The FTD/ALS-associated RNA-binding protein TDP-43 regulates the robustness of neuronal specification through microRNA-9a in Drosophila

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TDP-43 is an evolutionarily conserved RNA-binding protein currently under intense investigation for its involvement in the molecular pathogenesis of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). TDP-43 is normally localized in the nucleus, but translocated to the cytoplasm in diseased neurons. The endogenous functions of TDP-43 in the nervous system remain poorly understood. Here, we show that the loss of Drosophila TDP-43 (dTDP-43) results in an increased production of sensory bristles and sensory organ precursor (SOP) cells on the notum of some but not all flies. The location of ectopic SOPs varies among mutant flies. The penetrance of this novel phenotype is dependent on the gender and sensitive to environmental influences. A similar SOP phenotype was also observed on the wing and in the embryos. Overexpression of dTDP-43 causes both loss and ectopic production of SOPs. Ectopic expression of ALS-associated mutant human TDP-43 (hTDP-43M337V and hTDP-43Q331K) produces a less severe SOP phenotype than hTDP-43WT, indicating a partial loss of function of mutant hTDP-43. In dTDP-43 mutants, miR-9a expression is significantly reduced. Genetic interaction studies further support the notion that dTDP-43 acts through miR-9a to control the precision of SOP specification. These findings reveal a novel role for endogenous TDP-43 in neuronal specification and suggest that the FTD/ALS-associated RNA-binding protein TDP-43 functions to ensure the robustness of genetic control programs.

INTRODUCTION

TDP-43, an evolutionarily conserved RNA-binding protein, is currently under intense investigation for its contribution to the molecular pathogenesis of frontotemporal dementia and amyotrophic lateral sclerosis (ALS) (1–5). In both diseases, TDP-43 is depleted from the nucleus of affected neurons, implicating the loss of TDP-43’s normal nuclear function as a major pathogenic mechanism. However, the significance of TDP-43 in neural development and function is largely unknown.

During development, complex molecular interactions ensure the precision and reproducibility of patterning events, despite stochastic genetic and environmental fluctuations, a process called canalization (6). Sensory organ precursor (SOP) cells in Drosophila’s peripheral nervous system are one of the best-studied models for understanding the molecular pathways that control early neurogenesis and the robustness of developmental processes (7–9). A key process in SOP specification is lateral inhibition mediated by the Notch-Delta signaling pathway and complex feedback regulatory loops in which dynamic expression of the transcription factor Senseless (Sens) plays a central role (10,11).

Proper levels of gene expression during development are controlled through multiple mechanisms, including microRNAs (miRNAs). These small, non-coding RNAs regulate mRNA translation or stability, mostly by binding to 3′ untranslated regions (12,13). miR-9a ensures the precise specification of SOPs, which is in part through suppressing Sens expression and thus functions as a component of the regulatory circuit that confers robustness in lateral inhibition (14–16). Another miRNA involved in this developmental process is miR-7 that targets enhancer of split, a key transcription factor upstream of Sens (17). These findings support the hypothesis

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that miRNAs play a key role in canalization (18,19). Here, we show that Drosophila TDP-43 (dTDP-43) acts through miR-9a to regulate the precision of SOP specification in Drosophila. Ectopic expression of ALS-associated mutant human TDP-43, hTDP-43M337V and hTDP-43 Q331K (20) produces a less severe SOP phenotype than hTDP-43 WT, indicating a partial loss of function of mutant hTDP-43. These results provide novel insights into the molecular functions of TDP-43 and suggest that the loss of TDP-43’s normal function may disturb the robustness of genetic control programs.

RESULTS

dTDP-43 mutant flies produce ectopic SOPs on the notum

Previously, we obtained mutant flies with a nonsense mutation introduced into the codon encoding Glu-367 of the dTDP-43 gene and showed that dTDP-43 regulates dendritic branching (20). When examining viable adult dTDP-43Q367X mutant flies, we unexpectedly found that some of them had ectopic large sensory bristles called macrochaetes on the notum (Fig. 1B and C). The number (total 22) and position of these macrochaetes were precisely patterned in wild-type flies, an excellent example of canalization (9). For simplicity, our analysis focuses on scutellar (SC), dorsocentral (DC) and anterior postalar (aPA) bristles (Fig. 1A).

Among dTDP-43Q367X homozygous mutant flies, 26% had at least one extra macrochaete on the notum, and among them 90% had only one extra bristle (Fig. 1B and G). The positions of ectopic bristles might differ in different flies. Eighty-seven percent were near any one of the four SC bristles, and a few flies had ectopic bristles at one of the two aPA or four DC positions.

