Nuclear TDP-43 causes neuronal toxicity by escaping from the inhibitory regulation by hnRNPs

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Abstract

Dysregulation of transactive response DNA-binding protein-43 (TDP-43) is thought to be linked to the pathogenesis of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). TDP-43 normally localizes in the nucleus but its main localization shifts to the cytoplasm in most affected cells of ALS and FTLD patients. It is not yet known whether nuclear or cytoplasmic TDP-43 is responsible for TDP-43-induced neurotoxicity. In this study, we show that nuclear TDP-43 causes TDP-43 neurotoxicity. DNA/RNA-binding and dimerization of TDP-43 are both essential for TDP-43-induced cell death. Moreover, endogenous heterogeneous nuclear ribonucleoprotein-U (hnRNP-U) binds to TDP-43 and knocking-down of hnRNP-U induces neurotoxicity, whereas overexpression of hnRNP-U or hnRNP-A2 inhibits TDP-43-induced neurotoxicity. In addition, hnRNP-U inhibits TDP-43-mediated alterations in splicing of POLDIP3 mRNA. Altogether, these results suggest that nuclear TDP-43 becomes neurotoxic by escaping from the inhibitory regulation by hnRNPs.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron-specific neurodegenerative disease, characterized by a selective loss of upper and lower motor neurons (1). Approximately 5–10% of ALS cases occurs in a genetically inherited manner (familial ALS; fALS) whereas most ALS patients have no genetic background (sporadic ALS; sALS) (2). Frontotemporal lobar degeneration (FTLD) is the second most common cause of dementia with a typical onset of disease before 65 years of age, characterized by degeneration of the frontal and anterior temporal lobes of cerebral cortices (3). Transactive response DNA-binding protein-43 (TDP-43) is identified as a major component of ubiquitinated inclusions in the majority of ALS patients and a substantial portion of FTLD patients (4,5). Furthermore, a number of mutations in TARDBP, the gene encoding TDP-43, have been identified in ALS and FTLD patients (4,5). Based on these findings, dysregulation of TDP-43 is thought to be linked to the pathogenesis of these two neurodegenerative diseases even in the absence of TARDBP gene mutations (6,7).

TDP-43 predominantly localizes in the nucleus and is physiologically involved in the regulation of RNA metabolism including transcription, splicing, mRNA stability, mRNA transport, translation and miRNA biogenesis (8). In a context-dependent manner, TDP-43 is able to shuttle from the nucleus to the cytoplasm (9). In the affected areas in ALS and FTLD patients, TDP-43 and its cleaved derivatives mislocalize and aggregate in the cytoplasm of neurons and glial cells (4,5). Currently, it is unclear whether mislocalization and aggregation of TDP-43 and its cleaved derivatives in the cytoplasm is a cause of, an independent co-incidence to, or a result of neurodegeneration associated with the dysregulation of TDP-43 (10).

It is generally hypothesized that TDP-43 may exhibit neuronal toxicity in both gain-of-function and loss-of-function manners (10,11). The gain-of-function of a protein may be due to its overexpression. Multiple clinical studies have demonstrated that levels of TDP-43 expression are up-regulated in the spinal cords, cerebrospinal fluids, skin cells, and plasma of sporadic ALS cases, and expression of TDP-43 mRNA is also up-regulated in
the superficial frontal cortices of some sporadic FTLD patients (12–16). Moreover, a number of studies have shown that overexpression of TDP-43 results in cellular toxicity in pathological models including yeast, nematode, drosophila, zebrafish, rodents and non-human primates (17,18). In addition, a pathological mutation of TDP-43 increases stabilization of TDP-43 protein and the stability of TDP-43 is correlated to early disease onset (19–21). We previously found that the adenoviral vector-mediated low-level overexpression of TDP-43 (2–5-fold higher than the endogenous levels) causes caspase-mediated cell death in motor neuronal NSC34 cells and primary cortical neurons via up-regulation of a pro-apoptotic Bcl-2-related BH3-only protein Bcl-2-interacting mediator of cell death (Bim), the pro-apoptotic C/EBP homologous protein (CHOP), and c-Jun N-terminal kinase (JNK)/c-Jun signaling and the down-regulation of an anti-apoptotic Bcl-2-related protein Bcl-xL (22–24). Despite establishing an in vitro neuronal cell death model caused by low-level overexpression of TDP-43 and identifying several mediators that are responsible for the progression of neuronal cell death, it remains undefined whether nuclear or cytoplasmic TDP-43 plays a central role in exerting neuronal toxicity. In this study, we initially show that low-level overexpression of nuclear, but not cytoplasmic, TDP-43 is toxic to neurons. We also show that both DNA/RNA-binding and dimerization of TDP-43 are essential for TDP-43-induced neuronal cell death. Furthermore, we identify nuclear TDP-43-binding proteins including multiple heterogeneous nuclear ribonucleoproteins (hnRNPs). Knocking-down of endogenous hnRNP-U induces neuronal cell death whereas low-level overexpression of hnRNP-U or hnRNP-A2 inhibits TDP-43-induced neuronal cell death. In addition, hnRNP-U inhibits TDP-43-mediated increase in variant 2 of POLDIP3 mRNA. These data together suggest that nuclear TDP-43 becomes neurotoxic by overcoming the intrinsic cell death-preventing mechanism mediated by hnRNPs.

