-43 in Mushroom Bodies (MBs) Causes Axonal Loss and Neuronal Death. To test whether hTDP-43 expression had similar pathogenic effects in the central nervous system, we prepared flies expressing hTDP-43 in MBs under the control of OK107-Gal4. Axon projections from three subtypes of MB cells form the α/β , α'/β' , and γ lobes, respectively. These axon bundles were visualized by signals of the coexpressed membrane-localized GFP (mGFP) from UAS-mCD8::GFP construct. Three-dimensional structure of MB lobes was imaged by using confocal microscopy (Fig. 3). MBs in control flies showed typical lobe structure (Fig. 3A), whereas axon bundles were much thinner or completely lost as early as day one in hTDP-43-expressing MBs (Fig. 3C). There were remarkable variations in axonal defects among different flies with the same genetic background, involving different MB lobes. Interestingly,

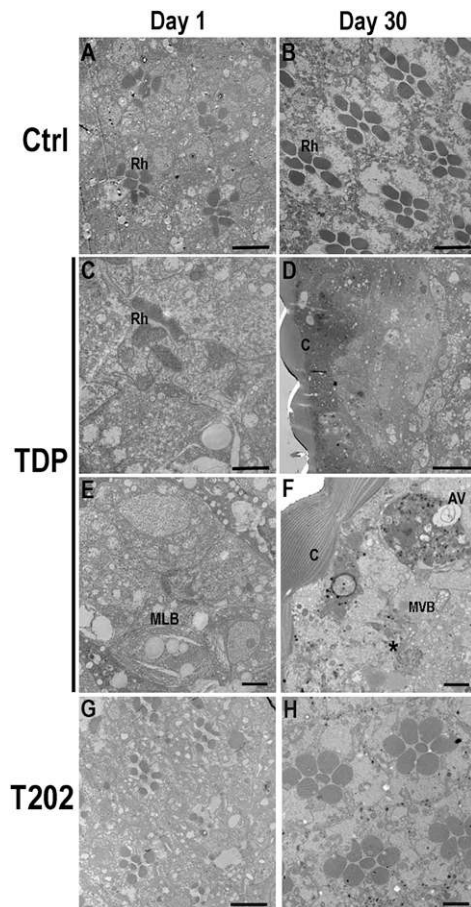


Fig. 2. Transmission EM reveals ultrastructural defects in the hTDP-43-expressing fly eyes. (A and B) Control flies (GMR-Gal4/UAS-RFP) showed normal rhabdomere (Rh) arrangement and ultrastructure at day 1 (A), and the intact rhabdomere structure is well maintained by day 30 (B). (C–F) At day 1, GMR-Gal4/UAS-hTDP-43-RFP flies showed severe losses of ommatidial structure with disrupted rhabdomeres (C) and the presence of multilamellar body (MLB). In 30-day-old flies expressing hTDP-43, the ommatidial arrangement under the cone (marked by "c") structure was completely disrupted (D), with the formation of autophagic vacuoles (AV) and multivesicular bodies (MVB) (F). The asterisk marks a degenerating mitochondrion. (G and H) The ommatidia were normal in transgenic flies expressing T202 mutant hTDP-43. (Scale bars: A–D and G, 10 μm ; E, F, and H, 2 μm .)

the remaining axons in each individual brain were often derived from one subtype of MB neurons (for example, α/β in C1, α'/β' in C2, and γ in C3 in Fig. 3). This suggests that the axon loss induced by hTDP-43 expression was not specific to one specific neuronal subpopulation, although it often occurred in a group manner. Furthermore, in hTDP-43-expressing MBs, these CNS neurons showed an age-dependent neurodegeneration, similar to that in hTDP-43-expressing photoreceptors cells. As compared with the normal lobe structures observed in the control MBs expressing RFP at day 30 (Fig. 3B), MBs expressing hTDP-43 showed only residual axon bundles with mGFP signals barely detectable (Fig. 3D). In addition, these hTDP-43-expressing MB neurons in aged flies showed obvious signs of axon degeneration, including a loss of axon integrity and increased abnormal axonal varicosities with uneven distribution of mGFP signals (marked by thin arrows in Fig. 3 D1 and D3). Such axonal pathology is not detectable in transgenic flies expressing T202 mutant TDP-43, suggesting the requirement of the amino-terminal domain for TDP-43 neurotoxicity in vivo.

We further tested whether reducing the expression of the endogenous *Drosophila* TBPH could affect the CNS neurons in

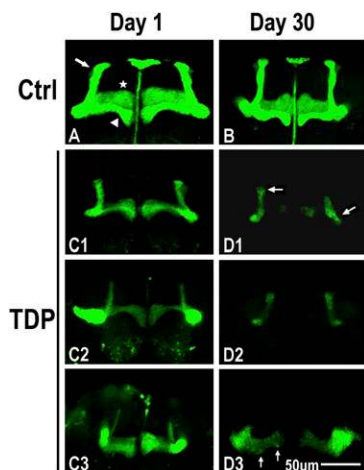


Fig. 3. Expression of hTDP-43 in mushroom bodies causes axonal loss. Axon bundles of MB neurons are visualized by membrane GFP (CD8::mGFP). Confocal images include all lobes in each brain obtained in Z-stacks and projected into single images. (A and B) Control flies (OK107-Gal4/UAS-mGFP/UAS-RFP) showed the normal MBs lobe structure at both day 1 and day 30. Arrow, α/α' lobes; arrowhead, β/β' lobes; star, γ lobe. (C) OK107-Gal4/UAS-mGFP/UAS-hTDP-43-RFP flies began to lose MB lobes at day 1. C1, only parts of α and β lobes were left; C2, only α' and β' lobes were left; C3, only γ and a few α'/β' lobes were left. (D) By day 30, flies expressing hTDP-43 had lost most axons, with few remaining in α and β lobes (D1 and D2) or in γ lobe (D3). The thin arrows in D1 and D3 mark the abnormal axonal varicosities with uneven distribution of mGFP signals.

