Fluorescence in-situ hybridization method reveals that carboxyl-terminal fragments of transactive response DNA-binding protein-43 truncated at the amino acid residue 218 reduce poly(A)$^+$ RNA expression

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Transactive response (TAR) DNA-binding protein 43 (TDP-43) has emerged as an important contributor to amyotrophic lateral sclerosis and frontotemporal lobar degeneration. To understand the association of TDP-43 with complex RNA processing in disease pathogenesis, we performed fluorescence in-situ hybridization using HeLa cells transfected with a series of deleted TDP-43 constructs and investigated the effect of truncation of TDP-43 on the expression of poly(A)$^+$ RNA. Endogenous and overexpressed full-length TDP-43 localized to the perichromatin region and interchromatin space adjacent to poly(A)$^+$ RNA. Deleted variants of TDP-43 containing RNA recognition motif 1 and truncating N-terminal region induced cytoplasmic inclusions in which poly(A)$^+$ RNA was recruited. Carboxyl-terminal TDP-43 truncated at residue 202 or 218 was distributed in the cytoplasm as punctate structures. Carboxyl-terminal TDP-43 truncated at residue 218, but not at 202, significantly decreased poly(A)$^+$ RNA expression by ~24% compared with the level in control cells. Our results suggest that the disturbance of RNA metabolism induced by pathogenic fragments plays central roles in the pathogenesis of amyotrophic lateral sclerosis and frontotemporal lobar degeneration. *NeuroReport* **29:846–851**

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Introduction

Transactive response (TAR) DNA-binding protein 43 (TDP-43) is a major component of proteinaceous inclusions observed in affected brain regions of patients with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) [1,2]. Missense mutations in the gene encoding TDP-43, *TARDBP*, were reported to be ambiguous causes of familial forms of FTLD and ALS [3,4]. Thus, TDP-43 has emerged as an important potential modifier of the pathogenic process underlying these diseases.

TDP-43 belongs to the group of heterogeneous nuclear ribonucleoproteins containing two RNA recognition motifs (RRM1 and RRM2), which allow the protein to bind double-stranded DNA, single-stranded DNA, and RNA. It is reported to be involved in several functions of RNA processing, including transcriptional regulation (repression and splicing), RNA transport, RNA stability, and translation [5]. In addition to TDP-43, mutations in genes encoding two other RNA-binding proteins, fused in sarcoma and T-cell intracytoplasmic antigen, have been identified to be genetic causes of familial forms of ALS and FTLD [6–8]. Thus, ALS/FTLD-associated proteins share similar structural and functional properties, with probable involvement in multiple transcriptional and translational steps.

TDP-43 is largely restricted to the nucleus in healthy tissues and organs, but is also involved in the nucleocytoplasmic shuttling of RNA for the functions described above. However, in the affected neurons of patients with the above mentioned diseases, TDP-43 is redistributed from the nucleus to the cytoplasm, with a reduction in nuclear expression [1,2]. Intriguingly, under stress conditions, these ALS/FTLD-associated RNA-binding proteins localize to stress granules (SGs), in which nontranslating mRNA, many translation initiation components, and RNA-binding proteins accumulate to limit protein synthesis, mostly by the inhibition of translation initiation [9]. It has thus been proposed that a disturbance of the RNA metabolism in which TDP-43 is involved may be central to the pathogenesis of ALS and FTLD.

In addition to RRM domains, TDP-43 possesses an N-terminal region including a nuclear localization signal and a glycine-rich domain at the carboxyl (C)-terminus, which are required for subcellular transport or interaction with other factors, respectively [5]. TDP-43 undergoes several post-translational modifications, such as hyperphosphorylation, ubiquitination, and truncation, to generate C-terminal fragments in the brain of patients with the disease [1,2]. Among these post-translational modifications, truncation of TDP-43 observed in diseased brains is

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speculated to affect protein function by dissecting certain domains, probably leading to disease initiation or progression. In the brains of patients, two major groups of truncation forms, C-terminal ~35 and 25-kDa fragments, were detected [10,11]. The ~35-kDa fragments contain RRM1, RRM2, and the C-terminal region, whereas the ~25-kDa fragments consist of partially truncated RRM2 and the C-terminal region. However, the exact mechanism by which these act in the brains of patients remains unresolved. In this study, to understand the involvement of TDP-43 protein truncation with RNA metabolism in disease pathogenesis, we applied a series of deleted TDP-43 constructs and investigated the expression of poly(A)$^+$ RNA.