Results

Nuclear TDP-43 is toxic to neurons

TDP-43 predominantly localizes in the nucleus under physiological conditions whereas it forms aggregation in the cytoplasm in affected areas of ALS and FTLD patients (4,5). To investigate whether nuclear or cytoplasmic TDP-43 is responsible for TDP-43-induced neuronal cell death, we constructed two adenoviral expression vectors encoding mutants of TDP-43, with the nuclear localization signal (NLS) of TDP-43, corresponding to the 82–98th amino acids of TDP-43, inactivated by incorporation of mutations (K82A/R83A/K84A) or complete deletion (Δ82–98) (Fig. 1A) (25). As expected, these mutants predominantly localized in the cytoplasm, whereas TDP-43-wt predominantly localized in the nucleus (Fig. 1B). Overexpression of TDP-43-wt caused cell death in motor neuronal NSC34 cells, shown by increased lactate dehydrogenase (LDH) release and cleaved-caspase-3 (Fig. 1C and D), and decreased cell viability of primary cultured cortical neurons (PCNs) (Fig. 1E and F), as shown in our previous study (22). The loss of TDP-43-wt, caused by treatment with siRNAs to TDP-43, did not cause toxicity in NSC34 cells (Supplementary Material, Fig. S1). This result confirmed that loss-of-function of TDP-43 from the nucleus, which partially mimics the localization shift of TDP-43 from the nucleus to the cytoplasm in ALS and FTLD patients, does not cause toxicity in NSC34 cells.

Both DNA/RNA-binding and dimerization of TDP-43 are required for TDP-43-induced neuronal cell death

DNA/RNA-binding of TDP-43 is mediated by two RNA recognition motifs (RRM) (27), and is necessary for several TDP-43 functions including cystic fibrosis transmembrane conductance regulator (CFTR) exon 9 skipping activity (28). To examine whether DNA/RNA binding of TDP-43 is required for TDP-43-induced neuronal cell death, we constructed two TDP-43 mutants, TDP-43-F147L/F149L (TDP-43-2FL) and TDP-43-deltaRRM1 that lacks RRM1 (TDP-43ΔRRM1). These mutants have been shown to be DNA/RNA binding-deficient and lack skipping activity of CFTR exon 9 (28) (Supplementary Material, Fig. S2A and B) and predominantly localizes in the nucleus (Supplementary Material, Fig. S2C). Overexpression of TDP-43-2FL or TDP-43-ΔRRM1 did not cause neurotoxicity in both NSC34 cells (Fig. 2A and B) and PCNs (Fig. 2C and D).

It has been recently reported that TDP-43 forms homodimers via its distal N-terminal region. Formation of TDP-43 homodimers is required for CFTR exon 9 skipping activity of TDP-43 (29). To determine whether the formation of TDP-43 homodimers is necessary for TDP-43 neuronal cell death, we overexpressed a previously reported (29) dimerization-deficient mutant of TDP-43, TDP-43-6GGGG (substitution of 6R7V8T9E with 6G7G8G9G), and examined its cell death-inducing activity in NSC34 cells. Using GST pull-down assays, we confirmed that both His6Xpress (HX)-tagged TDP-43-6GGGG and TDP-43-Δ6-9 did not bind to GST-tagged TDP-43-wt (Supplementary Material, Fig. S3A). We also confirmed that they are unable to promote skipping of CFTR exon 9 (Supplementary Material, Fig. S3B and C) and predominantly localize in the nucleus (Supplementary Material, Fig. S3D). Moreover, we found that overexpression of TDP-43-6GGGG did not cause toxicity in NSC34 cells (Fig. 2E and F).

Based on the finding that both DNA/RNA binding and dimerization of TDP-43 are required for TDP-43-induced cell death, we assumed that DNA/RNA-binding-deficient TDP-43 that dimerizes with TDP-43-wt may reduce TDP-43-induced cell death by forming less active or inactive TDP-43 dimers with TDP-43-wt. To examine this possibility, we co-expressed TDP-43-ΔRRM1 and TDP-43-wt and found that co-expression of TDP-43-ΔRRM1...
Figure 1. Nuclear TDP-43 is more neurotoxic than cytoplasmic TDP-43. (A) Schematic illustration of TDP-43 derivatives. The nuclear localization signal (NLS) of TDP-43 was mutated, deleted or replaced. Wt, wild-type; LT-NLS, SV40 virus large T antigen NLS; LT-NLSmut, LT-NLS mutant. TDP-43 contains three conserved domains, two RNA-recognition motif-containing domains (RRM1 and RRM2) and a glycine-rich domain (G rich). (B) NSC34 cells transiently overexpressing TDP-43 or TDP-43 NLS mutants were fixed and immunostained with anti-TDP43-C antibody (green). Nuclei were stained with Hoechst 33258 (blue). (C and D) NSC34 cells, seeded on six-well plates at 1×10^5 cells/well, were infected with the indicated adenoviruses at a multiplicity of infection (moi) of 400. All samples were co-infected with Cre-recombinase virus at a moi of 40. Twenty-four hours after infection, media of cells were replaced with DMEM/N2 supplement. Twenty-four hours after the replacement of media, LDH release from cells was measured (C). *P < 0.05. The cell lysates were immunblotted using the indicated antibodies (D). (E and F) PCNs, seeded on 96-well plates at 5×10^4 cells/well, were co-infected with the indicated adenoviruses at a moi of 100 and LacZ or Cre virus at a moi of 40. The WST-8 cell viability assays were performed at 4 days after infection (E). *P < 0.05. PCNs, seeded on six-well plates at 1×10^6 cells/well, were co-infected with the indicated adenoviruses at a moi of 100 and LacZ or Cre virus at a moi of 40. Four days after infection, the cell lysates were immunoblotted using the indicated antibodies (F).
Figure 2. Both DNA/RNA binding and dimerization of TDP-43 are required for TDP-43-induced neurotoxicity. (A and B) NSC34 cells, seeded on six-well plates at $1 \times 10^5$ cells/well, were infected with the indicated adenoviruses at a moi of 400. All samples were co-infected with Cre-recombinase virus at a moi of 40. Twenty-four hours after infection, media of cells were replaced with DMEM/N2 supplement. Twenty-four hours after the replacement of media, LDH release from cells was measured (A). * $P < 0.05$. The cell lysates were immunoblotted using the indicated antibodies (B). (C and D) PCNs, seeded on 96-well plates at $5 \times 10^4$ cells/well, were co-infected with the indicated adenoviruses at a moi of 100 and LacZ or Cre virus at a moi of 40. The WST-8 cell viability assays were performed at 4 days after infection (C). * $P < 0.05$. PCNs, seeded on six-well plates at $1 \times 10^6$ cells/well, were co-infected with the indicated adenoviruses at a moi of 200–800. All samples were co-infected with Cre-recombinase virus at a moi of 40. To maintain a constant total viral moi, an appropriate moi of LacZ-encoding virus was added for each infection. Twenty-four hours after infection, media of cells were replaced with DMEM/N2 supplement. Twenty-four hours after the replacement of media, LDH release from cells was measured (E). * $P < 0.05$. The cell lysates were immunoblotted using the indicated antibodies (F).
attenuated TDP-43-wt-induced cell death (Fig. 3A and B). However, unexpectedly, TDP-43-ΔRRM1 (and TDP-43-2FL) did not form dimers with TDP-43-wt (Supplementary Material, Fig. S4A). Furthermore, treatment with Rnase disrupted dimerization of TDP-43-wt (Supplementary Material, Fig. S4B). These data together suggest that homodimerization of TDP-43-wt is dependent on RNA binding and TDP-43-ΔRRM1 inhibits TDP-43-induced neuronal cell death by an unidentified mechanism. Given that TDP-43-ΔRRM1 inhibits the skipping activity of the CFTR exon 9 by TDP-43-wt (Fig. 3C and D), it appears that TDP-43-ΔRRM1 affects RNA splicing-regulatory factors that act downstream of TDP-43. It is also highly likely that TDP-43-ΔRRM1 attenuates TDP-43-wt toxicity by affecting the downstream mediators of TDP-43-induced neuronal cell death such as JNK/c-Jun and CHOP (Supplementary Material, Fig. S4C).