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We also examined dTDP-43 mutant fly lines generated independently by Feiguin et al. (21) (Supplementary Material, Fig. S1). Among dTDP-43D142 homozygous mutant flies, 81% had ectopic macrochaetes and 56% of those flies had only one extra bristle and 44% had two (Fig. 1E and G). Surprisingly, 81% of ectopic bristles were at one of the two aPA positions, in contrast to our findings in dTDP-43Q367X flies. When we examined mutant flies with dTDP-43Q367X and dTDP-43D142 alleles in trans, we found that the penetrance of the ectopic bristle phenotype was 21% (Fig. 1F and G), similar to that in dTDP-43Q367X flies, but most of the ectopic bristles were at the aPA position (Fig. 1G), more similar to the bristle location of the other fly lines.
in dTDP-43Δ/142 flies. Moreover, the ectopic bristle phenotype at the aPA position in dTDP-43Q367X/D142 mutants could be rescued by dTDP-43 expression. The differences in the penetrance and location of ectopic bristles between dTDP-43Q367X and dTDP-43D142 alleles remain after several generations of outcross with w1118 flies, raising the possibility that dTDP-43 function may be modified by other genetic factors near the dTDP-43 locus.

Each sensory bristle is accompanied by a sensory neuron and a few other support cells (Fig. 1H) that are derived from a single SOP through two rounds of asymmetric division (Fig. 1I). To examine whether the ectopic bristles we observed in dTDP-43 mutant flies are a reflection of increased SOP production, we performed immunostaining experiment using the embryonic lethal abnormal vision (ELAV) antibody and found that ectopic bristles (indicated by the asterisk in the white square in Fig. 1F) are accompanied by ectopic sensory neurons (Fig. 1J). Thus, the precise specification of neural progenitor cells is altered by the loss of dTDP-43 function in Drosophila.

**Ectopic expression of TDP-43 results in a mixed SOP phenotype**

To further examine TDP-43 function, we performed overexpression studies. Overexpression of dTDP-43 with Vg-Gal4 leads to not only the loss of SC bristles (black asterisk) but also ectopic production of DC or SC bristles (white asterisks). (D) Ectopic expression of hTDP-43 with Vg-Gal4 results in the ectopic production of SC bristles only. (E and F) The effects of ectopic expression of hTDP-43Q331K (E) or hTDP-43M337V (F) on ectopic SC bristle production is less potent than hTDP-43. The expression levels of wild-type and mutant hTDP-43 were comparable (Lu et al., 2009) (20). (G) Quantification of the penetrance of the ectopic SC bristle phenotype caused by expression of wild-type or mutant hTDP-43. Fifty flies were counted for each genotype and the average of results from three countings are presented here. *P < 0.05; **P < 0.01. The letters A, D, E and F indicate the genotypes of the flies presented in Panels A, D, E and F.

The effects of dTDP-43 on SOP specification are influenced by gender and environment

When we compared female and male flies, we found that in all dTDP-43 mutants with different allele combinations, the penetrance of ectopic SOP production was consistently higher in females (Fig. 3A). Environmental fluctuation can also affect the penetrance and expressivity of the ectopic SOP phenotype.
In some hemisegments of flies, about twice the rate in flies grown at 25 or 30\degree C positions at 18 and 25\degree C, further, determined whether miR-9a is downregulated in mutant flies as a consequence of misregulated SOP specification (14,15). Examination of the anterior margin of the wing revealed increased sensory bristle production in 8 of 52 $dTDP-43^{Q367X}/dTDP-43^{A142}$ transheterozygous mutant flies.

The precision of SOP specification of wild-type flies is not affected by environmental fluctuation. No difference was observed when wild-type flies were grown at 18, 25 or 30\degree C. In contrast, when grown at 18\degree C, 50% of $dTDP-43^{Q367X}/dTDP-43^{A142}$ mutant flies had one or more ectopic bristles, about twice the rate in flies grown at 25 or 30\degree C. Furthermore, ectopic bristles occurred preferentially at the aPA positions at 18 and 25\degree C, but at the SC positions at 30\degree C (Fig. 3B). Thus, dTDP-43 functions as a ‘gatekeeper’ to ensure the robustness of SOP specification, despite fluctuating environmental conditions. Without dTDP-43 function, SOP specification becomes more prone to environmental influence.