Materials and methods

Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal bovine serum. Transient transfection of cultured cells with each vector was performed using the Lipofectamine LTX Reagent (Invitrogen), in accordance with the manufacturer's instructions.

Plasmid constructs

pFLAG-CMV hTDP-43 plasmids for expressing wild-type (WT) TDP-43 or amino acid residues 2–104 ($TDP_{2–104}$), 101–272 ($TDP_{101–272}$), or 101–414 ($TDP_{101–414}$) of TDP-43 with a FLAG tag at the N-terminus, or pCI-neo hTDP-43 plasmids for expressing amino acid residues 202–414 ($TDP_{202–272}$) of TDP-43 with a FLAG tag at the C-terminus, as shown in Fig. 1, were prepared as described previously [12,13]. To prepare pFLAG-CMV hTDP-43 plasmids for expressing amino acid residues 101–190 ($TDP_{101–190}$) of TDP-43 with a FLAG tag at the N-terminus or pCI-neo hTDP-43 plasmids for expressing amino acid residues 218–414 ($TDP_{218–272}$) of TDP-43 with a FLAG tag at the C-terminus, truncated TDP-43 fragments were amplified by PCR using the following primers – forward: 5′-AAAAGCGGCCGCCAGAAAACATCCGATTTAATAGTGTTGG-3′ and reverse: 5′-AAAAGGATCCTTAGCTTCTCAAAGGCTCATCTTGGC-3′ for TDP101–190; and forward: 5′-AAAACTCGAGCCGCCACCATGGATGTCTTCATCCCCAAGCC-3′ and reverse: 5′-AAAAGCGGCCGCTTACTTGTCATCGTCGTCCTTGTAGTCCATTCCCCAGCCAGAAGACTTAG-3′ for $TDP_{218–414}$. The PCR products were digested with NotI and BamHI and cloned into the NotI-BamHI-digested pFLAG-CMV-6c expression vector (Sigma-Aldrich, St. Louis, Missouri, USA) or with XhoI and NotI and cloned into the XhoI/NotI-digested pCI-neo expression vector (Promega, Madison, Wisconsin, USA).

Antibodies

The following primary antibodies were used: rabbit polyclonal anti-TDP-43 raised to the C-terminus of TDP-43 [13]; mouse monoclonal anti-TDP-43 (Abnova Corporation, Taipei, Taiwan); and rabbit polyclonal anti-FLAG (Sigma-Aldrich).

Fluorescence in-situ hybridization

Fluorescence in-situ hybridization was carried out as described previously [12]. In brief, transfected or untransfected HeLa cells were prepared in two-chamber culture slides, rinsed with Tris-buffered saline, fixed with 4% paraformaldehyde for 10 min, permeabilized with 100% methanol for 10 min at − 20°C, and then rehydrated for 1 h at 4°C with 70% ethanol. After incubation in 1 M Tris-HCl (pH 8.0) for 5 min at room temperature (RT), cells were hybridized overnight at 37°C with 1 μg/ml Cy3-oligo-dT (30) (Eurofins genetics, Tokyo, Japan) in hybridization buffer (1 mg/ml yeast tRNA, 0.005% BSA, 10% dextran sulfate, 25% deionized formamide, 2× standard saline citrate (SSC)). After washing, sections were incubated for 3 h with primary antibody at an appropriate dilution in 0.1% Triton X-100 in 2× SSC at RT. After further washing, sections were incubated for 2 h with Cy2-conjugated secondary antibody at a 1 : 200 dilution in 0.1% Triton X-100 in 2× SSC at RT. After incubating with 4′,6-diamidino-2-phenylindole (DAPI) for 2 min, the coverslips were washed and mounted in mounting medium. Transfected cells from three independent experiments were viewed using Leica LAS X software (Leica Microsystems, Wetzlar, Germany), in accordance with the manufacturer's instructions.