TDP-43 interacts with hnRNP-U in the nucleus

Overexpression of TDP-43 induces neuronal cell death via activation of JNK/c-Jun signaling, up-regulation of CHOP and Bim expression and down-regulation of Bcl-xL expression (22–24). These mediators are responsible for TDP-43-induced neuronal cell death. To further elucidate the molecular mechanisms underlying TDP-43-induced neuronal cell death in the nucleus, we searched for proteins interacting with TDP-43 in nucleus using GST-TDP-43-wt pull-down assays and mass-spectrometry analysis of pulled-down proteins. To this end, NSC34 cell lysates were mixed with glutathione beads-bound recombinant GST (negative control) or GST-TDP-43-wt that was produced in bacteria. Washed pellets of the mixed lysates were subjected to 5–20% gradient-gel sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. Multiple TDP-43-binding proteins were isolated (Fig. 4A, lane 4) and identified by mass-spectrometry (Supplementary Material, Table S1). Multiple RNA binding proteins including hnRNPs were identified. HnRNPs predominantly localize in the nucleus and are involved in RNA metabolism including RNA transcription and mRNA splicing (30). We initially focused on hnRNP-U because it was the most abundant interactor among the co-precipitators of TDP-43 (Fig. 4, lane 4, band 6). Using the GST pull-down assays, we confirmed that FLAG-tagged hnRNP-U was co-precipitated with GST-TDP-43-wt (Fig. 4B). Reciprocally, TDP-43-wt was co-precipitated with GST-hnRNP-U (Fig. 4C). Furthermore, not only exogenously overexpressed FLAG-hnRNP-U (Fig. 4D) but also endogenously expressed hnRNP-U (Fig. 4E) was co-immunoprecipitated with exogenous or endogenous TDP-43, respectively. Finally, immunofluorescence analysis showed the co-localization of TDP-43 and FLAG-hnRNP-U in the nucleus (Fig. 4F). Altogether, these results suggest that TDP-43 interacts with hnRNP-U in the nucleus.

The C-terminal 90 amino acids of hnRNP-U are responsible for the interaction with TDP-43

Next, we determined the regions of hnRNP-U that are involved in the interaction with TDP-43. FLAG-tagged mutants of hnRNP-U lacking C-terminal regions, named as hnRNP-U-(1-776), hnRNP-U-(1-746) and hnRNP-U-(1-716), were co-expressed with TDP-43, and co-immunoprecipitation analysis was performed. HnRNP-U-(1-806) (hnRNP-U-wt), hnRNP-U-(1-776) and hnRNP-U-(1-746), but not hnRNP-U-(1-716), were co-precipitated with TDP-43 (Fig. 5A). Furthermore, GST pull-down analysis confirmed that TDP-43 was co-precipitated with GST-hnRNP-U-(717-806) (Fig. 5B, left panel), indicating that the region encoding the C-terminal 90 amino acids of hnRNP-U is centrally responsible for the interaction between TDP-43 and hnRNP-U, although it is possible that another region in the N-terminus and/or middle part of hnRNP-U may contribute to the interaction.

Both DNA/RNA-binding and dimerization of TDP-43 are required for the interaction with hnRNP-U

TDP-43-induced neuronal cell death depends on DNA/RNA binding and dimerization of TDP-43 (Fig. 2). If hnRNP-U functionally affects TDP-43-induced neuronal cell death by interacting with TDP-43, the interaction of hnRNP-U with TDP-43 may depend on DNA/RNA binding and dimerization of TDP-43. To test this, TDP-43-2FL or TDP-43-6GGGG was mixed with GST-hnRNP-U-(717-806). GST pull-down analyses demonstrated that neither TDP-43-2FL nor TDP-43-6GGGG co-precipitated with GST-hnRNP-U-(717-806) whereas TDP-43-wt did (Fig. 5B, middle and right panels). Another RNA binding-deficient mutant of TDP-43, TDP-43-ΔRRM1, was not co-precipitated with GST-hnRNP-U-(717-806) (Supplementary Material, Fig. S5). Furthermore, after the lysates were incubated with RNase, TDP-43-wt was no longer co-precipitated with GST-hnRNP-U-(717-806) (Fig. 5C). Overall, these results suggest that both DNA/RNA binding and dimerization of TDP-43 are required for the interaction with hnRNP-U.