MBs. Using the same TBPH RNAi lines, we showed that decreased TBPH expression in the MBs also resulted in axonal loss and neuronal death (Fig. S2), indicating that TBPH gene is

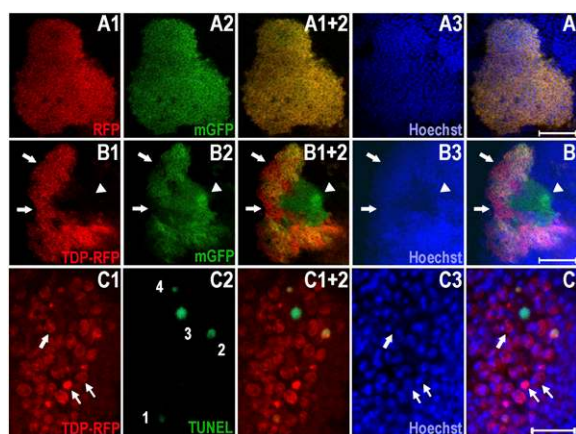


Fig. 4. Expression of hTDP-43 induces neuronal death in mushroom bodies. (A and B) Confocal sections of MB calyces in control flies (A, OK107-Gal4/UAS-mGFP/UAS-RFP) or hTDP-43-expressing flies (B, OK107-Gal4/UAS-mGFP/UAS-hTDP-43-RFP). MBs were well organized in the control, whereas hTDP-43-expressing brains showed much fewer neurons with underneath axons of residual neurons (arrowhead) visible in the area lacking cells. Note some neurons (arrows in B1+2) with hTDP-43-RFP signal did not have mGFP signal, whereas all control neurons showed normal morphology with both RFP and mGFP detected (A1+2). Nuclei were revealed by Hoechst dye staining in blue. (Scale bars: 50 μ m.) (C) TUNEL staining revealed cell death in hTDP-43-expressing cells. C1, hTDP-43-RFP; C2, TUNEL staining; C3, Hoechst staining; C1+2 and C, superimposed images. (Scale bars: 20 μ m.) The dying neurons show significant variations. Cell 1 showed relatively normal nuclear hTDP-43 distribution, whereas cell 3 had cytoplasmic hTDP-43-RFP signals with abnormal nuclear morphology as well as reduced nuclear TDP-43 signals. Cell 4 showed the condensed nucleus.

essential for normal development of *Drosophila* CNS neurons. However, axonal loss in TBPH RNAi flies was less severe and did not occur in the “group” manner as that in hTDP-43 overexpressing MBs (Fig. 3 C and D). No obvious abnormal axonal varicosities were detected in TBPH RNAi flies (Fig. S2 C and D).

We also examined morphological changes in cell bodies using confocal microscopy (Fig. 4). In normal flies, mushroom bodies have \sim 2,500 neurons in each calyx, organized in clusters. Such calyx structures were well preserved in control flies expressing RFP protein (Fig. 4A). In contrast, hTDP-43-expressing brains showed dramatic neuronal loss with significant variations in severity, similar to that in the fly eyes. For example, among 20 samples examined, 8 had severe neuronal loss with only a few neurons left, 10 lost more than half of their MB neurons (Fig. 4B), and 2 showed little neuronal defects. Interestingly, in some neurons, the mGFP signal was completely lost with only hTDP-43-RFP signal detectable (arrows in Fig. 4 B1–B3). Consistent with our observation in MB axons, MB neuronal death also occurred in groups, rather than in a random fashion, with regions of cell body loss and only residual axonal mGFP signals detectable (arrowhead in Fig. 4B).

We performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to examine the cell death. No TUNEL-positive cells were detected in control MBs expressing RFP (Fig. S3A) in 10-day-old flies. However, three to six TUNEL-positive cells were frequently found in each calyx of hTDP-43-expressing MBs from flies at the same age as control flies (Fig. 4C2), indicating that overexpression of hTDP-43 in these CNS neurons induced neuronal death. These dying neurons showed significant variations in the nuclear morphology as well as TDP-43-RFP fluorescence level and expression patterns. This may reflect dynamic changes of hTDP-43 expression and nuclear morphology during different stages of cell death.

Expression of hTDP-43 in Motor Neurons (MNs) Causes Aggregate Formation in Cell Bodies and Axons, Together with Axon Swelling.

To investigate potential roles of TDP-43 in MNs in vivo, we examined flies expressing hTDP-43 in MNs using a MN-specific driver, OK371-Gal4. Such transgenic flies expressing hTDP-43 showed apparently normal morphology at the larval stage but failed to hatch in the late pupa stage. We first examined the expression and localization of hTDP-43 in the transgenic flies using fluorescent microscopy. In most MNs, hTDP-43 was predominantly localized in the nucleus. When hTDP-43 was detected in the cytoplasm, neurons often showed signs of cell death: condensation and/or fragmentation of nuclei (marked by the arrowhead in Fig. 5A). TDP-43 protein aggregation was observed in both cell bodies (arrow in Fig. 5A) and axons (arrows in Fig. 5 B and C) in these MNs. Strikingly, axon swelling was frequently detected in MNs expressing hTDP-43 (Fig. 5C), similar in morphology to spheroid structures found in ALS patients (21, 22).