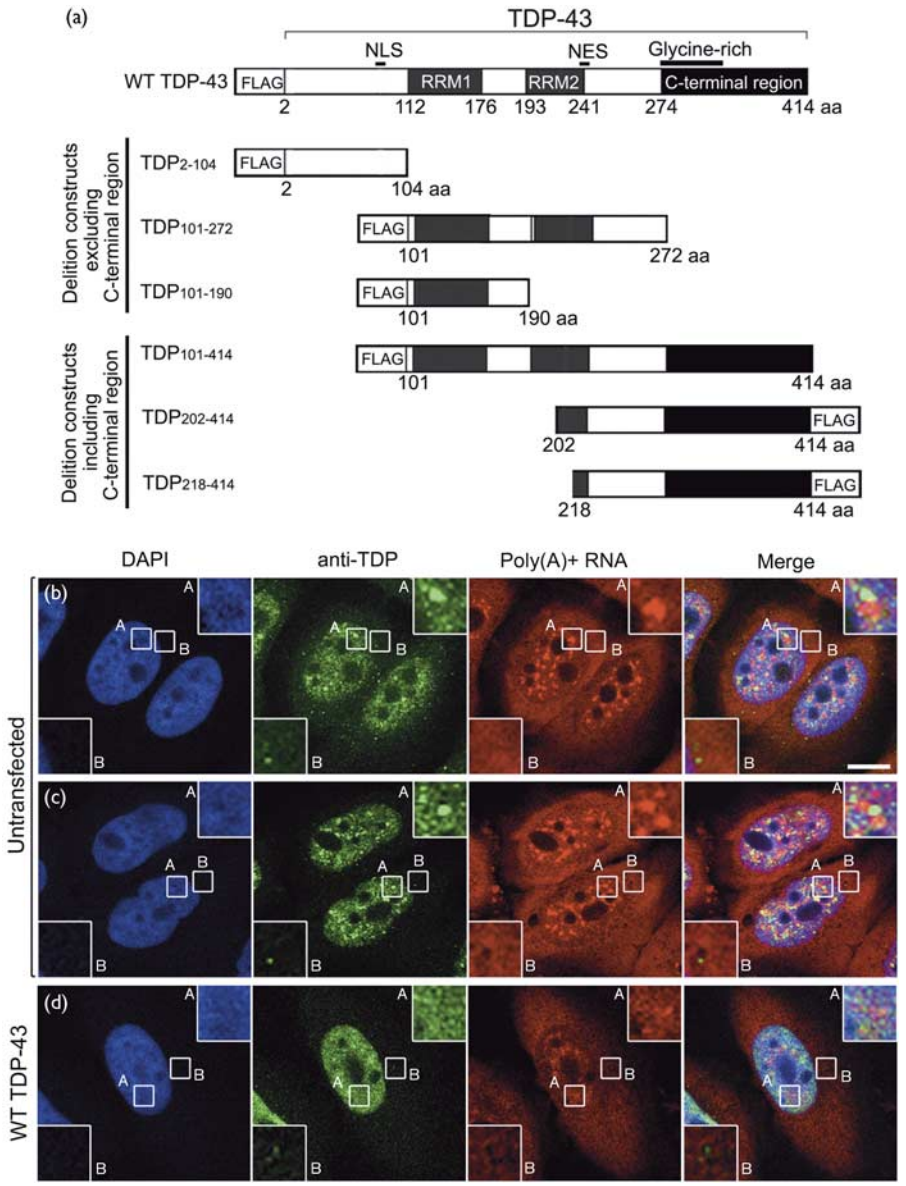
Studies were carried out according to the guidelines of the ethics committee.

Results

We first investigated the localization of the endogenous TDP-43 protein and poly(A)$^+$ RNA in the untransfected HeLa cells by fluorescence in-situ hybridization. Endogenous TDP-43 protein localized mainly to the nucleus in a speckled distribution, but also within the weakly stained cytoplasm as fine punctate structures (Fig. 1b and c). In these cells, TDP-43, poly(A)$^+$ RNA, and DAPI showing the distribution of DNA did not colocalize in the nucleus (Fig. 1bA and cA) and the cytoplasm (Fig. 1bB and cB), whereas TDP-43 and poly (A)$^+$ RNA were located next to each other in the nucleus. Next, we investigated the localization of overexpressed full-length WT TDP-43. WT TDP-43 localized to the nucleus as a speckled pattern as well as being distributed in the nucleoplasm more profoundly than endogenous TDP-43 (Fig. 2d). In addition, WT TDP-43 was distributed as fine dots in the cytoplasm, as found in the untransfected cells (Fig. 2d). WT TDP-43 did not colocalize to poly(A)$^+$ RNA and DAPI as observed in endogenous TDP-43 (Fig. 2dA and dB).