HnRNP-U negatively regulates TDP-43-induced neuronal cell death

To determine whether hnRNP-U functionally affects TDP-43-induced neuronal cell death, we next examined the effect of siRNA-mediated silencing of endogenous hnRNP-U expression. We constructed three types of siRNAs against hnRNP-U. Expression of all three hnRNP-U siRNAs caused cleavage of caspase-3 in LacZ-expressing cells and increased cleavage of caspase-3 in TDP-43-expressing cells (Fig. 6A). This finding suggests that the siRNA-mediated depletion of hnRNP-U alone causes neuronal cell death and increases TDP-43-induced neuronal cell death in an additive fashion. However, levels of increased cleavage of caspase-3 caused by the three siRNAs were different although levels of reduction in hnRNP-U expression induced by the three siRNAs are almost equal. Therefore, it appears that cleavage of caspase-3 may be artificially modified by some off-target effects and/or nucleic acid toxicities of siRNAs. Even if such artificial modifications associated with each siRNA are discounted, silencing of endogenous hnRNP-U consistently increases cleavage of caspase-3.

We previously demonstrated that low-level overexpression of TDP-43 causes caspase-mediated death of NSC34 cells via up-regulation of Bim, CHOP and c-Jun N-terminal kinase (JNK)/c-Jun-mediated signaling (22–24). Therefore, we also examined the effect of hnRNP-U siRNA on these signaling molecules. We found that siRNA-mediated depletion of hnRNP-U increased p-JNK (Fig. 6A). In contrast, we were unable to conclude that it increased the CHOP level or the Bim level because the majority of p-JNK siRNAs did not increase the CHOP level or the Bim level because the majority of siRNAs did not increase the CHOP level or the Bim level (Fig. 6A).

Reciprocally, overexpression of hnRNP-U attenuated TDP-43-induced neuronal cell death and decreased the cleavage of caspase-3 (Fig. 6B and C). Unfortunately, hnRNP-U-mediated reduction of TDP-43-induced neuronal death is ‘partial’ in the employed conditions (Fig. 6B and C). Once we increase the expression level of hnRNP-U to completely inhibit TDP-43-induced neuronal cell death, the toxicity of hnRNP-U appears (Fig. 6B, column 7) and tends to make the result unclear. Accordingly, we employed the indicated conditions and repeated the experiments. We confirmed hnRNP-U-mediated attenuation in the cleavage
of caspase-3 in another three independent experiments (Supplementary Material, Fig. S6A–C). Unlike hnRNP-U-wt, hnRNP-U-(1-716) (TDP-43-binding-deficient mutant of hnRNP-U) (Fig. 5A), did not inhibit TDP-43-induced neuronal cell death (Fig. 6D and E). Altogether, these results suggest that hnRNP-U negatively regulates TDP-43-induced neuronal cell death by interacting with TDP-43 via its C-terminal region.

Among the three cell-death signal pathways potentially underlying TDP-43-induced neuronal cell death (22-24), TDP-43-induced phosphorylation of JNK was partially attenuated by the overexpression of hnRNP-U (Fig. 6C). We confirmed hnRNP-U-induced attenuation of p-JNK in another three independent experiments (Supplementary Material, Fig. S6D–F). However, we were unable to assess potential hnRNP-U-mediated attenuation of CHOP and Bim induction caused by overexpression of TDP-43 because overexpression of hnRNP-U alone slightly increased the expression of CHOP and Bim (Fig. 6C).

HnRNP-U affects TDP-43-mediated modifications in mRNA splicing

It has been demonstrated that TDP-43 affects splicing of POLDIP3 mRNA (31,32). We confirmed that siRNA-mediated depletion of endogenous TDP-43 decreased the level of variant 1 and increased that of variant 2 of POLDIP3 mRNA (hPOLDIP3) (Supplementary Material, Fig. S7A and B) (31,32). In this study, we further found that overexpression of TDP-43 also decreased the level of variant 1 and increased that of variant 2 of human POLDIP3 mRNA (Fig. 7A and B, lanes 1 and 3) in a TDP-43 expression-dependent manner (Supplementary Material, Fig. S7C and D). Overexpression of hnRNP-U slightly, but significantly, increased both variants of hPOLDIP3 mRNA (Fig. 7A and B, lanes 1 and 2). Unexpectedly, co-expression of hnRNP-U and TDP-43 attenuated the TDP-43-mediated increase in variant 2 but has no effect on the decrease in variant 1 (Fig. 7A and B, lanes 3 and 4). Expression of TDP-43 was not altered by overexpression of

Figure 3. TDP-43-ΔRRM1 inhibits TDP-43-induced neuronal cell death and skipping activity of CFTR exon 9. (A and B) NSC34 cells, seeded on six-well plates at 1×10^5 cells/well, were co-infected with LacZ or TDP-43 adenovirus at a moi of 400 together with LacZ or TDP-43-ΔRRM1 adenovirus at a moi of 400. All samples were co-infected with Cre-recombinase virus at a moi of 40. Twenty-four hours after infection, media of cells were replaced with DMEM/N2 supplement. Twenty-four hours after the replacement of media, LDH release from cells was measured (A). *P<0.05. The cell lysates were immunoblotted using the indicated antibodies (B). (C and D) COS7 cells transiently overexpressing TDP-43-wt by adenoviral infection at a moi of 100 together with TDP-43-ΔRRM1 by plasmid transfection were harvested 48 h after transfection and the prepared total RNA were used for CFTR exon 9 skipping assays (C). All samples were co-transfected with pSPL3-CFTR9-TG11T7 reporter plasmid and co-infected with Cre-recombinase virus at a moi of 40. The cell lysates were immunoblotted using the indicated antibodies (D).
hnRNP-U (Fig. 7C). These results suggest that hnRNP-U negatively regulates TDP-43-mediated increase in the level of variant 2.

