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# Aggregate formation prevents dTDP-43 neurotoxicity in the *Drosophila melanogaster* eye

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ABSTRACT

TDP-43 inclusions are an important histopathological feature in various neurodegenerative disorders, including Amyotrophic Lateral Sclerosis and Fronto-Temporal Lobar Degeneration. However, the relation of these inclusions with the pathogenesis of the disease is still unclear. In fact, the inclusions could be toxic themselves, induce loss of function by sequestering TDP-43 or a combination of both. Previously, we have developed a cellular model of aggregation using the TDP-43 Q/N rich amino acid sequence 331–369 repeated 12 times (12xQ/N) and have shown that these cellular inclusions are capable of sequestering the endogenous TDP-43 both in non-neuronal and neuronal cells. We have tested this model *in vivo* in the *Drosophila melanogaster* eye. The eye structure develops normally in the absence of dTDP-43, a fact previously seen in knock out fly strains. We show here that expression of EGFP 12xQ/N does not alter the structure of the eye. In contrast, TBPH overexpression is neurotoxic and causes necrosis and loss of function of the eye. More important, the neurotoxicity of TBPH can be abolished by its incorporation to the insoluble aggregates induced by EGFP 12xQ/N. This data indicates that aggregation is not toxic *per se* and instead has a protective role, modulating the functional TBPH available in the tissue. This is an important indication for the possible pathological mechanism in action on ALS patients.



## Introduction

The human hnRNP TDP-43 has shifted from a modest player in obscure splicing mechanisms (Buratti and Baralle, 2001) to the main protagonist in the pathogenesis of neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis and Fronto-Temporal Lobar Degeneration. TDP-43 is mislocalized to the cytoplasm in the affected neurons of ALS patients, forming inclusions that contain ubiquitinated, hyperphosphorylated and cleaved TDP-43 (Neumann et al., 2006; Arai et al., 2006). This observation was followed by the identification in 2008 of mutations in the TARDBP gene in ALS patients (Sreedharan et al., 2008; Kabashi et al., 2008). However, it is important to notice that TARDBP gene mutations represent 1% of sALS cases and 4% of fALS cases, but TDP-43 is found aggregated in the 97% of all ALS cases (Ling et al., 2013). This means that TDP-43 aggregates in patients mostly in the absence of TARDBP mutations, making the understanding of the aggregation process more difficult. TDP-43 inclusions are the main histopathological feature of the disease, but their relation with the pathogenesis of ALS is still unclear. The possibilities that the inclusions could be toxic themselves induce loss of function by sequestering TDP-43 and hence depleting the nucleus of this essential factor or a combination of both these possibilities has been considered.

It is well known that lack of TDP-43 is deleterious for the cells and for the organisms in general (Ayala et al., 2008; Feiguin et al., 2009; L.-S. Wu et al., 2010). On the other hand, excessive production of TDP-43 is harmful for the cells and the whole organism, even when there is no detectable aggregation (Barmada et al., 2010; Hanson et al., 2010; Wegorzewska et al., 2009). Not surprisingly, a very tight self-regulation mechanism is in place to ensure the right level of TDP-43 in cells, whereby TDP-43 controls its mRNA production at post-transcriptional RNA processing level by binding to its own transcript and triggering a series of events that lead to degradation of the RNA (Ayala et al., 2011; Avendaño-Vázquez et al., 2012; Bembich et al., 2013).

The mechanisms that trigger aggregation and how the aggregates increase in size are unknown. Several attempts were done to mimic the TDP-43 aggregation in cells. It was early observed that the TDP-43 C-terminal tail contains a Q/N rich region that is involved in protein–protein interaction (D'Ambrogio et al., 2009). Moreover, it was shown that expression of C-terminal fragments of TDP-43 is sufficient to generate cytoplasmic aggregates (Igaz et al., 2009). The importance

*Abbreviations:* TDP-43, TAR DNA-binding protein-43; TBPH/dTDP-43, TAR DNA-binding protein-43 *Drosophila melanogaster* ortholog; ALS, Amyotrophic Lateral Sclerosis; 12xQ/N, Repeated TDP-43 amino acid sequence 331–369; UAS, upstream activating sequence.

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of the Q/N rich region within the C-terminal tail of TDP-43 in the self-aggregation process was also confirmed by Fuentealba et al. (Fuentealba et al., 2010). Based on this information and with the aim of looking for methodologies that could model the disease, we have developed a cellular model of aggregation using a 30 amino acid TDP-43 C-terminal peptide to promote TDP-43 aggregation (Budini et al., 2012a; Budini et al., 2012b). The inclusion bodies formed are predominantly cytoplasmic, ubiquitinated and phosphorylated like the ones found in patients. An animal model to extend these studies looking at *in vivo* phenotypes was needed and, in the first place, it was essential to test the aggregation profile in neurons and the effect it has on TDP-43 concentration in the target tissues.