We applied the deleted TDP-43 constructs shown in Fig. 1 to determine the effect of each domain of TDP-43 on poly(A)$^+$ RNA localization and expression. $TDP_{2–104}$, the N-terminal region of TDP-43 including the nuclear localization signal, localized mostly to the nucleus and its

Fig. 1

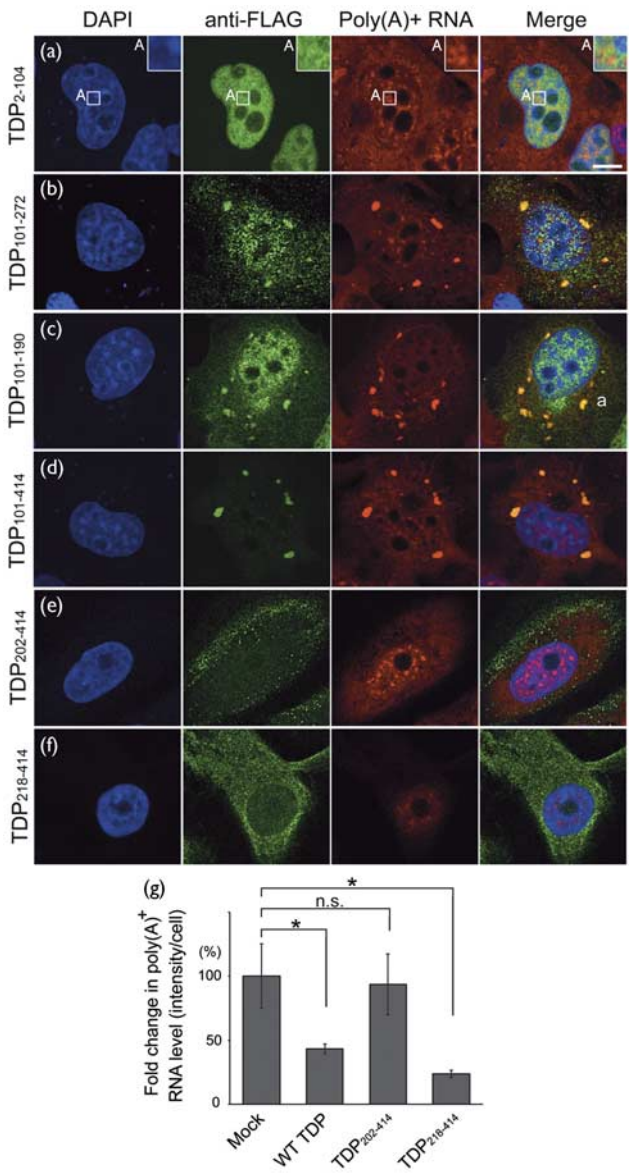


(a) Schematic representations of the protein domain architecture of full-length or deleted variants of transactive response DNA-binding protein (TDP)-43 are shown. The amino acid (aa) residues of TDP-43, the nuclear localization sequence (NLS), the nuclear export sequence (NES), and the glycine-rich region are indicated. (b–d) Untransfected (b, c) or full-length wild-type (WT) TDP-43 (d) transfected HeLa cells were analyzed by fluorescence in-situ hybridization (red) with immunocytochemistry with rabbit polyclonal (b) or mouse monoclonal (c) anti-TDP-43 or anti-FLAG antibody (d) (green), as indicated. The insets highlight that TDP-43 does not colocalize with 4′,6-diamidino-2-phenylindole (DAPI) (blue) to show the distribution of double-stranded DNA or poly(A)$^+$ RNA (red) in both the nucleus (bA, cA, and dA) and the cytoplasm (bB, cB, and dB). Scale bar in b represents 10 μm in b–d.