Expression of hTDP-43 in MNs Causes Neuronal Death and Functional Deficits.

We systematically studied our transgenic flies, focusing on MNs of late third instar larvae. Similar to the wild-type flies, MNs in the control RFP-expressing flies were well organized into clusters in different segments, as visualized by RFP and mGFP signals (Fig. 6A1 and A2). In contrast, MNs in hTDP-43-expressing flies were not organized in their normal clusters, showing cell loss. Such MN loss was more severe in the posterior abdominal segments as compared with the anterior ones. For example, in Fig. 6B1, two neurons expressing hTDP-43-RFP marked by arrowheads had lost mGFP signals, and another neuron (marked by the arrow) showed cell body swelling, suggesting of neuronal death. Such neuronal death was confirmed by TUNEL staining. There were no TUNEL-positive neurons in the controls, whereas TUNEL-positive cells were frequently detected among MNs expressing hTDP-43 (Fig. S4).

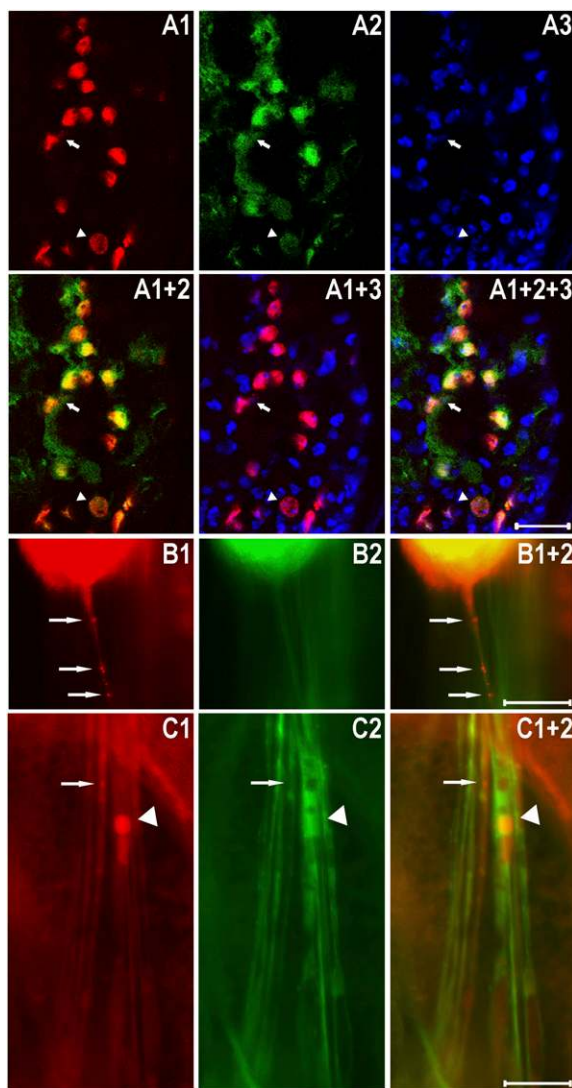


Fig. 5. Overexpression of hTDP-43 in motor neurons leads to protein aggregation in cytoplasm and axons, together with axonal swelling. (*A* and *B*) Overexpressing hTDP-43 using the motor neuron-specific driver (OK371-Gal4) resulted in protein aggregates detectable in the cytoplasm (*A*) and axons (*B*). Arrowheads in *A1–A3*, *A1+2*, *A1+3*, and *A1+2+3* mark cells containing condensed and fragmented nucleus. Arrows in *B1* and *B1+2* mark the hTDP-43-containing abnormal axonal varicosities. (*C*) All hTDP-43-overexpressing flies exhibited axon swelling with TDP-43-positive inclusions, which was absent in the control flies. Some regions with TDP-43 protein aggregates show reduced mGFP signals (marked by arrows), and other regions exhibit significant axonal swelling as marked by arrowheads. In all images: red, hTDP-43-RFP; green, mGFP; blue, Hoechst staining. (Scale bars: 50 μ m.)

Axonal and synaptic morphology of hTDP-43-expressing motor neurons were examined, especially at neuromuscular junctions (NMJ). Nerves from motor neurons project to their target muscles, branch a few times, and form synaptic boutons to innervate the muscle. MNs overexpressing hTDP-43 showed a significant decrease in the numbers of both big and small boutons, and of the axon branches (Fig. 6*D* and *E*) as compared to the control flies (Fig. 6*C*). Consistently, the locomotive ability of hTDP-43 flies was also significantly impaired at both larval and adult stages. The larval motility was examined from late third instar larvae, and the motility index was quantified as the number of movement waves during a period of 2 min. As shown in Fig. 6*F*, the motility index of control flies was 86.1 ± 3.1 . This index was reduced to 46.0 ± 2.3 in

