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TDP-35, a truncated fragment of TDP-43, induces dose-dependent toxicity and apoptosis in flies

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TAR DNA-binding protein 43 (TDP-43) is an essential 414 amino acid protein that regulates multiple aspects of RNA biogenesis, processing, and transport. It localizes primarily in the nucleus, but abnormal translocation and accumulation in the cytosol occur under pathological conditions (Tziortzouda et al., 2021). TDP-43 abnormalities are typical pathological hallmarks of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration. Mutations in the TDP-43-encoding gene TARDBP cause familial ALS, while wild-type TDP-43 is associated with almost all (~97%) of sporadic ALS cases and nearly half of frontotemporal lobar degeneration patients (~45%) (de Boer et al., 2020). Extensive research has identified post-translational modifications of TDP-43 such as phosphorylation, ubiquitination and truncation as major histopathological characteristics in TDP-43 proteinopathies. Substantial progress has occurred in studying protein aggregation involving phosphorylated and ubiquitinated TDP-43. However, as recently discussed by us, the relevance and pathological role of truncated TDP-43 forms are still poorly understood (Chhangani et al., 2021). Here we extend our discussion on truncation of TDP-43, present new experimental insights into the neurotoxic role of its cleaved TDP-35 fragment, and provide a perspective on new avenues of research in this field.

TDP-43 proteinopathies display prominent depletion of nuclear TDP-43, post-translational modifications, and cytosolic aggregation involving protein-protein and protein-RNA interactions. Truncation of TDP-43 may influence these processes, and different truncated forms may exert different degrees of toxicity (Igaz et al., 2009), suggesting a need to conduct thorough studies targeting individual TDP-43 fragments. Truncated TDP-43 species may range from 15–43 kDa in size. The 25 kDa and 35 kDa fragments, also known as TDP-25 and TDP-35, respectively, appear to be the most prominent ones. Three types of peptidases that include asparaginyl endopeptidase, Calpains (I, II) and Caspases (3, 4, 7) are known to cleave TDP-43 at a minimum of 28 different sites along the length of the protein. Cleavage by these peptidases can generate an array of C-terminal fragments (CTFs) and N-terminal fragments (NTFs). Igaz et al. (2009) reported the discovery of a wide range of CTFs in postmortem brain samples from patients with neurodegenerative conditions. Since then, multiple studies have confirmed the presence of TDP-43 truncated forms in patients with a variety of neurodegenerative maladies. A more recent mass spectrometry analysis suggested that the N-terminal end of TDP-43 is more accessible to proteases (Kametani et al., 2016). This property increases the likelihood of proteolytic cleavage at the N-terminus, making CTFs the primary products. As for NTFs, very little is known about their relevance in TDP-43 proteinopathies. Despite their lower abundance, NTFs may also play a significant role in disease progression. Hence, it is important to design and conduct studies to fully understand all truncated forms, including their origin and role in TDP-43 proteinopathies.

TDP-43 has two RNA binding domains (RRM1 and RRM2) and one C-terminal prion-like domain. Truncated forms often exclude one or more of these domains, and such exclusions lead to a loss-of-function. Also, the N-terminal end of TDP-43 is essential for its functional conformation. Consequently, truncated forms that lack the N-terminal end can interact to form amyloid-like structures and associated aggregates. Processes such as these that lead to the aggregation of truncated forms effectively result in a toxic gain-of-function. Since these aggregates also sequester full-length TDP-43, they may further obstruct the protein's normal regulatory functions. Regardless of the exact mechanisms involved, both N- and C-terminal fragments may lead to cellular toxicity by either a gain-of-function or a loss-of-function. Therefore, a comprehensive characterization of truncated TDP-43 forms is of paramount importance.