nuclear staining pattern was similar to that of WT TDP (Fig. 2aA). $TDP_{101–272}$, which consists of RRM1 and RRM2 of TDP-43, localized to the nucleus as a punctate staining pattern and to the cytoplasm as granular inclusions. In cells expressing $TDP_{101–272}$, most of the poly $(A)^+$ RNA was recruited to the cytoplasmic granular inclusions, in which poly(A)$^+$ RNA and $TDP_{101–272}$ colocalized (Fig. 2b), suggesting that granular localization of poly(A)$^+$ RNA could be induced by $TDP_{101–272}$ expression. The expression of $TDP_{101–190}$ consisting of only the RRM1 domain showed diffuse nuclear and granular cytoplasmic localization (Fig. 2c). $TDP_{101–190}$ did not colocalize to DAPI in the nucleus, but colocalized to poly(A)$^+$ RNA in the cytoplasmic inclusions (Fig. 2c).

We finally applied $TDP_{101–414}$, $TDP_{202–414}$, and $TDP_{218–414}$, resembling cleaved C-terminal fragments of TDP-43 found in affected regions of the brains of patients with FTLD/ALS.

Fig. 2



(a–f) HeLa cells transfected with the indicated deleted variants of transactive response DNA-binding protein (TDP)-43 were analyzed by fluorescence in-situ hybridization (red) and immunocytochemistry with the anti-FLAG antibody (green) 24 h after transfection, as indicated. The insets highlight that $TDP_{2–104}$ does not colocalize with 4′,6-diamidino-2-phenylindole (DAPI) (blue) or poly(A)$^+$ RNA in the nucleus (aA). Scale bar in A represents 10 μm in a–f. (g) The poly(A)$^+$ RNA level in cells transfected with empty vector (Mock) or indicated constructs was calculated by measuring the fluorescent intensity per cell, as described in the Methods section (mean ± SD, $n = 3$). *$P < 0.05$ (paired Student's *t*-test).

$TDP_{101–414}$ consisting of RRM1, RRM2, and the C-terminal region localized only to cytoplasmic granular inclusions in which $TDP_{101–414}$ and poly(A)$^+$ RNA colocalized (Fig. 2d). However, $TDP_{202–414}$, consisting of a partially deleted RRM2 domain and the C-terminal region of TDP-43, localized in the periphery of the cytoplasm as punctate structures (Fig. 2e). In the transfected cells, poly(A)$^+$ RNA localized to the nucleus with a reduction of cytoplasmic expression (Fig. 2e). $TDP_{218–414}$, in which the RRM2 domain was additionally deleted compared with $TDP_{202–414}$, diffusely localized in the whole cytoplasm with punctate structures (Fig. 2f). In the cells expressing $TDP_{218–414}$, the expression of poly(A)$^+$ RNA appeared to be reduced (Fig. 2f).

To compare the RNA expression levels between transfected cells, we calculated the poly(A)$^+$ RNA level on the basis of the fluorescence intensity per cell. The poly(A)$^+$ RNA level in WT TDP-43-transfected cells was decreased by ~43% compared with that in control cells (Fig. 2g). In addition, the poly (A)$^+$ RNA level was not altered in cells expressing $TDP_{202–414}$, whereas that in cells expressing $TDP_{218–414}$ was more dramatically decreased by ~24% compared with that in control cells (Fig. 2g). Thus, despite $TDP_{202–414}$ and $TDP_{218–414}$ had a similar structure, each effect on poly (A)$^+$ RNA level was different.

Discussion

In the present study, we investigated the localization of endogenous and overexpressed TDP-43 and deleted variants of TDP-43 consisting of each of its domains. In the nucleus, endogenous TDP-43 was stained with speckled distributions adjacent to poly(A)$^+$ RNA, but did not colocalize to both poly(A)$^+$ RNA and DAPI. A previous study showed that poly(A)$^+$ RNA was expressed in the perichromatin fibrils and interchromatin granule clusters [14,15]. The perichromatin region is a border zone of condensed chromatin, including dispersed chromatin and ribonucleoprotein perichromatin fibrils, and is the major site of newly synthesized RNA [16]. In contrast, interchromatin space is a nonchromatin domain for transcription, splicing, and DNA replication and repair. As endogenous TDP-43 and poly(A)$^+$ RNA did not colocalize, TDP-43 could associate with nascent RNA more than with mature RNA in the nucleus for transcriptional modifications. Consistent with our results, a previous study involving ultrastructural immunogold labeling showed that TDP-43 colocalized to nascent RNA in perichromatin fibrils [17]. Therefore, we speculated that endogenous TDP-43 might localize to the perichromatin region and the interchromatin space adjacent to poly(A)$^+$ RNA.