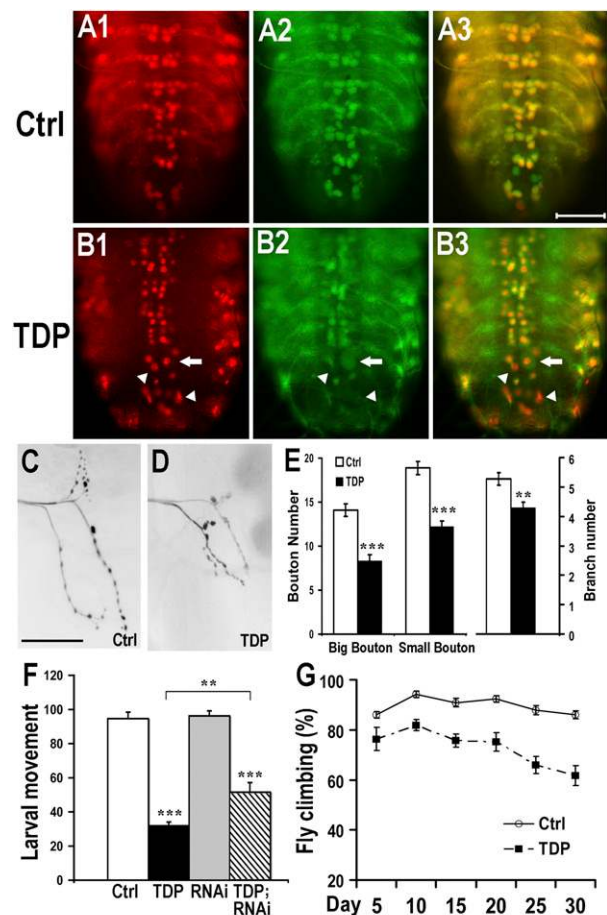


Fig. 6. Expression of hTDP-43 causes morphological and functional defects of motor neurons (MNs). (*A1–A3*) MNs in the dorsal clusters were well organized in the ventral nerve cord (VNC) in the control flies. Arrows and arrowheads mark three representative MNs showing healthy morphology. (Scale bars: 20 μ m.) (*B1–B3*) MNs expressing hTDP-43 showed cell death and morphological defects in dorsal clusters, especially in the last three segments of VNC. The arrow marks a swollen neuron with an enlarged mGFP area, and arrowheads mark MNs with fragmented or condensed nuclei and reduced mGFP signals. (*C* and *D*) Examples of axon branches and boutons at NMJ in the abdominal segment A2 of control or hTDP-43 transgenic flies, as revealed by mGFP signals. (Scale bars: 100 μ m.) (*E*) In the abdominal segments 2–4, hTDP-43 expression led to a significant reduction in the number of axon branches and boutons, as compared with the control flies. ***, $P < 0.0001$; **, $P < 0.001$. (*F*) The larval locomotion was significantly impaired in hTDP-43-expressing flies but not in TBPH-RNAi flies (labeled as “TDP,” “RNAi” groups, in black and gray bars, respectively), as compared to the control (Ctrl) flies. When hTDP-43-expressing flies were crossed to TBPH-RNAi flies, their larval movement was improved as shown in the cross-hatched bar. More than 30 larvae were tested in each group. ***, $P < 0.0001$; **, $P < 0.001$. (*G*) The climbing ability of adult flies showed progressive decrease during aging in hTDP-43-expressing flies, as compared with the controls, with the difference between the two groups significant beginning from day 15. The genotype of flies used in controls: *A–E*, OK371-Gal4/UAS-mGFP/UAS-RFP or UAS-TDP-43-RFP; *F*, OK371-Gal4/UAS-hTDP-43-RFP or UAS-RFP, or these flies crossed with TBPH-RNAi; *G*, RN2-Gal4/UAS-hTDP-43-RFP or UAS-RFP.

hTDP-43-expressing flies. Interestingly, the locomotive ability of TBPH-RNAi fly larvae was normal, indicating that reducing the endogenous TBPH expression did not affect larval movement. When hTDP-43 transgenic flies were crossed onto the TBPH-RNAi background, however, their larval movement was improved. The change in motility index was statistically significant (Fig. 6*F*). This suggests that the phenotype caused by hTDP-43 expression is

not due to a loss of function of TDP-43. Otherwise, we would expect exacerbation of the phenotype by the further reduction of the endogenous fly TDP-43 gene expression.

Most flies expressing hTDP-43 under the OK371-Gal4 driver died at or before eclosion. To examine adult fly motility, we used RN2-Gal4 to drive hTDP-43 expression in a small subset of MNs at the adult stage (23). Adult fly locomotive ability was examined at different ages by using a climbing assay as described in ref. 24. As compared to the control flies, hTDP-43-expressing flies showed a reduction in their motility, becoming progressively worse with aging and with the difference significant from day 10 after eclosion (Fig. 6G).

We further investigated whether hTDP-43 expression in fly neurons led to the formation of insoluble TDP-43 species by fractionating protein lysates prepared from heads of adult flies with the GMR driver. Sarkosyl-insoluble high-molecular-weight TDP-43 immunoreactive protein aggregates were detected in these flies (Fig. S5, lane 6), again mimicking the pathological TDP-43 species detected in patient tissues (6).

Discussion

The concept of TDP-43 proteinopathy has emerged as TDP-43-positive lesions have been discovered in a range of neurodegenerative diseases including ALS. TDP-43 proteinopathy is characterized by TDP-43 immunoreactive neuronal and glial inclusion bodies together with a progressive loss of function of the affected neurons. A recent study using an adeno-associated virus vector to express hTDP-43 in substantia nigra (SN) in rats revealed a loss of SN neurons, gliosis, and changes in amphetamine-stimulated rotational behavior (25). Here, we report the generation and characterization of transgenic flies expressing human TDP-43. Our hTDP-43 transgenic flies have amorphologic features strikingly similar to neuropathological characteristics of TDP-43 proteinopathy, and functional defects in these flies mimic those in TDP-43 proteinopathy patients. Several findings make our study particularly intriguing (*SI Discussion*). First, this animal model displays a wide range of neuropathological, biochemical, and functional features of human TDP-43 proteinopathy. Other currently available animal models recapitulate a limited repertoire of features of this syndrome. Second, our data suggest that overexpression of hTDP-43 or accumulation of hTDP-43 gene products may lead to morphological changes and functional deficits of TDP-43 proteinopathy. Third, similar to ALS and FTLN cases with TDP-43 pathology, our transgenic flies show significant variations in both neuropathology and functional changes. Fourth, our model may be useful for studying both sporadic and familial forms of TDP-43 proteinopathy, especially those without detectable mutations in the TDP-43 gene. Fifth, the neuropathological and functional features of our transgenic flies make them a powerful model for identifying both genetic modifier genes and chemical compounds that can either improve or exacerbate phenotypes in TDP-43 proteinopathy patients.