**MiR-9a is substantially downregulated in $dTDP-43$ mutant larvae**

The SOP phenotype in $dTDP-43$ mutant flies is remarkably similar to that in miR-9a knockout flies as a consequence of misregulated SOP specification (14,15). Examination of the anterior margin of the wing revealed increased sensory bristle production in 8 of 52 $dTDP-43^{Q367X}$. In some hemisegments of $dTDP-43^{Q367X}$ mutant embryos, ectopic sensory neurons were also produced (Fig. 4E and F). Both the wing and embryo phenotypes also resembled those in miR-9a knockout flies (14,15). We, therefore, determined whether miR-9a is downregulated in $dTDP-43$ mutants.

Because TDP-43 overexpression in the notum resulted in a complex SOP phenotype (Fig. 2), we focused our biochemical analysis on $dTDP-43$ loss of function mutant flies. Indeed, the expression of miR-9a, but not of the muscle-specific miR-1, was reduced in $dTDP-43^{Q367X}$ mutant larvae, as shown by northern blot analysis (Supplementary Material, Figs. S2 and 5A). Similar results were obtained and confirmed in $dTDP-43$ mutant larvae with other allele combinations (Fig. 5A). Of note, miR-9a expression was not completely abolished in $dTDP-43$ mutants. Indeed, unlike miR-9a knockout flies (14,15,24), $dTDP-43$ mutants did not have a notching defect at the posterior margin of the wing. To examine how dTDP-43 regulates miR-9a biogenesis, we measured the levels of pri-miR-9a and pre-miR-9a and found that both were reduced as shown by quantitative real-time PCR (qRT-PCR) (Fig. 5B). Thus, the lower level of miR-9a in $dTDP-43$ mutants is partially due to a function of dTDP-43 in pri-miR-9a transcription and/or stability. Indeed, it has been reported that the complex containing TDP-43 and overexpressed Drosha in cultured cells does not possess miRNA processing activity (25).

To further examine the role of dTDP-43 in miR-9a biogenesis, we co-transfected pri-miR-9a and dTDP-43 into HEK293 cells. dTDP-43 overexpression increased the production of both pre-miR-9a and mature miR-9a (Fig. 5C and Supplementary Material, Fig. S3); and the level of pri-miR-9a was also higher and a biochemical interaction between TDP-43 and pri-miR-9a was detected with GAPDH mRNA as the negative control (Fig. 5D). Thus, these results raise the possibility that dTDP-43 regulates the transcription and/or stability of pri-miR-9a.

dTDP-43 regulates SOP specification in part through miR-9a

To further demonstrate the involvement of miR-9a in the dTDP-43 genetic pathway controlling the robustness of SOP specification, we first performed genetic interaction experiments using only female flies because their SOP phenotype is stronger than that in males. $dTDP-43$ heterozygous female flies exhibited an ectopic bristle phenotype at a low penetrance (Fig. 6A and F), indicating that dTDP-43 itself functions in a dose-dependent manner in SOP specification. Loss of one
Figure 5. MiR-9a is downregulated in dTDP-43 mutants. (A) miR-9a expression level is decreased in dTDP-43 mutant third instar larvae with combinations of different alleles. (B) Downregulation of pri-miR-9a and pre-miR-9a in dTDP-43 mutants. (C) Overexpression of dTDP-43 in HEK293 cells increases the levels of pre-miR-9a and mature miR-9a. (D) dTDP-43 binds to pri-miR-9a as shown by co-immunoprecipitation experiments and increases the steady-state level of pri-miR-9a.

Figure 6. Genetic interactions between dTDP-43 and miR-9a. (A–E) Representative images of female flies with different genotypes as indicated in each panel. (F) Penetrance of the ectopic bristle phenotype in flies with the genotypes listed in (A–E). Only female flies for each genotype were used in this analysis; thus, the penetrance of the phenotype in dTDP-43^{Q367X} mutant flies is higher than that in Figure 1.
copy of miR-9a, either the miR-9a\textsuperscript{122} or miR-9a\textsuperscript{E39} allele, significantly enhanced the SOP phenotype of dTDP-43 heterozygous flies (Fig. 6D–F). Only one extra bristle was observed in dTDP-43, miR-9a transheterozygous flies. We repeated this experiment using both male and female flies and obtained a similar result (Supplementary Material, Fig. S4). This result supports the notion that the two genes function in the same pathway.