TDP-43 is a highly conserved protein, which has a very close ortholog in *Drosophila melanogaster*, coded by the TBPH gene. As regards their binding activity and their role in splicing, the human and *Drosophila* proteins were shown to be interchangeable (Ayala et al., 2005). Furthermore, the deletion of the TBPH gene results in a locomotion defective fly (D23 and D142 strains), whose climbing ability could be restored with the expression in motor neurons of both *Drosophila* and human TDP-43 (Feiguin et al., 2009). The D23 and D142 flies do not present any evident external morphology alteration, and the eye structure is completely conserved (Feiguin et al., 2009). It follows that while TBPH plays a fundamental structural and functional role in locomotion (*i.e.*: neuromuscular junction development and maintenance), it is not essential for the development of a normal eye structure. This organ is then an ideal testing ground to study the interplay between TBPH overexpression and aggregation.

The results described here analyze the effect of TBPH overexpression in the eye, its toxic effects and their reduction by the formation of TBPH aggregates. Our results show that aggregation is not toxic *per se*. Furthermore, in the retinal tissue we demonstrated that the aggregates have a protective role, as they capture and insolubilize the excess of TBPH, blocking its toxic effect.

## Materials and methods

### *Transgenic flies*

Endogenous TBPH, its truncated form TBPHΔC (1–332) and human Tau cDNA (4N2R isoform) were Flag tagged and cloned in pKS69. EGFP-12xQ/N (12 repetitions of the TDP-43 331–369 sequence) and EGFP constructs were cloned in the pUASTattB vector (Bischof et al., 2007). All the constructs have been sequenced and subsequently used to create transgenic flies by standard embryo injections (Best Gene Inc.). While random insertions in yellow-white stain have been chosen for TBPH, TBPHΔC and human Tau, a specific insertion using strain 24486 was chosen for EGFP-12xQ/N and EGFP. All transgenic flies have been subsequently balanced on the required chromosome.

W1118, Oregon-R and GMR-Gal4 were obtained from Bloomington Drosophila Stock Center at Indiana University. Flies were fed on standard cornmeal (2.9%), sugar (4.2%), yeast (6.3%) fly food, maintained and crossed in a humidified incubator at 25 °C with a 12 hour–12 hour light–dark cycle.

### *Light microscopy of fly eyes*

Eye phenotypes of 1 day-old flies were analyzed with a stereomicroscope (Leica MZ75) and photographed with a camera (Leica DFC420C).

For the quantitative analysis of the induced eye phenotypes we defined arbitrarily 3 categories: (1) normal eye, (2) loss of pigmentation and small regions of necrosis and (3) loss of pigmentation and massive regions of necrosis. At least 50 1 day-old flies were analyzed for each genotype.

### *Immunoblot*

*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, 4 M urea, and protease inhibitors (Roche Diagnostic # 11836170001)). Proteins were separated by 8% SDS-PAGE, transferred to nitrocellulose membranes (Whatman # NBA083C) and probed with primary antibodies: rabbit anti-TBPH (1:3000, home-made) and mouse anti-tubulin (1:4000, Calbiochem # CP06). The membranes were incubated with the secondary antibodies: HRP-labeled anti-mouse (1:1000, Thermo Scientific # 32430) or HRP-labeled anti-rabbit (1:1000, Thermo Scientific # 32460). Finally, protein detection was assessed with Femto Super Signal substrate (Thermo Scientific # 34095).

### *Immunostaining*

Immunostaining was performed according to standard protocols (J. S. Wu and Luo, 2006). Briefly, wandering third instar larvae were dissected in phosphate buffer and fixed in ice-cold 4% paraformaldehyde (Alfa Aesar # 30525-89-4) for 20 min, washed in Phosphate Buffer with 0.1% Tween 20 (PBT) and blocked with Normal Goat Serum (NGS, Chemicon # S26-100 ML) 30 min at room temperature. The samples were incubated with primary antibodies mouse anti-FlagM5 (1:200, Sigma # F4042), rabbit anti-GFP (1:250, Life technologies # A11122) and rat anti-ELAV (1:300, Hydridoma bank # 7E8A10) over night at 4 °C with agitation, and treated with fluorescent conjugated secondary antibodies Alexa 555 anti-mouse (1:500, Invitrogen # A21422), Alexa 488 anti-rabbit (1:500, Invitrogen # A11008) and Alexa 647 anti-rat (1:500, Invitrogen # A21472) for 2 h at room temperature. All primary and secondary antibodies were diluted in PBT-5% NGS. SlowFade Gold Antifade reagent (Life technologies # S36936) was used as a mounting medium. The samples were imaged under a confocal laser-scanning microscope (LSM 510 META; Carl Zeiss, Inc.). Images were acquired by using 63× oil immersion objective and 2× zoom. Image processing was done with ImageJ software. All images were displayed as a single section of 0.5 μm.