TDP-43 fragments are also present in several other neurodegenerative conditions: Alzheimer's disease, corticobasal degeneration, Parkinson's disease, Pick's disease, and traumatic brain injury (Chhangani et al., 2021). While TDP-43 truncated forms are not the primary causative agents in these diseases, TDP-43 truncated forms may play an essential secondary role by exacerbating disease progression. Indeed, recent studies suggest that truncated TDP-43 species can serve as biomarkers for certain TDP-43 proteinopathies, even if the precise pathogenic role of these fragments is unclear (Feneberg et al., 2021). Furthermore, several *in vitro* and *in vivo* studies suggest that TDP-43 fragments are neurotoxic (Igaz et al., 2009; Medina et al., 2014; Zhang et al., 2009). However, insights on their pathomechanisms remain elusive and controversial since cleavage of TDP-43 could be an irrelevant byproduct of other cellular processes. For instance, cells may activate apoptotic pathways in response to various stressors, activating caspases that cleave TDP-43.

We recently published a review on TDP-43 fragmentation that highlights its relevance and potential implications in neurodegeneration (Chhangani et al., 2021). We deduced that the heterogeneity of TDP-43 fragments leaves us with two fundamental questions. First, how are these different truncated forms produced? Second, what roles do these TDP-43 fragments play in neurodegeneration? Apart from the proteolytic cleavage of full-length TDP-43, we know very little about the origin of these truncated forms. A combination of protease-dependent and -independent mechanisms are potentially involved since there are several TDP-43 cleavage products associated with ALS and frontotemporal lobar degeneration without candidate proteases. Either investigators have not found the relevant proteases or non-enzymatic mechanisms are also involved. Recent studies have found that alternative splicing can also produce TDP-35 (Xiao et al., 2015; Weskamp et al., 2020). Interestingly, these studies describe at least two different splice variants of the TDP-43 transcript that produce different neurotoxic TDP-35 species. These findings introduce another layer of complexity by showing that both post-transcriptional and post-translational modifications are involved.

Several *in vitro* and *in vivo* studies have shown that cleaved TDP-25 fragments are toxic and can induce neuronal defects in animals, but only a few have addressed the role of TDP-35 (Chhangani et al., 2021). In one such study, Crippa et al. (2016) created transgenic flies that expresses full-length TDP-43 and its truncated TDP-35 product from the same genomic locus, allowing similar expression levels to facilitate proper comparison of phenotypes. They found that TDP-35 is significantly more toxic than TDP-43 when expressed in the *Drosophila* eye, resulting in pupal lethality (Crippa et al., 2016). Motivated by this finding, we obtained these flies (a gift from Serena Carra) to compare the effects of different expression levels. These fly models employ the binary UAS-Gal4 system to control the expression of TDP-43 and TDP-35 transgenes. Furthermore, the UAS-Gal4 system induces expression in a temperature-dependent manner, with higher Gal4 activity in flies cultured at higher temperatures. Therefore, we used the eye-specific gmr-Gal4 driver to activate expression of TDP-43, TDP-35 and the innocuous LacZ control transgene in photoreceptor neurons at 22°C, 25°C and 27°C. As shown in **Figure 1**, we found that LacZ and wild-type TDP-43 did not affect eye morphology at any temperature, except for minor depigmentation patches at the highest temperature (27°C) for TDP-43-expressing flies (**Figure 1A**, top and middle rows). In contrast, TDP-35 was increasingly toxic with a direct relationship to temperature and its associated expression level. Specifically, TDP-35 triggered disorganization of the retinal ommatidial array at 22°C, extensive necrotic patches at 25°C, and pupal lethality with complete necrosis of the eye structures at 27°C (**Figure 1A**, arrows in bottom row). To test whether TDP-35 induces apoptosis in *Drosophila* photoreceptor neurons, we

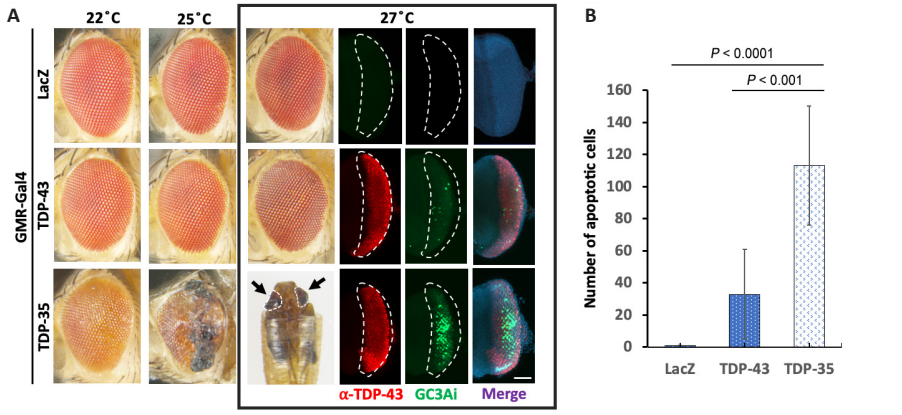

Figure 1 | **Overexpression of TDP-35 causes dose-dependent neurodegeneration and apoptosis in the *Drosophila* eye.**