The reduced miR-9a expression in dTDP-43 mutant larvae (Fig. 5) raises the possibility that dTDP-43 regulates SOP specification in part through miR-9a. To further support this hypothesis, we performed rescue experiment and expressed miR-9a in dTDP-43 mutant background. To this end, we recombined Vg-Gal4 and dTDP-43\textsuperscript{G367X} onto the same chromosome. Vg-Gal4/+ (Fig. 2A) or UAS-pre-miR-9a/+ by itself had no SOP phenotype (Fig. 7C). Expression of UAS-pre-miR-9a by Vg-Gal4 in the dTDP-43\textsuperscript{G367X} mutant background could largely rescue the SOP phenotype (Fig. 7A and B). Moreover, our previous studies showed that sens mRNA is a key target of miR-9a in SOP specification and loss of one copy of sens could partially rescue miR-9a mutant phenotype (14). sens\textsuperscript{E38}/+ by itself had no SOP phenotype (Fig. 7G) but loss of one copy of sens markedly suppressed the ectopic SOP phenotype in dTDP-43 mutant flies (Fig. 7E and F), further supporting the importance of miR-9a in the regulation of SOP specification by dTDP-43.

DISCUSSION

In this study, we identified a novel function for the evolutionarily conserved RNA-binding protein TDP-43 in neural specification in Drosophila. Unlike several well-studied transcription factors in the Notch-Delta lateral inhibition pathway (e.g. 9,26), TDP-43 seems to function as a ‘robustness’ factor in a manner similar to that of miR-9a (14,16). In the absence of TDP-43 activity, ectopic bristles appear at one of several locations and SOP specification becomes more sensitive to environmental influences. Thus, TDP-43 seems to serve as a ‘gatekeeper’ to ensure the reproducible execution of genetic control programs and to canalize developmental phenotypes.

Since mRNA synthesis can fluctuate (27), regulation of mRNA metabolism plays a central role in gene expression. miRNAs are a key class of regulatory molecules that ensure the robustness of developmental programs and may play a key role in canalization (18,19), which is partly due to the modest effect of each miRNA on expression levels of multiple mRNA targets (13). Hundreds of RNA-binding proteins are encoded by the Drosophila genome (28) or in other species, and many simultaneously regulate multiple miRNAs (29). It is likely some other RNA-binding proteins also serve as robustness factors through direct interactions with target miRNAs.

Our findings show that the ‘robustness’ function of TDP-43 is mediated in part by miR-9a in one specific neural developmental process. The molecular functions of TDP-43 in development as revealed here may provide a new perspective on its endogenous role in neurodegeneration. Although the loss of TDP-43 leads to an early lethal phenotype in mouse embryos (30–32), loss of nuclear TDP-43 does not necessarily result in rapid neuronal cell death (33). Gene expression in individual cells is tightly regulated and also varies significantly, in part due to stochastic biochemical events (34). Chronic loss of nuclear TDP-43 or compromise of its buffering function by genetic mutations may cause an imbalance in protein homeostasis in human neurons before eventual neurodegeneration, during which miRNAs may play an essential role downstream of TDP-43 (35). Both TDP-43 and miR-9 are highly conserved through evolution and their interaction may occur in
mammalian cells as well. It is interesting to note that miR-9 is significantly downregulated in Huntington’s disease (36) and a mouse model of spinal motor neuron disease (37). Thus, downregulation of miR-9 and possibly other miRNAs as well in stressed neurons in which TDP-43 has been depleted from the nucleus may be a common contributing factor in different neurodegenerative disorders.

**MATERIALS AND METHODS**

**Fly strains and genetics**

All flies were maintained at 25°C on standard medium except for those in the experiment in Figure 3B. The w^{1118} strain served as a wild-type control. The following dTDP-43 mutant lines were studied: dTDP-43Q367X/Cy0 (20), dTDP-43Δ142/Cy0 (20), miR-9a^{J22}, miR-9a^{E39} and sens^{E58} flies were as described (14). For dTDP-43 overexpression, vg-Gal4 (on the second chromosome) and UAS-dTDP-43 (on the third chromosome) lines were crossed to generate vg-Gal4/+; UAS-dTDP-43/+ flies. For dTDP-43 knockdown, actin-Gal4/Cy0 flies were crossed with UAS-dTDP-43 RNAi line 38377 (Vienna Drosophila RNAi Center) to generate actin-Gal4/+; 38377/+ and Cy0/+; 38377/+ flies. UAS-hTDP-43WT, UAS-hTDP-43M337V and UAS-hTDP-43Q331K were described before (20).

