Supplementary Information for

An ALS-associated mutation in the TDP-43 gene enhances protein aggregation, fibril formation and neurotoxicity

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METHODS

Plasmids, peptides, antibodies

The cDNA encoding TDP-43 wild type and fragments were subcloned into the pCS2 vector with corresponding tags (GFP or RFP or HA) at the carboxyl terminus as described previously[1]. Affinity purified rabbit polyclonal antibody to TDP-43 was obtained from ProteinTech Group. Anti-HA, His, GFP antibodies were obtained from Covance.

The peptides were synthesized either containing the wild type TDP-43 sequence (Wt Q286-Q331:QGGFGNSRGGGAGLGNNQGSNMGGGMNFGAFSINPAMMAAAQAALQ) or mutant sequence (phosphoA315T mutant Q286-Q331: QGGFGNSRGGGAGLGNNQGSNMGGGMNFGT(p)FSINPAMMAAAQAALQ) or control sequence (Control peptide:DEAFAIVVGGVMLGIIAGKNSGVDEAFFVLKQHHVEYGSDHRFEAD).

Abeta(1-42) peptide was purchased from Invitrogen.

Postmortem samples collection and analyses. Human brain samples were collected from autopsied tissues at the Neuropathology Core of the Northwestern University following NIH and institutional guidelines. Brain samples were evaluated for atrophy, for pathology by hematoxylin-eosin staining and immunostaining using multiple antibodies including anti-TDP-43 (ProteinTech Group, Chicago).

RIPA soluble protein lysates were prepared in the presence of 5 mM EDTA from post-mortem brain tissues from seven control cases and seven TDP-43 immunoreactive FTLD cases. Both the control and FTLD-TDP groups contain 4 females and 3 males with ages ranging from 66-89 and 61-87, respectively. Control samples were from non-cognitively impaired subjects with minimal AD pathology containing Braak & Braak tangle stages II to III, except one with mild cognitive impairment and pathological diagnosis of early AD (see lane 7, Fig 3). The samples were analyzed by Western blotting using specific anti-TDP-43. In addition to the predicted band migrating at 43 kDa, additional bands migrating around 74 kDa and faster than 37 kDa (marked by the arrowhead) were detected by anti-TDP-43 antibody. Actin was used as a loading control.

Primary neuronal culture, Transfection, TUNEL assay, immunostaining and fluorescent microscopy

Primary neuronal culture was carried out using embryonic day 15 (E15) mice following published protocols [5,6]. Neurons were transfected with lipofectamine 2000 (Invitrogen) 48 hours after plating. Culture medium was changed into fresh B27 supplemented Neurobasal media (Invitrogen) 6 hours later. At different time points, cells were fixed for immunostaining or TUNEL staining.

For peptide treatment, Abeta(1-42) peptide or 46mer TDP-43 synthetic peptides (Wt or A315T; Q286-Q331) or control peptides were dissolved in dimethyl sulfoxide (DMSO) at 2.5 mM and stored at −80°C until use. For cell treatment, corresponding peptides were diluted in fresh neurobasal/B27 medium to final concentrations of 5 uM, 10 uM or 20 uM and added onto cultured cortical neurons on day 6 in vitro in Neurobasal/B27 medium. After 48 hour incubation, media was changed to fresh Neurobasal/B27 medium with the same concentrations of corresponding peptides, and incubation was continued for another 48 hours before fixation, immunostaining and microscopy.

HEK (human embryonic kidney) 293 cells were cultured in DMEM containing 10% FBS. Transfections were carried out with lipofectamine 2000 (Invitrogen) or using the calcium phosphate method. To establish stable expression cell lines, HEK293 cells were transfected with HA- or GFP tagged TDP-43 (Wt or A315T) in a modified CS2 vector containing a G418 resistance cassette[1]. G418 was added at 1mg/ml to cells 48hrs after transfection; individual clones with G418 resistance were identified by immunofluorescent staining or GFP fluorescence and maintained in G418 containing media. For treatment with okadeic acid (OA), 40 µM OA was added to stable expression cells at 37°C for 6 hours and cells were then lysed in PBS containing 1% SDS; 1 mM EDTA; 2 mM Na_3VO_4, followed by Western blotting with anti-HA antibody.