HnRNP-A2 negatively regulates TDP-43-induced neuronal cell death

HnRNP-A1 and hnRNP-A2B1 were also identified as TDP-43 interactors (Supplementary Material, Table S1) (33) and as ALS and multi-system proteinopathy (MSP)-causative genes (34).

Interaction of TDP-43 with hnRNP-A2 was confirmed by co-immunoprecipitation assays (Fig. 8A and B). Similar to overexpression of hnRNP-U, low-level overexpression of hnRNA-A2 inhibited the TDP-43-induced neuronal cell death (Fig. 8C and D).

Discussion

It is possible that the gain-of-function of cytoplasmic TDP-43, rather than that of nuclear TDP-43, may be toxic to neurons.
However, it has been also demonstrated that TDP-43 is toxic without the shift of its localization to the cytoplasm (18,20,35–38), and, in support of this, reducing cytoplasmic aggregation of TDP-43 using synthetic peptides does not prevent cell death (39). Our results reinforce that the gain-of-toxic function of nuclear TDP-43 (using low-level overexpression of TDP-43) causes toxicity and cytoplasmic localization of TDP-43 may be a result of TDP-43-induced neuronal cell death rather than a cause. In accordance with this notion, our previous studies show that the activation of the caspase cascade, associated with TDP-43-induced and/or non-TDP-43-induced neuronal cell death, results in cleavage of TDP-43, generating C-terminal fragments of TDP-43 that tend to localize and aggregate in the cytoplasm as less toxic or non-toxic TDP-43 derivatives (22). In addition, besides functioning as a non-toxic TDP-43 derivative, TDP-43-CTF27, corresponding to TDP-43-(170–414), has a dominant-negative effect on TDP-43-wt-induced neuronal cell death when co-expressed with TDP-43-wt (22).

TDP-43 has a variety of nuclear functions, especially related to RNA metabolism, and it is highly likely that the loss-of-function of TDP-43 in the nucleus, caused by cytoplasmic localization, contributes to the toxicity in TDP-43 proteinopathy in vivo. This has been supported by multiple studies using animals ranging from worms to mice demonstrating that the disruption of the TDP-43 gene results in ALS-like phenotype and pathology (11,40). In mammalian cells, the loss-of-TDP-43 also leads to a decrease in cell viability (41). However, in our study, siRNA-mediated reduction of endogenous TDP-43 expression did not cause neuronal cell death in NSC34 cells (Supplementary Material, Fig. S1), suggesting that short-term disruption of TDP-43 does not cause toxicity to motor neuronal cells. Alternatively, it could be assumed that the difference in the employed cell types and/or the cell-death-monitoring methods give rise to difference in results.

TDP-43 binds to DNA/RNA and forms homodimers (27,29). Both DNA/RNA binding and dimerization are required for TDP-43-induced CFTR exon 9 skipping (28,29) and cell death induction (Fig. 2). In agreement, RNA-binding of TDP-43 is necessary for TDP-43-mediated neurotoxicity in vivo in C. elegans, drosophila and chick models of TDP-43 proteinopathy (38,42,43). Combined with our findings showing that dimerization of TDP-43 does not

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**Figure 5.** C-terminal 90 amino acids of hnRNP-U interact with TDP-43 and this interaction is dependent on both RNA binding and dimerization of TDP-43. (A) NSC34 cells transiently co-overexpressing FLAG-hnRNP-U-(1-806) (=wt), -(1-776), -(1-746) or -(1-716) together with TDP-43-wt were harvested at 48 h after transfection and the prepared cell lysates were used for immunoprecipitation with TDP-43-N antibody or normal rabbit IgG (Control). The washed precipitates were fractionated by SDS-PAGE and immunoblotted using the indicated antibodies. (B) All recombinant proteins were separately overexpressed in NSC34 cells. The GST and GST-hnRNP-U-(717-806)-containing cell lysate was mixed with the cell lysates containing TDP-43-wt (left panel), -2FL (middle panel) or -6GGGG (right panel), and incubated for 3.5 h, followed by pull-down with glutathione sepharose. The washed precipitates were fractionated by SDS-PAGE and immunoblotted using TDP-43-N antibody. TDP-43-N antibody also detects GST proteins. (C) GST, GST-hnRNP-U-(717-806) and TDP-43-wt were all separately overexpressed in NSC34 cells and the cell lysates were pre-incubated with or without 20 μg/ml RNase A at room temperature for 2 h. The GST or GST-hnRNP-U-(717-806)-containing lysate was then mixed with the cell lysates containing TDP-43-wt and incubated for 2 h, followed by pull-down with glutathione sepharose. The precipitates were fractionated by SDS-PAGE and immunoblotted using the TDP-43-N antibody. TDP-43-N antibody also detects GST proteins.
Figure 6. hnRNP-U negatively regulates TDP-43-induced neuronal cell death. (A) NSC34 cells, seeded on six-well plates at $7 \times 10^4$ cells/well, were transfected with 5 nM control siRNA (Cont.), hnRNP-U-#1, -#2 or -#3 siRNA using Lipofectamine 2000 reagent. Sixteen hours after transfection, cells were infected with LacZ or TDP-43 virus at a moi of 200. All samples were co-infected with Cre-recombinase virus at a moi of 40. Forty-eight hours after infection, cell lysates were prepared and were immunoblotted using the indicated antibodies. Intensities of immunodetected signals were densitometrically estimated with an ImageJ software. (B and C) NSC34 cells, seeded on six-well plates at $1 \times 10^5$ cells/well, were co-infected with the indicated adenoviruses and Cre-recombinase virus at a moi of 40. Twenty-four hours after infection, media of cells were replaced with DMEM/N2 supplement. Twenty-four hours after the replacement of media, LDH release from cells was measured (B). *$P < 0.05$. The cell lysates were immunoblotted using the indicated antibodies (C). p-JNK, phosphorylated JNK; t-JNK, total JNK. Intensities of immunodetected signals were densitometrically estimated with an ImageJ software. (D and E) NSC34 cells, seeded on six-well plates at $1 \times 10^5$ cells/well, were co-infected with LacZ, hnRNP-U-wt or hnRNP-U-(1-716) adenovirus at a moi of 200 together with LacZ or TDP-43 adenovirus at a moi of 400. All samples were co-infected with Cre-recombinase virus at a moi of 40. Twenty-four hours after infection, media of cells were replaced with DMEM/N2 supplement. Twenty-four hours after the replacement of media, LDH release from cells was measured (D). *$P < 0.05$. The cell lysates were immunoblotted using the indicated antibodies (E). Intensities of immunodetected signals were densitometrically estimated with an ImageJ software.
occur when TDP-43 lacks DNA/RNA binding activity (Supplementary Material, Fig. S4A) or when TDP-43 is incubated with RNase (Supplementary Material, Fig. S4B), it can be assumed that two (or more) TDP-43 molecules dimerize (or multimerize) via RNA molecule(s), with the resulting TDP-43/RNA complex inducing TDP-43-induced neurotoxicity in vitro. Swarup et al. reported that TDP-43 behaves as a co-activator of p65 NF-κB and overexpressed TDP-43 stimulates the p65 NF-κB pathway via the direct interaction with p65 NF-κB and causes neurotoxicity (12). It is likely that this mechanism does not depend on RNA binding of TDP-43 and is therefore thought to play a minor role in the cell death model employed in this study.