**Northern blot and qRT-PCR analysis**

Northern blot analysis was performed as described (38), using digoxigenin-labeled probes from Exiqon as recommended by the manufacturer. Total RNA was extracted from third instar larvae with the miRNAeasy mini kit (Qiagen). For northern analysis, larvae were homogenized in 200 μl of lysis buffer (0.137 M NaCl, 20 mM Tris, pH 8.0, 10% glycerol, 1% NP-40, 0.1% sodium deoxycholate, 1 mM dithiothreitol and Pierce protease inhibitor). Protein (10 μg) was separated by SDS-PAGE with 10% polyacrylamide gel gels, transferred to PVDF membranes and immunoblotted with antibodies against the pan-neuronal marker ELAV (Developmental Hybridoma Study Bank) and the second antibody was hybriodoma Study Bank) and the second antibody was Alexa Fluor 488 goat anti-rat IgG (1:400, Invitrogen). The protein extracts were centrifuged for 1 h at 4°C. The supernatant was incubated with protein-G-agarose beads conjugated with anti-Flag antibody (Sigma) at 4°C for 4 h. The precipitated complex was washed three times with RNase-free phosphate-buffered saline. The associated RNA was isolated with Trizol (Invitrogen) and detected with primers specific for pri-miR-9a (5’-TGTCGCTGTCGCAGGAC-3’ and 5’-TGTCGCTGTCGCAGGAC-3’).

**Generation of expression constructs**

*Drosophila* miR-9a pri-miRNA (>1.6 kb) was cloned into pSuper-GFP vector from the wild-type genomic DNA with primers 5’-TATAAGCTTGGAGTCGAC-3’ and 5’-TATCTCGAGAAGCCTGTTGGTTTATAATGCCC-3’. cDNA of dTDP-43 protein coding region was cloned into p3 × Flag-CMV-7.1 vector.

**Cell culture and immunoprecipitation assay**

HEK293 cells were maintained in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and penicillin/streptomycin and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Immunoprecipitation was performed as described (38) with some modifications. Briefly, 36 h after transfection with pri-miR-9a and Flag-dTDP-43 or control vector, HEK293 cells were lysed in cold RNase-free lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.5% NP-40, 1 mM dithiothreitol, protease inhibitor and RNase inhibitor). The protein extracts were centrifuged for 10 min at 4°C. The supernatant was incubated with protein-G-agarose beads conjugated with anti-Flag antibody (Sigma) at 4°C for 4 h. The precipitated complex was washed three times with RNase-free phosphate-buffered saline. The associated RNA was isolated with Trizol (Invitrogen) and detected with primers specific for pri-miR-9a (5’-TGTCGCTGTCGCAGGAC-3’ and 5’-TGTCGCTGTCGCAGGAC-3’).

**Immunostaining**

In Figure 1, aPα bristles in late pupae were immunostained with antibodies against the pan-neuronal marker ELAV (Developmental Hybridoma Study Bank; 1:20) as described (14). The pupae were dissected and fixed in 4% formaldehyde in PBT (0.3% Triton in PBS) overnight at 4°C.

Standard protocols were used to immunostain *Drosophila* embryos. Briefly, *Drosophila* stage-15 embryos were collected, dechorionated in 50% sodium hypochlorite and fixed in 100% methanol for 1–2 h at room temperature. The primary antibody was anti-ELAV (1:200; Developmental Hybridoma Study Bank) and the secondary antibody was Alexa Fluor 488 goat anti-rat IgG (1:400, A11006, Invitrogen). All samples with fluorescent signals were imaged by confocal microscopy (Nikon, D-Eclipse C1).

**Western blot analysis**

Western blot analysis was performed according to the standard protocol. Briefly, protein was extracted from the heads of 60 adult flies (30 females and 30 males) of each genotype and homogenized in 200 μl of lysis buffer (0.137 M NaCl, 20 mM Tris, pH 8.0, 10% glycerol, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM dithiothreitol and Pierce protease inhibitor). Protein (10 μg), as measured with the Bio-Rad reagent, was separated by SDS-PAGE with 10% gels, transferred to PVDF membranes and immunoblotted with anti-dTDP-43 rabbit polyclonal antibody (1:3000; a gift of Dr Baralle, Italy) as described (21). The second antibody
was horseradish peroxidase-conjugated IgG antibody (Jackson Laboratory). β-actin served as a loading control and was detected with antibodies from Cell Signaling (1:5000).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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