For the TUNEL assay, cells were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 15 min at room temperature. Samples were washed twice with PBS and blocked in PBS with 5% BSA at 37°C for 1 hour. The TUNEL reaction mixture (Roche, In Situ Cell Death Detection Kit, TMR Red) was applied to the samples following the manufacturer's instruction.

For immunostaining, cells were fixed in 4% paraformaldehyde in PBS for 15 min and then permeabilized and blocked in PBST/BSA (PBS with 0.1% Triton and 3% BSA) at 37°C for 1 hour. Following incubation with primary antibodies, Cy2 or Cy3-conjungated

secondary antibodies were used with nuclei stained by Hoechst dye 33342.

Confocal images were collected on a LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss) using a 63x/1.4 Plan-APOCHROMAT oil immersion objective.

Size-exclusion chromatography was carried out using a gel-filtration column (Superdex-200 10/300 GL, Amersham Biosciences) calibrated with thyroglobulin (667 kDa), ferritin (440 kDa), bovine serum albumin (67 kDa), β-lactoglobulin (35 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa). Protein lysates were prepared from TDP-43 expressing stable HEK293 cell lines by lysing cells in cold RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail (Roche), 50 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4)]. Protein extracts were centrifuged at 13,000 g for 15 min to remove insoluble particles before loading and eluted at 0.5ml/min. Fractions (0.5 ml each) were collected and analyzed by Western blotting with different antibodies.

ThT binding, Fibril formation and Electron microscopy (EM)

The control peptide or Wt or A315T mutant synthetic TDP-43 peptides were dissolved in PBS buffer (pH 7.0) at a concentration of 500μM. Amyloid fibril formation was detected by fluorescence enhancement upon binding to thioflavin T (ThT). ThT binding reactions were set up in triplicates in 96-well Corning Costar black plates. Each well had one 1/8-inch glass sphere (Fisher Scientific) and 150 ul of peptide in PBS buffer supplemented with 20 uM ThT. Plates were sealed with a Greiner EASYseal (Greiner Bio-one) and continuously shaken at 37°C except for 1 min each 10min as required for data acquisition. ThT fluorescence changes were monitored at 450 nm (excitation) / 480 nm (emission) on a Varioskan Flash (Thermo Scientific).

For EM detection of fibrils, control peptide or TDP-43 peptides (Wt or A315T) were incubated at 1.25mM in PBS buffer (pH 7.0) at 37°C for 48 hours followed by further incubation at 4°C for two weeks or at 37°C for 10 days with rotation at 240rpm. Following incubation, peptide solutions were diluted to 250uM with PBS (pH 7.0) as fibril solutions. 5ul aliquots of fibril solutions were placed on a carbon-coated EM copper grids stained with 1% uranyl acetate. After drying, samples were examined on a FEI

Tecnai 20 electron microscope at 200 kV.

Atomic force microscopy (AFM).

Twenty microliter of aqueous solution of Wt and A315T mutant TDP-43 synthetic peptides at the concentration of 0.05 mM was incubated at 37°C for 0, 7, 13 or 17 hours and then drop-cast on freshly cleaved mica surface. After 10 min adsorption, excessive solutions are withdrawn from the mica surface followed by observation using a Dimension 3100 AFM (Veeco Metrology, USA) with tapping mode. Commercial silicon tips with a nominal spring constant of 2.0 N/m and resonant frequency of 69.6 kHz were used in all the experiments.

Statistical Analyses.

Statistical analyses were carried out with either ANOVA or Student's T-test. For details, see corresponding figure legends.