### *Solubility test*

24 adult fly heads were dissected and homogenized in 192 ul of RIPA buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% Nonidet-P40 (v/v), 0.1% SDS, 1% Na-deoxycholate and a cocktail of protease inhibitors (Roche Diagnostic # 11836170001)). The samples were incubated under agitation for 1 h at 4 °C and then centrifuged at 1000 *g* for 10 min at 4 °C. An aliquot was taken at this point as the input, and after a further centrifugation step at 100,000 *g* for 30 min at 4 °C, the supernatant was collected as the soluble fraction. The remaining pellet was re-extracted in 60 ul of urea buffer (9 M urea, 50 mM Tris–HCl, pH 8, 1% CHAPS and a cocktail of protease inhibitors (Roche # 04693159001)) and spun down to remove any precipitate, while the 9 M urea soluble material was collected as the insoluble fraction. Proteins were separated by 8% SDS-PAGE. The different samples were loaded in a proportion 1:1:1 for the input, soluble and insoluble fractions. Proteins were electro-blotted to a nitrocellulose membrane (Whatman # NBA083C) and probed with the following primary antibodies: rabbit anti-TBPH (1:3000, home-made), mouse anti-GFP (1:2000, Roche # 11814460001) and mouse anti-tubulin (1:4000, Calbiochem # CP06). The membranes were incubated with the secondary antibodies: HRP-labeled anti-mouse (1:1000, Thermo Scientific # 32430) or HRP-labeled anti-rabbit (1:1000, Thermo Scientific # 32460). Finally, protein detection was assessed with Femto Super Signal substrate (Thermo Scientific # 34095).

*Climbing assay*

1 day-old flies were transferred without anesthesia to a 15 ml conical tube, tapped to the bottom of the tube, and their subsequent climbing activity was quantified as number of flies that reach the top of the tube in 15 s. At least 100 flies of each genotype were tested. In each set of experiments 20 flies were introduced in the cylinder and tested three times. The number of top climbing flies was converted into % value, and the mean % value (±SEM) was calculated for at least 5 experiments.

*Phototaxis assay*

Phototaxis assay was performed in a Y-maze with one arm exposed to violet light (peak wavelength 400 nm) and the other arm completely in the dark. Flies from each genotype were independently introduced into the stem of the Y-maze, and had the choice between violet light and the dark. After 30 s the number of flies that moved towards the illuminated chamber was determined. In each test 50 flies were analyzed. At least 100 flies of each genotype were tested. The number of phototactic flies was converted into % value, and the mean % value (±SEM) was calculated.

*Statistics*

One-way ANOVA followed by Bonferroni's multiple comparison was used to compare measures among 4 groups. Unpaired t-test analysis was used to compare measures between 2 groups. The significance between the variables was shown based on the p-value obtained (ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$). Values are presented as a mean and error bars indicate standard error of the mean (SEM).

## Results and discussion

*EGFP-12xQ/N expression forms insoluble aggregates in retinal cells with no consequence for the eye structure*

Our previously developed cellular model of TDP-43 aggregation showed that repeated TDP-43 amino acid sequence 331–369 (12xQ/N) is capable of interacting with TDP-43 inducing its aggregation both in non-neuronal and neuronal cells (Budini et al., 2012a).

To determinate whether similar interactions occur *in vivo*, we created transgenic *D. melanogaster* lines encoding either the *Drosophila* TDP-43 ortholog, TBPH, or the 12 repetitions of the 331–369 sequence of TDP-43 tagged with EGFP (EGFP-12xQ/N), under the control of the upstream activating sequence (UAS) (Brand and Perrimon, 1993).

After crossing the transgenic line EGFP-12xQ/N with the eye-specific GMR-Gal4 driver, we analyzed biochemically the solubility of the expressed protein and observed that EGFP-12xQ/N is mainly in the insoluble fraction, while EGFP alone is a soluble protein (Fig. 1a). Moreover, we found that neither the expression of the EGFP-12xQ/N, nor the expression of EGFP alone affect the external structure of the eye (Fig. 1b), meaning that EGFP-12xQ/N aggregates, *per se*, are not neurotoxic.