We identified multiple TDP-43 interactors, including hnRNPs, some of which were already known from prior reports (21,44). We found that siRNA-mediated reduction of hnRNP-U causes neuronal cell death and that overexpression of hnRNP-U attenuates TDP-43-induced neuronal cell death by binding to TDP-43 in the nucleus (Fig. 6). These observations suggest that TDP-43 is not toxic when TDP-43 is associated with and negatively regulated by hnRNP-U and other hnRNPs, but once the level of TDP-43 expression exceeds normal levels, negative regulation by hnRNPs is no longer effective and TDP-43 becomes neurotoxic.

The mechanism underlying inhibition of TDP-43-induced neuronal toxicity by hnRNP-U has not yet been determined. HnRNP-U does not bind to TDP-43 that lacks DNA/RNA binding activity and dimerization (Fig. 5B). Moreover, hnRNP-U does not inhibit dimerization of TDP-43 (Supplementary Material, Fig. S8). These results suggest that hnRNP-U directly binds to the TDP-43/RNA complex and inhibits TDP-43-mediated regulation in splicing of mRNA and neuronal cell death.

In previous studies, low-grade overexpression caused neuronal cell death by increasing Bim and CHOP levels and activating the JNK signaling (22–24). Co-expression of hnRNP-U attenuates TDP-43-induced up-regulation in the p-JNK level (Fig. 6C and Supplementary Material, Fig. S6D–F) and siRNA-mediated depletion of endogenous hnRNP-U increased the p-JNK level (Fig. 6A). On the other hand, we are unable to conclude that either CHOP or Bim level is significantly affected by hnRNP-U expression (Fig. 6A and C). These results suggest that at least the JNK-mediated signaling is a downstream target for hnRNP-U-mediated inhibition of TDP-43-induced neuronal death. However, it is highly likely that the JNK-mediated signaling is not the central mediator of hnRNP-U-mediated inhibition of TDP-43-induced neuronal death, because overexpression of hnRNP-U inhibited the JNK signaling only partially (Fig. 6C and Supplementary Material, Fig. S6D–F).

At least 20 abundant and major hnRNPs (named from A to U), as well as less abundant and minor hnRNPs, have been identified (30). Among them, missense mutations in the prion protein-like domain of hnRNP-A1 and hnRNP-A2/B1 cause multi-system proteinopathy (MSP) and/or ALS with cytoplasmic aggregation and the loss-of-function of the proteins (34). Depletion of hnRNPs including hnRNP-A1 and hnRNP-A2B1 from myonuclei, combined with their sarcoplasmic aggregation, may contribute to sporadic inclusion body myositis that is also regarded as a TDP-43 proteinopathy (45). These findings are consistent with our conclusion that the loss-of-function of hnRNP-A2 may contribute to decreased total hnRN function that prevents TDP-43-induced neuronal cell death. In addition to hnRNP-U and hnRNP-A2, many other hnRNPs may also contribute to the prevention of TDP-43-induced neuronal cell death.

Previous reports showed that increased expression of variant 2 of POLDIP3 in motor neurons in ALS patients, and siRNA-mediated knocking-down of endogenous TDP-43 decreases the...
level of variant 1 and increases the level of variant 2 of POLDIP3 (31,32). We confirmed this result (Supplementary Material, Fig. S7A and B). In the present study, low-level overexpression of TDP-43 decreased the level of variant 1 and increased the level of variant 2 of POLDIP3 (Fig. 7 and Supplementary Material, Fig. S7C and D). These results together suggest that abnormal levels of TDP-43, lower or higher, cause similar modifications in splicing of POLDIP3 mRNA. Interestingly, hnRNPU reversed the TDP-43-mediated increase in the level of variant 2 but did not affect the decrease in the level of variant 1 (Fig. 7). The mechanisms underlying these modifications by TDP-43 and hnRNPU in splicing of POLDIP3 mRNA remain speculative. Furthermore, either overexpression of TDP-43 or hnRNPU increases the level of the variant 2 whereas the co-expression of hnRNPU with TDP-43 inhibits TDP-43-induced increase in the level of the variant 2. One interpretation is that hnRNPU slightly increases the level of the variant 2 via non-TDP-43-mediated mechanisms whereas its overexpression of TDP-43 increases the level of variant 2 of POLDIP3 mRNA. Interestingly, hnRNPU reversed the splicing of TDP-43 (46–49), some of which may be linked to neuronal cell death. The relationship between TDP-43 and regulation of mRNAs of CHOP and Bim has not been fully examined due to the technical problems (Fig. 6C). TDP-43-induced phosphorylation of JNK was partially attenuated by overexpression of hnRNPU (Fig. 6C).