Progressive and age-dependent neuronal loss is a major pathologic hallmark of TDP-43 proteinopathy, including FTLN and ALS. In our study, when wild-type hTDP-43 protein was expressed in specific neuronal subpopulations in vivo, progressive and age-dependent neuronal loss was found in photoreceptors (Figs. 1 and 2), MBs (Figs. 3 and 4 and Fig. S2), and motor neurons (Figs. 5 and 6 and Fig. S4), suggesting that simply increasing TDP-43 expression is sufficient to initiate progressive neuronal death and functional deficiencies. ALS neuropathology is characterized by progressive degeneration of motor neurons, with synaptic loss and the presence of chromatolytic neurons ("balloon-like cells") together with axonal spheroids (swellings) (26, 27). The synaptic reduction in anterior horns of the spinal cord in ALS patients is correlated with the degree of neuronal loss (27). In some cases, axonopathy with giant axon swellings in the corticospinal tracts was detected, suggesting possible distal axonal degeneration during the early stages of this disease (21, 22, 26). In our transgenic flies, motor neurons expressing hTDP-43 show both syn-

apse reduction and abnormal axon swelling, mimicking the synaptic loss and axonal spheroids/axon swellings found in ALS patients. In addition, these transgenic flies exhibited age-dependent deficits in locomotion, similar to the clinical disability in ALS patients. Interestingly, a very recent study published while this article was under review reported the comparison of nontransgenic mice and transgenic mice expressing A315T mutant form of hTDP-43, showing a reduction in motor neuron axon numbers in A315T-TDP-43 transgenic mice (28). It remains unclear whether overexpressing wild-type hTDP-43 in mice could lead to similar pathology. However, deleting fly TBPH gene or reducing its expression by RNAi has been reported to decrease dendritic branching in sensory neurons, uncovering an important role of TBPH in dendritic development (29).

It is unclear whether dysfunction of TDP-43 is the major cause of TDP-43 proteinopathy and whether gain or loss of function of TDP-43 protein is the underlying pathogenic mechanism. We examined the effects of down-regulating fly TDP-43 homolog TBPH on the formation and function of the nervous system. As a result of alternative splicing, *Drosophila* TBPH gene is predicted to produce four RNA transcripts encoding two protein isoforms (Fig. S1). With TBPH-specific primers, the long isoform (TBPHI) was detected as the predominant isoform in the fly heads, and it was significantly reduced in TBPH-RNAi-expressing flies as compared to that in the control flies (Fig. S1B). These RNAi flies show axonal and synapse losses as well as impaired locomotion in the adult stage, suggesting that TBPH is required for function and maintenance of the nervous system in *Drosophila*, consistent with a recent study (29). It will be interesting to test whether overexpressing either long or short isoform of fly TBPH gene may also cause neurodegeneration phenotypes. Our data show that motor neuron phenotype caused by hTDP-43 expression was partially rescued by knocking down the expression of the endogenous fly TDP-43 homolog (Fig. 6F), consistent with the possibility of gain-of-function toxicity of hTDP-43 overexpression. If loss-of-function of TDP-43 is a major cause of MN phenotype caused by hTDP-43 expression in flies, it would be anticipated that reducing the endogenous fly TDP-43 expression by RNAi should further exacerbate, not partially rescue, MN loss. Therefore, our results support the gain-of-function model for TDP-43 proteinopathy. It is worth noting that the carboxyl-terminal domains of *Drosophila* TBPH (in both splicing isoforms) show little similarity to that of hTDP-43, a region where most human disease mutations have been located. Accumulation of carboxyl-terminal fragments of TDP-43 in affected tissue has been reported (6). One proteolytic cleavage site has been mapped to arginine residue 208, and expression of the carboxyl-terminal fragment in transfected cells leads to the formation of cytoplasmic aggregates in vitro (16). In our parallel study, overexpression of a similar carboxyl-terminal fragment, T202, did not affect either neuronal morphology or neuronal function (Figs. 1 and 2). This indicates that neurotoxicity caused by hTDP-43 expression requires its amino terminus, including the first RNA recognition motif.

Taken together, our hTDP-43 transgenic fly model recapitulates critical features of human TDP-43 proteinopathy and should serve as a powerful animal model for TDP-43 proteinopathy. In addition, our hTDP-43 transgenic fly data indicate that simply increasing hTDP-43 expression may be sufficient to cause neurotoxicity in vivo. This suggests that aberrant regulation of TDP-43 gene expression or decreased clearance of hTDP-43 in the adult stage may contribute to the pathogenesis of TDP-43 proteinopathy. Consistent with this is the observation that TDP-43 pathology is indistinguishable between sporadic and familial ALS cases and that TDP-43 proteinopathy can occur in the absence of TDP-43 mutation (ref. 30, reviewed in refs. 31 and 32). Our hTDP-43 transgenic flies can be used for studying both sporadic and familial forms of TDP-43 proteinopathy without detectable mutations in TDP-43 gene. Furthermore, our fly model will be useful for identifying genetic interacting factors that may modify the disease phenotypes and screening for chemical com-

pounds that could delay or reduce neuromuscular defects caused by aberrant TDP-43 protein expression, thus facilitating future development of effective therapy for TDP-43 proteinopathy.