*EGFP-12xQ/N expression rescues TBPH-induced neurodegeneration*

As reported for the human protein (Li et al., 2010; Miguel et al., 2011), the overexpression of TBPH with the GMR-Gal4 driver induced a remarkable degeneration of the external surface of the eye, with the formation of necrotic patches, followed by the consistent loss of eye pigmentation (*compare* Figs. 2a and b). Surprisingly, such a strong neurodegeneration was completely reverted by the co-expression of TBPH together with EGFP-12xQ/N (Fig. 2c). We assumed that this improvement was due to the modulation of the TBPH cellular levels by sequestration of the protein in the EGFP-12xQ/N aggregates.

Such recovery of the degenerated phenotype is 12xQ/N related and cannot be due to a non-specific effect since the co-expression of EGFP, without the 12xQ/N tail, and TBPH did not prevent the retinal degeneration induced by TBPH expression (Fig. 2d). A quantitative analysis of the phenotypes was performed examining at least 50 flies per genotype (Fig. 2e).

*The TBPH C-terminal amino acids are essential for the interaction with the EGFP-12xQ/N aggregates*

In previously published results (Budini et al., 2012a) we demonstrated that the C-terminal tail of human TDP-43 is the part of the protein required for the protein–protein interaction. The expression of TBPH lacking the C-terminal tail under GMR-Gal4 driver induced also a strong

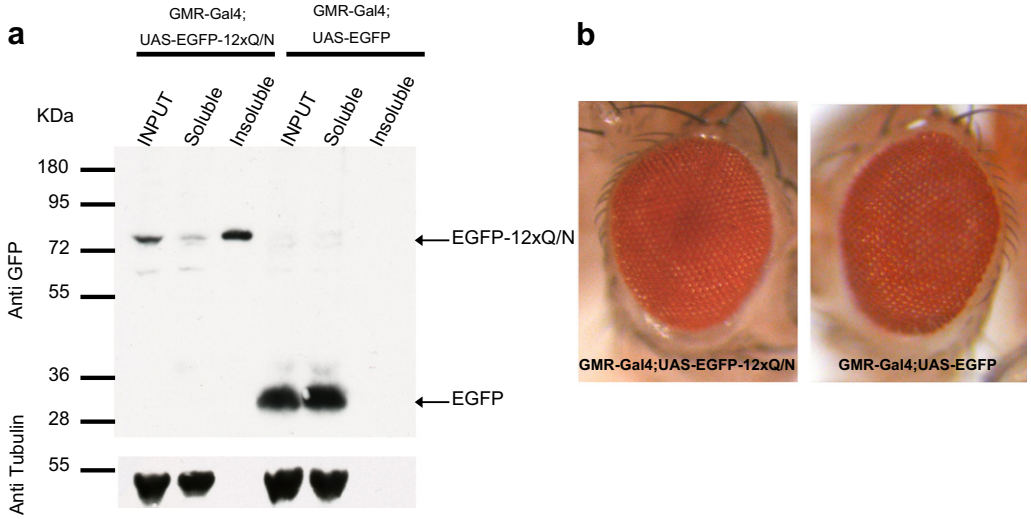


**Fig. 1.** (a) Western blot of fractionated proteins obtained from GMR-Gal4;UAS-EGFP-12xQ/N and GMR-Gal4;UAS-EGFP adult fly heads. Total proteins were fractionated into soluble and insoluble fractions. The proteins were detected using an anti-GFP antibody. EGFP-12xQ/N is mainly an insoluble protein, whereas EGFP alone is found only in the soluble fraction. Tubulin served as loading control. (b) External eye phenotype of flies GMR-Gal4;UAS-EGFP-12xQ/N and GMR-Gal4;UAS-EGFP, the external eye phenotype of both flies is completely normal.