Additional Discussion

In this study, we systematically compared the wild type TDP-43 and an ALS mutant TDP-43 *in vivo* in transgenic flies, in cultured cells and *in vitro* in biochemical assays. Expression of the A315T mutant TDP-43 in cultured neurons and transgenic flies led to significantly increased neurotoxicity compared to the wild type protein. Our data demonstrate similarity between TDP-43 and other amyloid proteins.
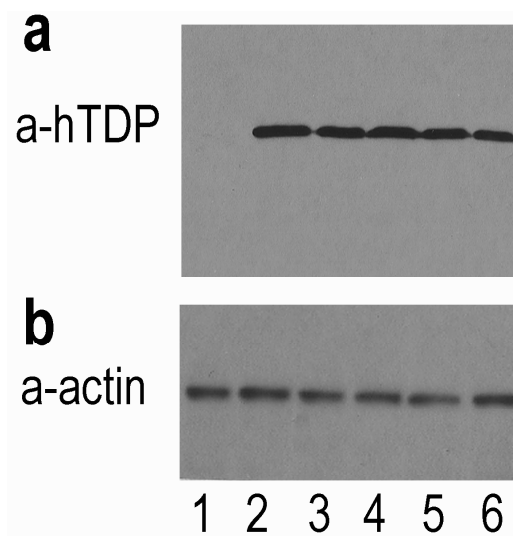
First, the carboxyl terminal domain of TDP-43 shows a sequence similar to the human prion protein and is predicted to form β-sheets and β-turns (**Fig. 6**; **Fig. 7**). Second, the A315T mutant TDP-43 forms heat-stable, detergent-insoluble aberrant species and protease-resistant fragments, similar to the amyloid forms of the prion protein. Third, TDP-43 exists in multiple conformations or oligomeric forms. Fourth, molecular dynamics simulation suggests that synthetic peptides flanking amino acid residue A315 of TDP-43 have a high propensity to form β-sheets. ThT binding assay and EM reveal that synthetic TDP-43 peptides corresponding to this region forms amyloid fibrils. Time-lapse AFM demonstrates that the Wt and A315T mutant TDP-43 peptides have distinct

dynamic properties in forming protofibrils and fibrils, with A315T mutant progressing faster to the protofibril stage and eventually forming thicker fibrils. Fifth, these synthetic TDP-43 peptides caused neurotoxicity when added to neuronal cultures with A315T mutant showing enhanced neurotoxicity. This suggests the possibility of horizontal transmission of neuronal damage in TDP-43 proteinopathy. Consistent with this, in transgenic flies expressing human TDP-43, axonal loss often occurs in a "group" fashion, suggesting the non-cell autonomous nature of neuronal death in such transgenic animals[2].

Understanding the precise role of TDP-43 phosphorylation in the pathogenesis of TDP-43 proteinopathy may require years of effort, as is the case with the role of tau protein hyperphosphorylation in tauopathy, another major group of FTLD. There are more than 60 potential phosphorylation sites in hTDP-43, including Ser, Thr, and Tyr residues. ALS-linked mutations create additional potential phosphorylation sites: 7 Ser and 2 Thr (including A315T and A382T). The alanine 315 residue in TDP-43 is highly conserved, suggesting that it is crucial for the physiological function of TDP-43. The missense mutation of Ala to Thr, A315T, may lead to hyperphosphorylation of TDP-43, disrupting the normal conformation of Wt TDP-43 protein. Alteration in TDP-43 protein conformation may affect its interactions with other protein/RNA partners by either "disruption of the normal function" or "gain-of-function" mechanisms.