We next investigated the influence of each domain of TDP-43 on poly(A)$^+$ RNA localization. The localization of overexpressed WT TDP-43 and the N-terminal fragment of TDP-43, namely, $TDP_{2–104}$, in the nucleus was similar to that of endogenous TDP-43, suggesting that N-terminal regions might be crucial for the localization of TDP-43 in the perichromatin regions and interchromatin space. In contrast, deleted variants of TDP-43 containing RRM1 and truncating N-terminal regions, $TDP_{101–272}$, $TDP_{101–190}$, and $TDP_{101–414}$, formed cytoplasmic inclusions in which poly(A)$^+$ RNA localized, reminiscent of

SGs, despite no cellular stress being applied in this study. A previous study showed that both the RRM1 domain and amino acid residues 216–315 in the C-terminal region are necessary for the incorporation of TDP-43 into SGs [18]. However, in this study, cytoplasmic inclusions harboring poly(A)$^+$ RNA were induced only by the RRM1 domain, indicating that the RRM1 domain in TDP-43 *per se* has the ability to form RNA-containing aggregations when it is overexpressed.

In contrast to $TDP_{101–414}$, C-terminal TDP-43 with partially truncated RRM2, $TDP_{202–414}$, and $TDP_{218–414}$ showed cytoplasmic distributions with punctate structures rather than inclusion body formation. Of these constructs, $TDP_{218–414}$ induced a significant reduction in the expression of poly(A)$^+$ RNA. In contrast to $TDP_{218–414}$, $TDP_{202–414}$ did not show any reductions in the level of poly(A)$^+$ RNA. Previous studies showed that amino acid residues 208, 218–219, or 246–247 in TDP-43 purified from FTLD brains constituted pathogenic cleavage sites [10,19]. Of the above two constructs, $TDP_{218–414}$ was reminiscent of pathogenic C-terminal fragments, whereas $TDP_{202–414}$ was longer than them [19]. Therefore, our results showing that $TDP_{218–414}$ reminiscent of pathogenic fragments is a cause of the reduction of RNA expression indicate that the disturbance of RNA metabolism plays a central role in the pathogenesis of ALS and FTLD. Although we could not identify a cause for this difference between $TDP_{218–414}$ and $TDP_{202–414}$, we speculate that it might have been because of a difference in interaction with other factor(s) by residue-specific conformations because C-terminal regions might be crucial for protein–protein interaction [20].

$TDP_{218–414}$ showed diffuse distributions with punctate structures in the transfected cells, which is different from the localization of C-terminal fragments existing in the brains of patients with cytoplasmic inclusion bodies. A previous study showed that toxicity in TDP-43-expressing cells is associated with diffuse cytoplasmic distribution rather than cytoplasmic inclusion body formation [21], which is consistent with our results. There is also growing evidence indicating that inclusion body formation can function as a coping response by cells expressing aggregation-prone proteins [22]. Therefore, the effect of $TDP_{218–414}$ on RNA metabolism might represent the pathogenesis of the disease, at least in part, irrespective of whether or not it is associated with the formation of cytoplasmic inclusions.

In addition to $TDP_{218–414}$, overexpressed WT TDP-43 also induced a reduction in the expression of poly(A)$^+$ RNA to a significant but smaller extent than $TDP_{218–414}$. This result appears to be reasonable because TDP-43 is well known to function as a transcriptional repressor [23,24]. However, as the degree of this reduction was extensive, the overexpression of WT TDP-43 might be deleterious to cells. Consistent with this, previous studies showed that the overexpression of WT TDP-43 *per se* was toxic to cells [21,25].

Conclusion

We showed that the effect of C-terminal fragments of TDP-43 on RNA metabolism is dependent on their length, and that those reminiscent of pathogenic fragments found in the affected brains of patients with ALS/FTLD are more disruptive of poly(A)$^+$ RNA expression. This suggests the involvement of a toxic gain of function in the pathogenesis of these diseases, consistent with the autosomal dominant inheritance of nearly all TDP-43 mutations associated with familial forms of these diseases.

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Conflicts of interest

There are no conflicts of interest.

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