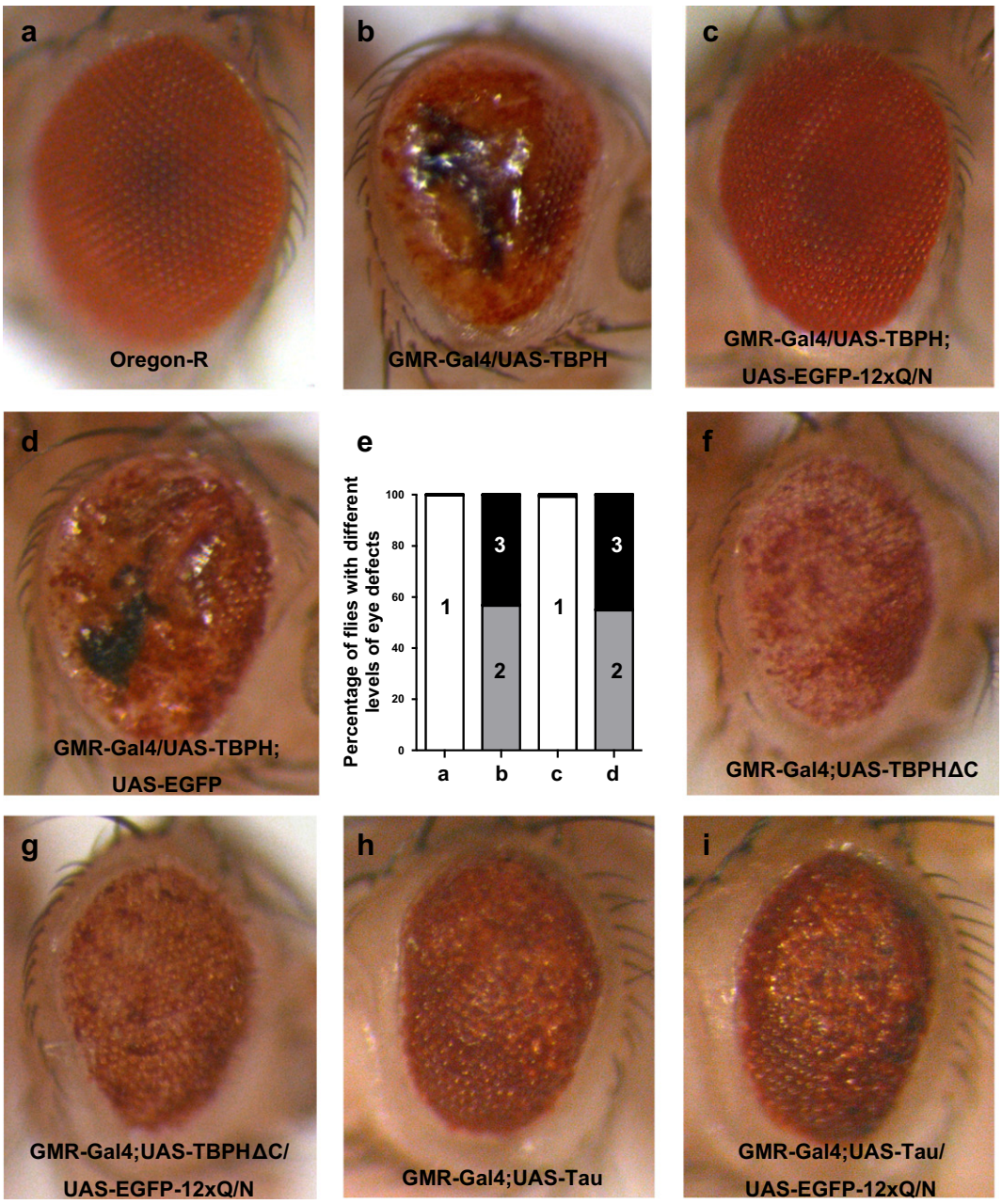


**Fig. 2.** External eye phenotype of flies: (a) Oregon-R, (b) GMR-Gal4/UAS-TBPH, (c) GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N, (d) GMR-Gal4/UAS-TBPH;UAS-EGFP, (f) GMR-Gal4;UAS-TBPHΔC, (g) GMR-Gal4;UAS-TBPHΔC/UAS-EGFP-12xQ/N, (h) GMR-Gal4;UAS-Tau, (i) GMR-Gal4;UAS-Tau/UAS-EGFP-12xQ/N. (e) Quantification of eye defects for Oregon-R, GMR-Gal4/UAS-TBPH, GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N, GMR-Gal4/UAS-TBPH;UAS-EGFP flies. We defined arbitrarily 3 categories: (1) normal eye, (2) loss of pigmentation and small regions of necrosis and (3) loss of pigmentation and massive regions of necrosis. Expression of TBPH induces degeneration in Drosophila eye (b). The co-expression of EGFP-12xQ/N rescues the eye degeneration (c), whereas the co-expression of EGFP alone does not change the phenotype induced by TBPH expression (d). On the other hand, expression of TBPHΔC also induces degeneration in *Drosophila* eye (f). The co-expression of EGFP-12xQ/N does not rescue this degeneration (g). The expression of Tau also induces degeneration in *Drosophila* eye (h), and the co-expression of EGFP-12xQ/N does not rescue this degeneration (i).

phenotype in the eye that includes rough degeneration of the eye surface and extensive depigmentation of the retina (Fig. 2f). Although this phenotype was milder compared to the expression of the TBPH full-length protein, we did not observe any modification of the phenotype upon co-expression of TBPHΔC and EGFP-12xQ/N (*compare* Figs. 2f and g). This result suggests that the direct interaction between these proteins is required to prevent TBPH-mediated neurodegeneration.