(A) The indicated transgenes were crossed with the eye-specific gmr-Gal4 driver at three different temperatures to elicit an increasing expression gradient. Compared to the innocuous LacZ and TDP-43 transgenes, TDP-35 causes mild disorganization of the ommatidial array at 22°C, large necrotic areas at 25°C, and complete necrosis of the eye (black arrows) at 27°C resulting in pupal lethality. While the GFP-based apoptosensor GC3Ai detected high levels of apoptosis in the eye primordium (dotted line) of TDP-35-expressing larvae, GFP signal was absent in LacZ specimens and only marginal levels of GC3Ai were found in TDP-43-expressing flies. Scale bar: 50 µm. The anti-TDP-43 (C-terminal) antibody (Proteintech:12892-1-AP; 1:300 dilution) was used, which recognizes both TDP-43 and TDP-35. (B) Quantification of apoptotic cells was performed with ImageJ software using micrographs from third instar larval eye imaginal discs carrying the indicated transgenes, prior to pupal lethality. Unpaired *t*-test was used to calculate the *P*-values indicated in the figure. Unpublished data. GC3Ai: GFP-based caspase 3-like proteases activity indicator; GMR-Gal4: glass multiple reporter promoter elements driving yeast GAL4; LacZ: beta-galactosidase; TDP-35:35 kDa fragment cleaved from TDP-43; TDP-43: TAR DNA-binding protein 43.

used the GC3Ai apoptosensor to quantify the extent of apoptosis in larval eye imaginal discs expressing each transgene. The GC3Ai apoptosensor is a sensitive GFP-based marker that responds specifically to apoptosis (Schott et al., 2017) and thus we used it in larval stages prior to pupal lethality. As expected, we detected higher levels of apoptosis in the eye primordium of TDP-35-expressing eye imaginal discs compared to TDP-43 or LacZ specimens (**Figure 1A** and **B**). These results are not due to differences in expression levels as the transgenes are inserted as single copies in the same genomic landing site. Moreover, all the flies were grown and treated in parallel under shared culture conditions to rule out batch effects and other artifacts. Therefore, these results demonstrate the robust ability of TDP-35 to hamper cellular homeostasis, resulting in cell death.

These results and the flexibility of our experimental system open the possibility of conducting a systematic comparative analysis of all truncated TDP-43 variants. To do this, one would generate additional transgenic lines encoding other TDP-43 fragments, either by enzymatic cleavage or by alternative transcriptional splicing. Importantly, all transgenic flies would be created by inserting transgenes at the same chromosomal landing site used for the existing TDP-35-expressing flies (51D) to ensure similar transgene expression levels for a given set of conditions. Furthermore, it would also be possible to create flies with transgenes that express multiple TDP-43 fragments to evaluate potential synergistic or additive effects, although this will require the use of conditional expression systems to bypass developmental toxicity. A variety of mechanistic studies are also anticipated to understand the molecular basis of TDP-35-induced cell death.

In conclusion, this study provides additional experimental evidence demonstrating the neurotoxic potential of TDP-35 *in vivo*. This toxicity seems to be dose-dependent; therefore, the presence of TDP-35 fragments beyond certain thresholds in humans could affect neuronal health and may aggravate the course of TDP-43 proteinopathies. Of note, the TDP-35 transgene discussed here could also be used to investigate its role in the context of other fly models of human neurodegenerative disorders characterized by proteinopathies that involve TDP-43 fragments, such as Alzheimer's disease. While *Drosophila* provides excellent experimental platforms to study neurodegenerative conditions, we encourage others to use different model organisms to verify our findings in flies and to fully understand the enigmatic role of truncated TDP-43 fragments in neurodegeneration.

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