Our results suggest that hnRNPs, involved in the physiological RNA metabolism in association with TDP-43 in the nucleus, behave as endogenous defending molecules that prevent the gain-of-toxic function of TDP-43. Moreover, a small increase in TDP-43 expression in the nucleus may enable a fraction of TDP-43 to escape from the inhibitory regulation of hnRNPs, causing neuronal cell death by slightly altering the expression patterns of target mRNAs.

Materials and Methods

Antibodies and compounds

The following antibodies were purchased from suppliers: TDP-43-C (12892-1-AP), TDP-43-N (10782-2-AP), hnRNPU-N (14599-1-AP) and hnRNPU-A2B1 (14813-1-AP), ProteinTech Group, Inc. (Chicago, IL, USA); cleaved-caspase-3, GAPDH, Bim and phosphorylated-JNK, Cell Signaling TECHNOLOGY (Beverly, MA, USA); GST, CHOP, JNK1 and normal rabbit IgG, Santa Cruz (Santa Cruz, CA, USA); FLAG, Horseradish peroxidase (HRP)-conjugated anti-FLAG antibody and actin, Sigma (St Louis, MO, USA); hnRNPU-C, Cosmo bio (Tokyo, Japan); Xpress, Invitrogen (Carlsbad, CA, USA); HRP-conjugated goat anti-rabbit secondary antibody, Bio-Rad (Hercules, CA, USA); hnRNPA2B1-protein A secondary antibody, Bio-Rad, Santa Cruz, CA, USA). Staurosporine and RNase A were purchased from Calbiochem (Darmstadt, Germany) and Wako (Osaka, Japan), respectively.

Plasmid constructs

The vector encoding FLAG-tagged human hnRNPU was provided by Dr Hiroshi Handa (Tokyo Institute of Technology). Human TDP-43 cDNA was provided by Dr Randall S. Tibbetts (University of Wisconsin). The hnRNPU cDNA was subcloned into the pE8G vector to construct the GST-tagged hnRNPU expression vector. The TDP-43 cDNA was subcloned into the pEF1/Myc-His vector (Invitrogen), the pEF4/HisC vector (Invitrogen) or the pE8G vector with a native stop codon to construct non-tagged TDP-43, HisXpress (HX)-tagged TDP-43 or GST-tagged TDP-43 expression vector, respectively. The FLAG-tagged TDP-43 cDNA was subcloned into the pcDNA3 vector (Invitrogen). TDP-43-K32A/R33A/K64A, -Δ(82–98), -ΔLT-NLS, -ΔLT-NL5mut, -ΔF147L/F149L (2FL), -ΔRRM1, -ΔGGGG, Δ(6–9), hnRNPU-U-(1–776), -(1–746), -(1–716) and -(717–806) were generated by KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). Primer sequences for mutagenesis are available on request. The FLAG-tagged hnRNPU-A2 encoding vector was provided by Dr Michael Fry (Technion-Israel Institute of Technology). The pSPL3-CTFR-TG11T7 vector for CTFR exon 9 skipping assay was provided by Dr Masato Hasegawa (Tokyo Metropolitan Institute of Medical Science).

Adenoviral vector-mediated expression

The systems of adenovirus expression vectors were purchased from TaKaRa (Shiga, Japan). LacZ, Cre and Cre-TDP-43 wt adenoviruses were as described previously (22). cDNAs encoding TDP-43-K32A/R33A/K64A, -Δ(82–98), -ΔLT-NLS, -ΔLT-NL5mut, -ΔF147L/F149L (2FL), -ΔRRM1, -ΔGGGG, hnRNPU-U, -(1–716) and hnRNPU-A2 were inserted into the SwaI site of a cosmid adenoviral vector.
pAxCALNLw. In this vector, a stuffer DNA fragment, sandwiched by two loxP sequences, is located just upstream of cDNA and interferes with gene expression. If an adenosivirus expressing Cre-recombinase is co-introduced into the cells, the stuffer is removed and gene begins to be expressed. All viruses were grown in HEK293 cells and purified by CsCl2 gradient ultracentrifugation.

Cell culture and transfection
NSC34, a hybrid cell line established from a mouse neuroblastoma cell line and mouse embryonic spinal cord cells, was a kind gift from Dr Neil Cashman (University of Toronto). NSC34, CO57, HeLa and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% of fetal bovine serum (FBS) (Invitrogen). Transfection was performed using Lipofectamine (Invitrogen) and PLUS reagent (Invitrogen) under the manufacturer’s protocol.

Primary cultured cerebral cortical neurons
Primary cultured cerebral cortical neurons (PCNs), obtained from embryonic Day 14 ICR mice, were seeded on poly-l-lysine-coated 96-well plates (Sumitomo Bakelite, Tokyo, Japan) at 5 × 10^4 cells/well or poly-l-lysine-coated 6-well plates (Sumitomo Bakelite). Purity of neurons by this method was >98%. PCNs were infected with adenosviruses at the indicated multiplicities of infection (moi) in Neuron medium.

Cell death assay and cell viability assay
Cells, seeded on six-well plates, were incubated with virus-containing media at the indicated multiplicity of infection (moi) at 37°C for 1 h with agitation. Twenty-four hours from infection, media of cells were replaced by DMEM with N2 supplement (DMEM, GIBCO, Waltham, MA, USA). Cell viability was measured by a multilabel reader 2030 ARVO (Perkin Elmer, Waltham, MA, USA). Cell death assay and/or cell viability assay was measured by WST-8 cell viability assays. The WST-8 assay, performed using Cell Counting kit-8 (Dojindo, Osaka, Japan), was based on the ability of cells to convert a water-soluble 2-(2-methoxy-4-nitrophenyl)-(4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt into a water-soluble formazan. Cells were treated with WST-8 reagent at 37°C, and 450 nm absorbance was measured.