Moreover, we investigated if the effect seen with EGFP-12xQ/N was specific for the neurodegeneration induced by TBPH overexpression. For this purpose, we focused on Tau protein, which is involved in other proteinopathies. Tau expression in *Drosophila* eye caused degeneration of the retinal tissue, which could not be rescue by the co-expression of EGFP-12xQ/N (*compare* Figs. 2h and i).

### *EGFP-12xQ/N expression promotes TBPH aggregation in retinal cells*

Importantly, EGFP-12xQ/N expression does not suppress the TBPH-induced eye phenotype simple through down-regulation of transgene expression, as reveled by western blot analysis (Fig. 3). Co-expression of TBPH and EGFP-12xQ/N or EGFP did not change the total TBPH level, pointing to a different functionality of the protein, most probably due to its physical status.

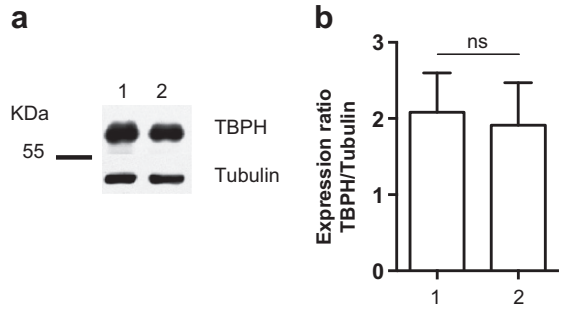


**Fig. 3.** (a) Quantification of TBPH level on total protein extracts prepared from adult fly heads GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N (1) and GMR-Gal4/UAS-TBPH;UAS-EGFP (2). A representative western blot is shown. Tubulin was used as a loading control. (b) Image J quantification of TBPH was performed from three independent experiments. ns indicates $p > 0.05$ (not significant) calculated by unpaired t-test. Error bars indicate SEM.

In order to further explore the mechanism behind the recovery of the TBPH-induced eye phenotype by EGFP-12xQ/N expression, we looked for potential modifications in the intracellular pattern of TBPH by performing a biochemical fractionation of the *Drosophila* adult head proteins extracted from GMR-Gal4/UAS-TBPH;UAS-EGFP and GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N expressing flies. Fig. 4 shows a clear shift in the TBPH solubility pattern when it is co-expressed with EGFP-12xQ/N compared with EGFP control. In the case of GMR-Gal4/UAS-TBPH;UAS-EGFP flies, TBPH appears mainly in the soluble fraction, whereas in GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N flies, the majority of the TBPH is present in the insoluble fraction.

*EGFP-12xQ/N and TBPH co-localize in the Drosophila eye discs*

The fact that the TBPH-induced degeneration was completely rescued when EGFP-12xQ/N was co-expressed with TBPH (Fig. 2c), taken together with the solubility data reported above (Fig. 4), implied that

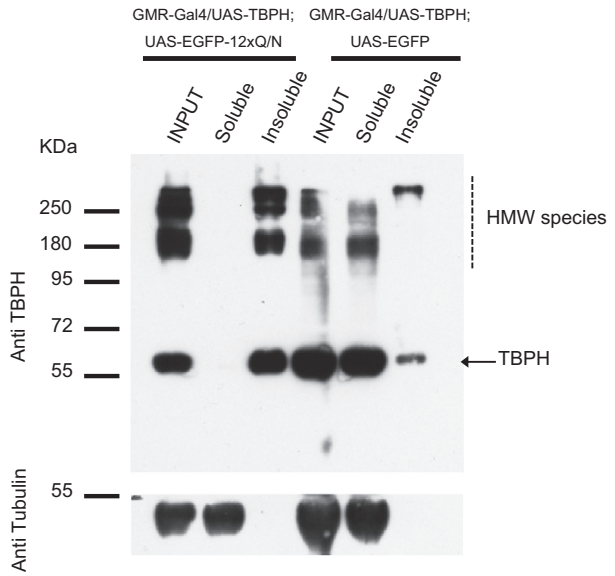


**Fig. 4.** Western blot of fractionated proteins obtained from GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N and GMR-Gal4/UAS-TBPH;UAS-EGFP adult fly heads. Total proteins were separated into soluble and insoluble fractions. Proteins were detected with anti-TBPH antibody (arrow and high molecular weight (HMW) species). Tubulin served as a loading control. Co-expression of EGFP-12xQ/N and TBPH leads to formation of insoluble protein aggregates. In contrast, TBPH remains mainly soluble when it is co-express with EGFP.