Materials and Methods

Constructs, Fly Stains, Histology, Immunostaining, Fluorescent Microscopy, and Transmission Electron Microscopy. For a detailed description, see *SI Text*.

Fly Motility Assays. The larval motility was measured as the number of peristaltic waves in 2 min in the late third instar larvae expressing control or hTDP-43 under the driver of OK371-Gal4, as described in ref. 20. The adult motility was determined by using the climbing assay (24). Briefly, 35 virgin

female flies were collected in each group. Fly climbing ability was examined every 5 days as the number of flies climbing above a 5-cm line in 18 seconds after they were tapped to the bottom of an empty vial. The experiment was repeated 10 times for each group.

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Supporting Information

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SI Discussion

The formation of TDP-43-containing inclusion bodies is a prominent histopathological feature of TDP-43 proteinopathy. It remains controversial whether TDP-43 protein aggregation is a cause or consequence of neuronal loss, and whether it accelerates or prevents neurotoxicity. It has not escaped our attention that axon swelling and movement defects were found in all flies expressing hTDP-43. On the other hand, only ~10–20% of these flies showed TDP-43-containing cytoplasmic aggregates in the cell body and axons of the motor neurons, although larvae with aggregates showed more severe locomotive defects than their siblings without detectable aggregates. It is possible that early neurotoxic species of TDP-43 protein exist in forms below the microscopic detection level, and that the large TDP-43 protein aggregates may represent the late-stage byproducts, similar to Alzheimer disease with amyloid beta oligomers (reviewed in ref. 1). Alternatively, as in Huntington disease and other trinucleotide repeat disorders (2), such large TDP-43 protein aggregates may represent reactive or even protective responses by the neurons. We are currently developing biochemical assays to characterize hTDP-43 in neurons by examining soluble versus insoluble TDP-43 pool(s) and their relationship to neuronal death as well as their interactions with other proteins including chaperone proteins. These results will be reported in separate papers. In any case, generation of such transgenic flies provides a powerful system for us to address these issues when more sophisticated imaging techniques and biochemical assays are developed.

A number of studies reveal the ubiquitin-positive staining of pathological lesions in tissues affected by TDP-43 proteinopathy (for recent reviews, see ref. 3). Our initial data suggest that hTDP-43 expressed in transgenic flies colocalized with ubiquitin-immunostaining signals; however, immunoprecipitated hTDP-43 did not cross-react with commercially available antibodies specific to mono- or poly-ubiquitin, consistent with the finding that ubiquitin staining did not colocalize with TDP-43 staining in A315T-TDP-43-expressing transgenic mice (4). Additional studies are required to address the question of whether ubiquitinylation of hTDP-43 plays a critical role in the development of TDP-43 proteinopathy or, alternatively, whether the ubiquitin-positive feature of TDP-43 proteinopathy-associated neuropathological lesions simply reflects noncausative events during TDP-43 protein clearance. We have also begun to examine whether TDP-43 associates with chaperon proteins including Hsp70. So far, we have not detected direct interaction between hTDP-43 and Hsp70. Furthermore, it will be interesting to determine in our future study whether the neurodegeneration phenotypes associated with hTDP-43 overexpression are dependent on or independent of the RNA-binding activity of hTDP-43. These studies may require more sophisticated combinations of biochemical and imaging approaches.

In both ALS and FTLD cases with TDP-43 pathology, there are significant variations in both clinical manifestations and neuropathological changes, even among the patients from the same family. The disease duration can vary significantly, ranging from months to decades. For example, the age of onset for sporadic ALS (sALS) is generally between 55 and 65 years, with a median age of onset of 64 years and ~5% of cases having onset before the age of 30 (reviewed in refs. 5 and 6). Muscle weakness and atrophy can occur either symmetrically or asymmetrically. Likewise, our hTDP-43 transgenic flies exhibit variations in neuronal death, axonal changes, and functional deficits, recapitulating the phenotypic variations in TDP-43 proteinopathy

patients. Such pathologic and clinical variations suggest possible involvement of environmental and epigenetic factors as well as genetic modifiers in influencing phenotypic manifestations and clinical outcomes of TDP-43 proteinopathy.