Western blot analysis
Cells were homogenized with a cell lysis buffer [10 mM Tris–HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, protease inhibitors, phosphatase inhibitors] by a freeze–thaw cycle or solubilized by sonication in a 4% SDS-containing sample buffer. The samples in the SDS-containing sample buffer were boiled for 5 min at 95°C, fractionated by SDS–PAGE and blotted onto polyvinylidene fluoride membranes. Immunoreactive bands were detected with ECL western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA). After washing four times with the lysis buffer, the precipitates were fractionated by SDS–PAGE, followed by immunoblotting analysis.

GST pull-down assay
NSC34 and CO57 cells, transiently transfected with indicated vectors, were harvested at 48 h after transfection and lysed in a pull-down buffer [150 mM NaCl, 20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, protease inhibitors] by sonication. After centrifugation at 12 000g for 15 min, the cell lysates were pre-cleared with sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) for 1.5 h. The cleared supernatants were then incubated for 2–3 h with TDP-43-N, FLAG, or hnRNP-U-N antibody and precipitated for 1–2 h with protein G-Sepharose (Amersham Biosciences) at 4°C. After washing four times with the lysis buffer, the precipitates were fractionated by SDS–PAGE, followed by immunoblotting analysis.

Immunocytochemistry
NSC34 cells were transfected using Lipofectamine2000 (Invitrogen) under the manufacturer’s protocol. Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde-PBS and immunostained using the TDP-43-C antibody and/or FLAG antibody and the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit polyclonal antibody and/or TexasRed-conjugated goat anti-mouse monoclonal antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclei were stained with Hoechst 33258 (Sigma). The cells were observed with a confocal microscope LSM510 (Carl Zeiss).

siRNA-mediated knock-down
siRNAs against mouse TDP-43, mouse hnRNP-U and non-targeting control siRNA were purchased from RNAi Co., Ltd. (Tokyo, Japan). The siRNA sequence for mouse TDP-43-#1 and #2 are 5′-GGAAUCGCGGCUAUAUAGC-3′ and 5′-GUCCGUAUGGCGAAAGAG-3′, respectively. The siRNA sequence for mouse hnRNP-U#1, -#2 and -#3 are 5′-GGCGUCUGAGUCUACUC-3′, 5′-GUUGAACUCUCUUAUGCGAAG-3′ and 5′-CAGAAUUGCGUGCAAAU-3′, respectively. siRNAs against human TDP-43 (L-012394-00) and negative control (D-001810-10) were purchased from Dharmacon (Waltham, MA, USA). NSC34 cells or HeLa cells were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer’s reverse transfection protocol. Briefly, 7 × 10^4 cells for NSC34 cells or 5 × 10^4 cells for HeLa cells per well on six-well plates were combined with the 5 nM siRNA and Lipofectamine2000 reagent complexes.

CFTR exon 9 skipping assay
Total RNA was extracted from COS7 cells that had been transfected with TDP-43-encoding vector together with a reporter vector, pSPL3-CFTR9-TG11T7 (50), using the ISOGEN (Wako, Osaka, Japan). First-strand cDNAs were synthesized from total RNA
CBB staining and mass-spectrometry analysis
Bacteria-derived recombinant GST-fused TDP-43-wt was prepared as a bait. NSC34 cell lysates, solubilized with lysis buffer [150 mM NaCl, 20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, protease inhibitors], were mixed with glutathione-beads-bound recombinant GST or GST-TDP-43-wt overnight by rotation. After washing five times using the lysis buffer, the precipitates were fractionated by 5%–20% gradient gel (Wako) SDS–PAGE and stained with Coomassie brilliant blue (CBB) (Sigma). The CBB-stained protein bands were carefully excised from the gel, washed and destained with acetonitrile (ACN). After reduced with 10 mM DTT, proteins were alkylated with 55 mM iodoacetamide and digested for 16 h at 37°C with sequence-grade trypsin (Promega, Madison, WI, USA). The resulting peptides were sequentially extracted from the gel with 0.1% trifluoroacetic acid (TFA)/2% ACN, 0.1% TFA/33% ACN, and 0.1% TFA/70% ACN. The combined solutions were evaporated, and the peptides were analyzed by a nano-LC-ESI-MS/MS system consisting of DiNa nano-LC (KYA Technologies) and QSTAR Elite hybrid liquid chromatography tandem mass spectrometry (LC/MS/MS) (Applied Biosystems, Carlsbad, CA, USA). Proteins were identified using ProteinPilot software version 3.0 (Applied Biosystems) with default parameters using the UniprotKB database (mouse).

Quantitative real-time PCR analysis
Total RNA was extracted from HeLa cells infected with indicated adenovirus vectors or transfected with TDP-43 siRNA using ISOGEN (Wako). Reverse transcription and PCR reactions were performed on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems) using the Taqman RNA-to-CT 1-Step Kit (Applied Biosystems). The pairs of primers and the Taqman probes for target mRNAs were designed based on human mRNA sequences using TaqMan Gene Expression Assays (Applied Biosystems, Assay ID: POLDIP3 variant 1, Hs00993978_m1; POLDIP3 variant 2, Hs00994038_m1; GAPDH, Hs02758991_g1). Data analysis was performed using StepOne Software ver. 2.0.2 (Applied Biosystems). Relative mRNA expression was analyzed by the relative standard curve method. Data were normalized to the mRNA expression of GAPDH. GenBank Accession Number of POLDIP3 variant 1 and variant 2 is NM_032311.4 and NM_178136.2, respectively.

Statistical analysis
All values in the figures are shown as means ± SD. All experiments that were statistically analyzed were performed with N = 3. Statistical analysis was performed with Student’s t-test. *P < 0.05.

Supplementary Material
Supplementary Material is available at HMG online.

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