TBPH may be sequestered in the EGFP-12xQ/N aggregates, allowing a reduction in TBPH availability and avoiding the relative degeneration induced by its high level. To test this hypothesis we analyzed the localization of both proteins in the retinal cells. Immunohistochemistry of the third instar larvae eye discs was performed in order to examine the cellular distribution of the proteins, in GMR-Gal4/UAS-TBPH;UAS-EGFP and GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N transgenic lines. In the case of the larvae expressing TBPH and EGFP, both proteins were homogeneously distributed (Fig. 5a), while TBPH was found aggregated and co-localizing with the EGFP-12xQ/N aggregates when both proteins were co-expressed (Fig. 5b). Moreover, we confirmed that the observed co-localization of TBPH and EGFP-12xQ/N in the aggregates was abrogated when the C-terminal tail of TBPH was not present (Fig. 5c).

It can be concluded that the neurotoxicity caused by increased level of TBPH *in vivo* is modulated by its aggregation. In fact, the co-expression of EGFP-12xQ/N in *Drosophila* eye is protective by titrating the excess of the active TBPH.

This effect differs from the protective role postulated for Lewy bodies (present in Parkinson's disease and composed mainly by mutant α-synuclein), hyperphosphorylated Tau aggregates (Alzheimer's disease) and huntingtin inclusions that result from polyglutamine expansions (Huntington's disease). In all these cases, it has been proposed that the toxicity is a consequence of a mutation or abnormal modification of the protein, and that the aggregation is able to reduce this toxicity (Arrasate et al., 2004; Cowan and Mudher, 2013; Tanaka et al., 2004). However, in our case, the aggregates have a protective effect as just remove the wild type TBPH in excess.

*EGFP-12xQ/N expression restores eye functionality in TBPH expressing flies*

To determinate whether the suppression of TBPH toxicity by EGFP-12xQ/N is correlated with eye functionality, phototaxis assay was performed with 1 day-old flies of the different genotypes (Fig. 6a). TBPH overexpressing flies were not attracted to the light source, meaning that they were blind, consistent with the defective morphology of the eye structure. In flies co-expressing TBPH and EGFP-12xQ/N the vision was restored to normal levels, as there was not significant difference compared to the wild type flies, consistent with the recovery of the eye morphology. Moreover, the visual deficit was still present in flies co-expressing EGFP and TBPH.

Fig. 6b shows that the climbing ability of the flies of all the genotypes analyzed was comparable to the wild type flies, meaning that the negative phototaxis result is not due to a motor impairment.

## Conclusions

We have shown here that the neurotoxicity caused by increased level of TBPH *in vivo* can be modulated by its aggregation. The co-expression of EGFP-12xQ/N, an inducer of TBPH aggregation, in *Drosophila* eye is protective, because the excess TBPH becomes a non-functional insoluble protein as part of the induced inclusions.

TDP-43 has a natural tendency to aggregate and in normal circumstances a small fraction of the protein is insoluble in the tissues. During aging, the clearance of TDP-43 aggregates by, for example, the autophagy pathway or by the proteasomes may become more difficult. The increasing capture of TDP-43 by growing cytoplasmic aggregates may lower the amount of TDP-43 returning to the nucleus. This drop in levels will be sensed by the self-regulation mechanism, and may in turn increase the TDP-43 mRNA levels and hence the protein levels. This overexpression of TDP-43 could be initially modulated by the aggregates that capture the protein in excess, maintaining acceptable levels of active protein in the cell. However, continued addition to the inclusions will make them grow to a point in which they will capture so much protein that the nuclear capacity to produce TDP-43 mRNA will be overcome. This situation will lead to a lack of TDP-43, and its nuclear function will be lost, leading to neuronal loss.