It is clear from our results that the phenotypes of TBPH-RNAi flies are different from pathological findings in TDP-43 proteinopathy patients. TBPH-RNAi flies and the hTDP-43 transgenic flies show a number of distinct pathological and functional features. First, no TDP-43 protein aggregates were detected in TBPH-RNAi flies. Second, some neuron populations, such as photoreceptors, are not affected by decreased expression of TBPH, suggesting that TBPH may be not essential for eye development. Consistent with these results, a recent study showed that a TBPH knockout fly has normal eye morphology (7). Overexpression of hTDP-43, however, induces severe degeneration of photoreceptors but leaves the external cone structure largely unaffected, indicating the relative specificity of the effects of TDP-43. Decreased expression of TBPH in MBs and motor neurons leads to a significant neuronal loss. Interestingly, in TDP-43 proteinopathy patients, dementia and motor neuron degeneration may coexist. Third, although the bouton number and axon branching of motor neurons are decreased in both cases, hTDP-43-expressing flies have significantly fewer small boutons and axon branches than TBPH RNAi flies. Fourth, the neuronal loss and axonal swelling in hTDP-43-expressing larvae were not detected in TBPH RNAi larvae. Correlating with these morphologic findings, no locomotive defects were found at the larval stage in TBPH RNAi flies, and they hatched normally when OK371-Gal4 was used as the driver. Larvae of hTDP-43 transgenic flies, on the other hand, did not hatch and exhibited severely impaired motility when hTDP-43 expression was driven by OK371-Gal4 driver. Interestingly, adult flies of either TBPH RNAi or TDP-43 transgenic lines exhibited progressive reduction in locomotion, suggesting that the endogenous TBPH may not be essential for larval motor neuron development, but it becomes critical after eclosion. This may also provide an explanation for the observation that TBPH knockout flies began to die at the age of 3 days after hatching (7). Fifth, MBs expressing hTDP-43 show axonal loss in groups corresponding to the neuronal loss. Such lobular fashion of axonal degeneration was particularly noticeable during early stages, for example, α/β in C1, α'/β' in C2, and γ in C3 as shown in Fig. 3. This is reminiscent of “denervation atrophy” seen in ALS patients. Skeletal muscles of ALS patients often undergo chronic denervation atrophy in which the normal random distribution of fiber types I and II is lost with “fiber-type grouping,” and then a bundle of skeletal muscle fibers becomes innervated by sprouts from neurons innervating adjacent fibers. When that innervating neuron degenerates, the muscle fibers undergo “group atrophy.” In contrast, this phenotype is not detected in TBPH RNAi flies, in which all three subtypes of neurons survived and retained their axons, although these axons were frequently shortened (Fig. S2). These comparisons suggest that mechanisms underlying neuronal phenotypes caused by decreasing endogenous TBPH gene expression and by overexpressing hTDP-43 are distinct. As compared with TBPH RNAi flies, the hTDP-43 transgenic flies show more pathological and functional deficits that resemble those in TDP-43 proteinopathy patients. Consistent with this, mutations reported in TDP-43 proteinopathy patients are predominantly missense mutations in TDP-43 rather than gene deletion or loss of expression of TDP-43 gene, although both excessive TDP-43 expression and insufficient TDP-43 function (for example, as a result of sequestration of TDP-43 protein in inclusion bodies) may

contribute to neuronal dysfunction in TDP-43 proteinopathy. It should be noted that the gain-of-function toxicity hypothesis and loss-of-function hypothesis for TDP-43 proteinopathy may not be necessarily exclusive of each other, because TDP-43 overexpression or aberrant TDP-43 expression may lead to protein aggregation that may in turn cause sequestration and reduction of functional TDP-43 in the nucleus. Further investigation is necessary for us to understand nuclear versus cytoplasmic functions of TDP-43 and to elucidate pathogenic mechanisms underlying neuronal loss and functional deficits in TDP-43 proteinopathy.

As compared to the reported rat/AAV system that reproduces some aspects of TDP-43 diseases (8), our hTDP-43 transgenic fly model offers unique features and advantages as a model for TDP-43 proteinopathy. This transgenic fly system has the stable germline transmitted UAS-hTDP-43 gene that allows expression of hTDP-43 protein in selected regions and developmental stages upon crossing into different Gal4 driver lines. A large number of well characterized driver lines are commercially available. The selected driver lines used in this study have already demonstrated the powerful spatial and temporal resolutions that our system can achieve for detailed characterization of pathologic effects of hTDP-43 expression in corresponding subpopulations of neurons. For example, the phenotypes observed in hTDP-43-expressing motor neurons and NMJ have revealed striking similarities to those of ALS patients. Our animal model will enable us to carry out systematic and thorough studies on the *in vivo* pathologic effects of hTDP-43 as well as the resulting changes in neuronal morphology and function in distinct neuronal populations affected in human TDP-43 proteinopathy patients. Application of this TDP-43 proteinopathy fly model in the future may also allow us to establish the temporal sequence and possible causal relationships of different events during neurodegeneration in TDP-43 proteinopathies, such as axonal swelling, synapse loss, and neuronal death.

SI Materials and Methods

Constructs and Fly Lines. The ORF encoding human TDP-43 was cloned to express TDP-43 protein fused at its carboxyl terminus to RFP with a hemagglutinin (HA) tag using pUAST vector, which contains UAS sequence in the promoter region (Fig. S1C). The expression of the control RFP or hTDP-43-RFP was tested in S2 cell lines by transient transfection. The control RFP and hTDP-43-RFP constructs were then sent for customer fly injection service

(Rainbow Transgenic Flies) for generating transgenic flies in W1118 background. TBPH-RNAi flies were obtained from the Vienna Drosophila Research Stock Center (VDRC, stock no. 38377 and 38379). Different Gal4 driver lines were obtained from the Bloomington Stock Center and used to drive hTDP-43 or RFP expression in all cells (Act-Gal4), all neurons (elav-Gal4), eye (GMR-Gal4), mushroom bodies (OK107-Gal4), or subtypes of motor neurons (OK371-Gal4 and RN2-Gal4). The expression of hTDP-43 was examined by RT-PCR, Western blotting, and fluorescent microscopy by standard protocols.

Histology, Immunostaining, Fluorescent Microscopy, and Transmission Electron Microscopy. Fly heads were dissected at days 0, 10, 20, and 30 after eclosion and fixed for 48 h in 2% glutaraldehyde at 4 °C. After washing with PBS and after fixation in 1% OsO₄ buffer for 2 h, samples were rinsed in distilled water and dehydrated through a series of graded alcohols. Heads were placed in a transition solvent of propylene oxide and then a 1:1 solution of propylene oxide/resin for 30 min each, and infiltrated with pure resin overnight in a 60 °C oven. Samples were subjected to semithin or ultrathin sectioning.