Taken together, our study uncovers previously unknown biochemical characteristics, sequence/structural features and pathogenic behavior of TDP-43 derivatives. Our data also suggest that decreasing the formation of neurotoxic TDP-43 species, increasing the clearance of such TDP-43 derivatives or blocking the spread of the disease phenotype in affected tissues may provide therapeutic benefits.
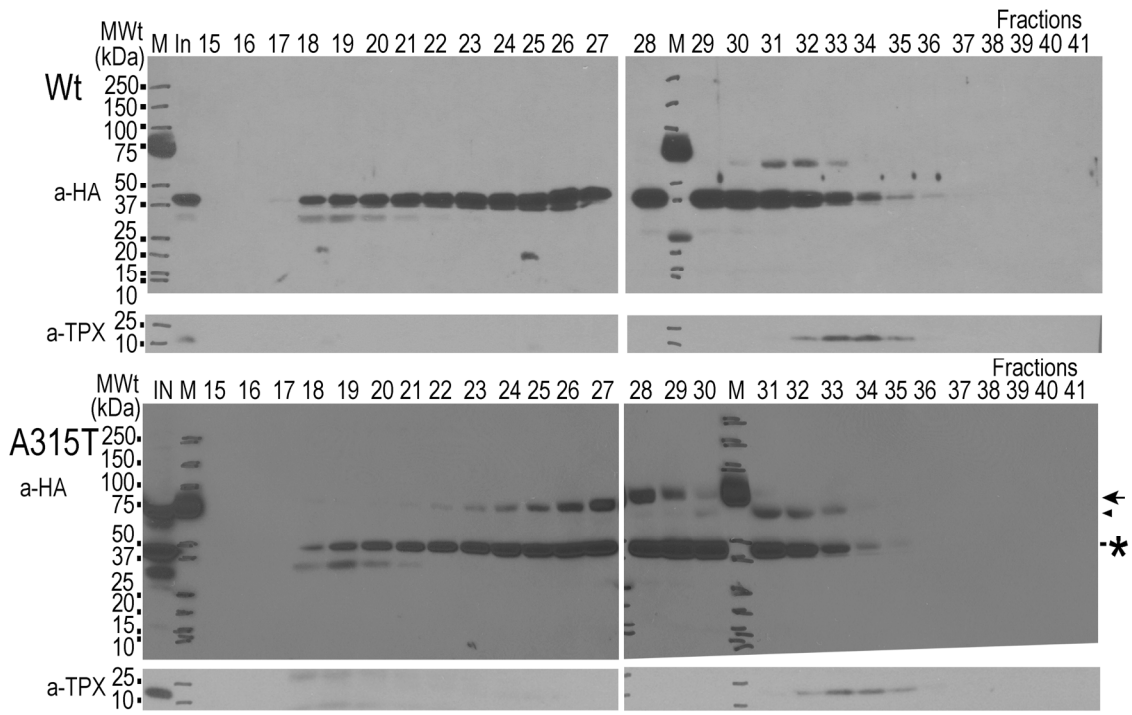
Supplementary Figure S1. The human TDP-43 protein was expressed at equivalent levels in the wild type and A315T mutant transgenic fly lines. Protein lysates were prepared from day 3 adult fly heads from different lines of UAS-RFP (lane 1), UAS-Wt-hTDP-43 (lanes 2, 3: lines #A and B respectively, corresponding to Wt fly lines shown in Fig. 1d) and UAS-A315T-hTDP-43 flies (lanes 4, 5, 6: lines #A, B and C respectively, corresponding to A315T fly lines shown in Fig. 1d) crossed with a GMR-Gal4 driver line. Western blotting was carried out using anti-hTDP-43 (**a**) or anti-actin as a loading control (**b**).

Supplementary Figure S2. (a). A schematic representation of the domain structure of TDP43: RNA recognition motifs (RRM1 and RRM2) and the glycine-rich carboxyl terminal domain with the vertical bars indicating positions of mutations associated with TDP-43 proteinopathy. (**b).** The expression pattern of GFP-tagged wild type TDP-43 is identical to that of the endogenous TDP-43 (Endo). Expression of the carboxyl terminal domain (T202) or A315T mutant TDP-43-GFP led to the formation of the protein aggregates in the cytoplasm, either in axons (arrows) or perinuclear region (arrowhead). Scale bar, 4 μm. **(c, d).** Expression of the carboxyl terminal domain T202 or A315T mutant TDP-43-GFP led to neuronal death as shown by the TUNEL staining (c) and quantification of percentage of TUNEL positive neurons in different groups 8 hrs or 32 hrs following transfection of corresponding plasmid: control (Ctrl), Wt TDP-43, T202 or A315T mutant TDP-43 (**d).**
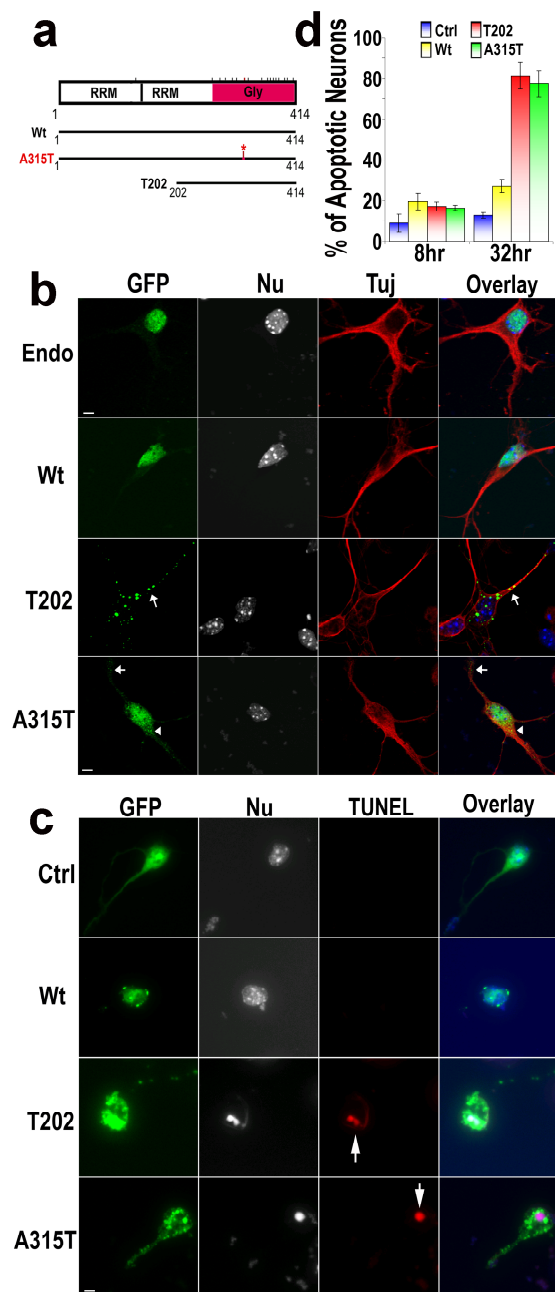
Supplementary Figure S3. Original images for the gels shown in Figure 4.