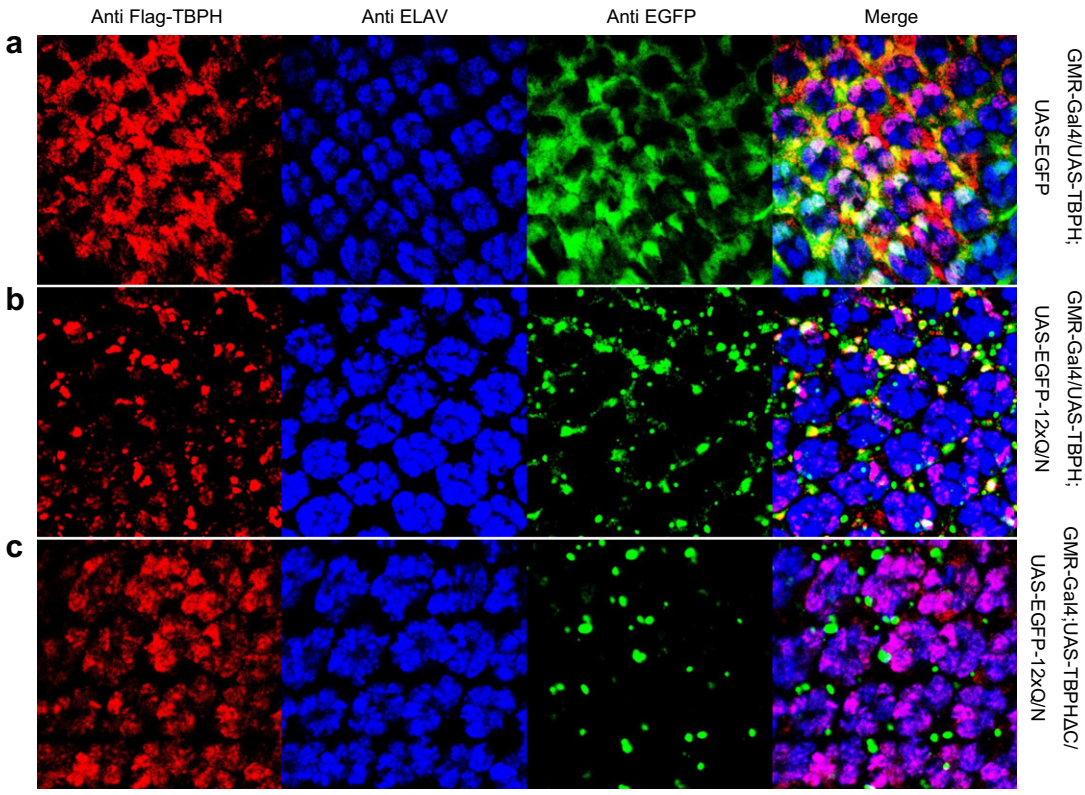


**Fig. 5.** Confocal images of third instar larvae eye disc co-expressing TBPH and EGFP (a), TBPH and EGFP-12xQ/N (b), TBPHΔC and EGFP-12xQ/N (c). Samples were stained for ELAV, Flag-TBPH, and EGFP. All the figures correspond to a single confocal section.

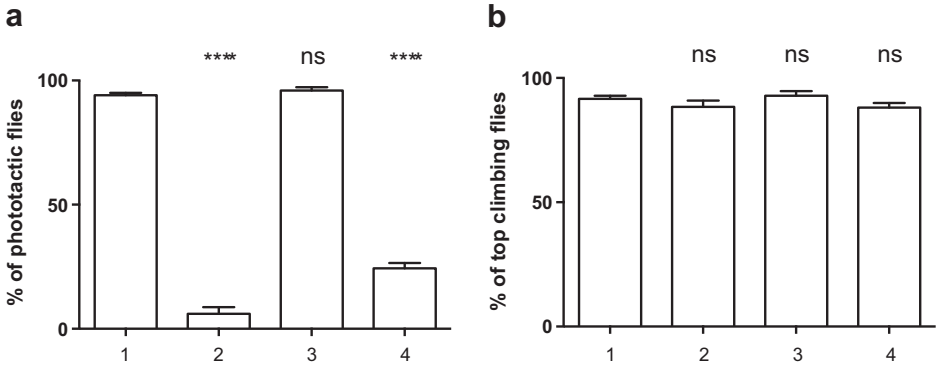


**Fig. 6.** Phototaxis assay of 1-day-old flies of different genotypes indicated as follows: (1) Oregon-R, (2) GMR-Gal4/UAS-TBPH, (3) GMR-Gal4/UAS-TBPH;UAS-EGFP-12xQ/N, (4) GMR-Gal4/UAS-TBPH;UAS-EGFP. Flies expressing TBPH are not attracted to the light, whereas flies co-expressing TBPH and EGFP-12xQ/N are attracted in the same proportion as wild type flies. On the contrary, the co-expression of TBPH and EGFP does not restore the vision. **** indicates $p < 0.0001$ and ns indicates $p > 0.05$ (not significant) calculated by one-way ANOVA followed by Bonferroni's multiple comparison. Error bars indicate SEM. (b) Climbing assay of 1-day-old flies of different genotypes. The climbing ability of all the genotypes was comparable to the wild type. ns indicates $p > 0.05$ (not significant) calculated by one-way ANOVA followed by Bonferroni's multiple comparison. Error bars indicate SEM.

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