Oriented vertically, heads were cut for semithin sections (1.0 μm each for ~20 μm per head) and stained with 0.5% toluidine blue. Light microscope images were obtained with an Olympus BX60 microscope. Alternatively, the ultrathin sections (50–60 nm) were cut on a Leica Ultracut UCT 54 Ultramicrotome and examined on a Phillips CM12 transmission electron microscope (TEM). Digital TEM images were taken of cross-sections of the entire head and retina.

Fly brains and larval ventral nerve cords were dissected and fixed in 4% paraformaldehyde. After wash, Hoechst was added into the mounting buffer to label nucleus. For TUNEL staining, samples were fixed in 4% formaldehyde and permeabilized in 50 mM sodium citrate. After blocking with BSA, samples were treated with TUNEL (In Situ Cell Death Detection Kit) with green fluorescence (Roche, catalog no. 11684795910). Boutons at the same anatomic position in dorsal side were examined in the third instar larvae. The nerves and boutons in live animals were revealed by the expression of mGFP. Fluorescent microscopy was performed by using a Leica DM5500B or a confocal microscope (Zeiss LSM 510). Z-stacks were obtained by confocal photo-sectioning and projected into single images for MB lobe structure.

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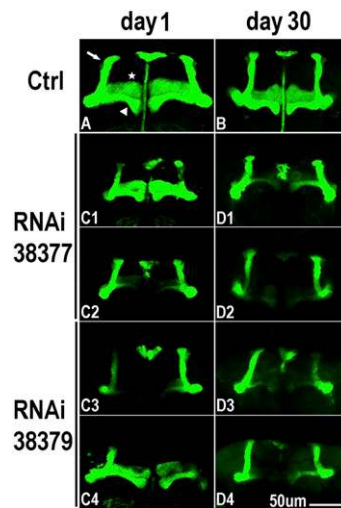


Fig. S2. Decreased expression of *Drosophila* TBPH gene in mushroom bodies causes MB lobe defects. (A and B) OK107-Gal4/UAS-mGFP/UAS-RFP flies showed normal MB lobe structure at both early and late adult stages (days 1 and 30). Arrow, α/α' lobes; arrowhead, β/β' lobes; star, γ lobe. (C and D) Reducing the expression of endogenous TBPH by RNAi led to axonal loss in MBs. Two independent TBPH-RNAi fly lines (38377 and 38379) were examined, showing similar axonal defects.

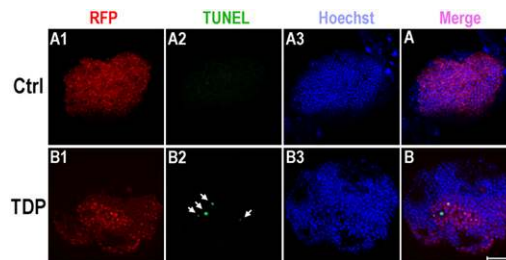


Fig. S3. TUNEL staining revealed apoptosis in hTDP-43-expressing MB neurons (B), as marked by arrows in B2. Such TUNEL-positive cells are not detectable in the control flies expressing RFP (A). OK107-Gal4 was used to drive hTDP-43-RFP or RFP expression in MBs. (Scale bars: 20 μm.)

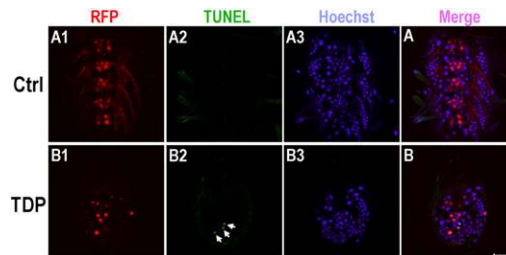


Fig. S4. TUNEL staining revealed apoptosis in hTDP-43-expressing motor neurons (B), as marked by arrows in B2. Such TUNEL-positive cells were not detectable in the control flies expressing RFP (A). OK371-Gal4 was used to drive hTDP-43-RFP or RFP expression in motor neurons. (Scale bars: 20 μm.)

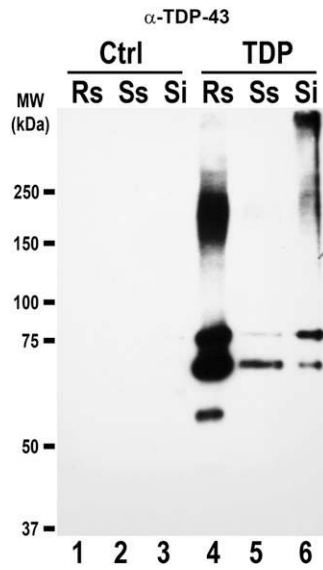


Fig. S5. Expression of hTDP-43 led to the formation of insoluble protein aggregates with high-molecular-weight species. GMR-Gal4/UAS-RFP (Ctrl) or GMR-Gal4/UAS-hTDP-43-RFP (TDP) flies were used. Total protein lysates were prepared from fly heads collected from control RFP- or hTDP-43-RFP-expressing groups in RIPA buffer and separated into RIPA-soluble fraction (Rs) or RIPA-insoluble pellets, which were further separated into sarkosyl soluble (Ss) or sarkosyl insoluble (Si) fractions after centrifugation. Sarkosyl insoluble fractions were dissolved in 6M urea containing sample buffer. These fractions were then separated by SDS/PAGE, followed by Western blotting with anti-hTDP-43 antibody.