Supplementary Figure S4. Size-exclusion chromatography shows that Wt and A315T mutant TDP-43 proteins form high molecular weight complexes and that A315T TDP-43 protein forms stable degradation products. (a) UV280/260 profiles of size-exclusion chromatography analysis of purified GST-tagged Wt or A315T proteins. **(b, c, d)** Different fractions were analysed by SDS-PAGE followed by Coomassie blue staining (b) or Western blotting using either anti-TDP-43 (c) or anti-GST (d) antibodies. A significant fraction of Wt or A315T TDP-43 was detected in the high molecular weight fractions, ranging from 667-440 kDa. As compared to Wt TDP-43, a ladder of A315T TDP-43 protein derivatives (e.g., the 29 kDa band marked by the arrowhead) were detected by anti-TDP-43 antibody that were not detectable in Wt TDP-43 group (compare left and right panels in c), suggesting that degradation products of A315T mutant TDP-43 may be more stable. The 29 kDa A315T mutant TDP-43 species (marked by the arrowhead) was detectable by TDP-43 antibody recognizing the carboxyl terminus of TDP-43 but not by the monoclonal anti-GST antibody, suggesting that this 29 kDa fragment was generated from the carboxyl terminal region of TDP-43.
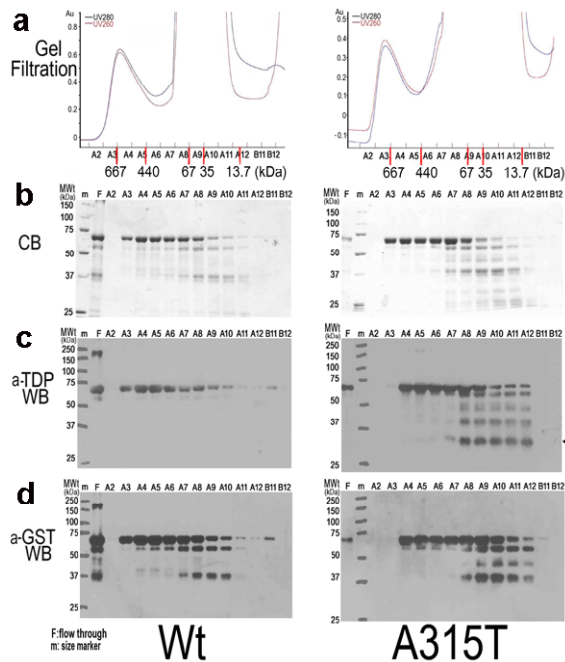
Supplementary Figure S5. The 75 kDa species in A315T mutant TDP-43 expressing cells is not ubiquitinylated. Immunoprecipitation was carried out with anti-HA antibody using cell lysates from stable HEK293 cells either expressing wt or A315T mutant TDP-43 as HA-tagged protein. The immunoprecipitated proteins were analysed together with control cell lysate (Ctrl), by Western blotting using either anti-TDP-43 (lanes 1-6) or anti-Ub (lanes 7-12) antibodies.
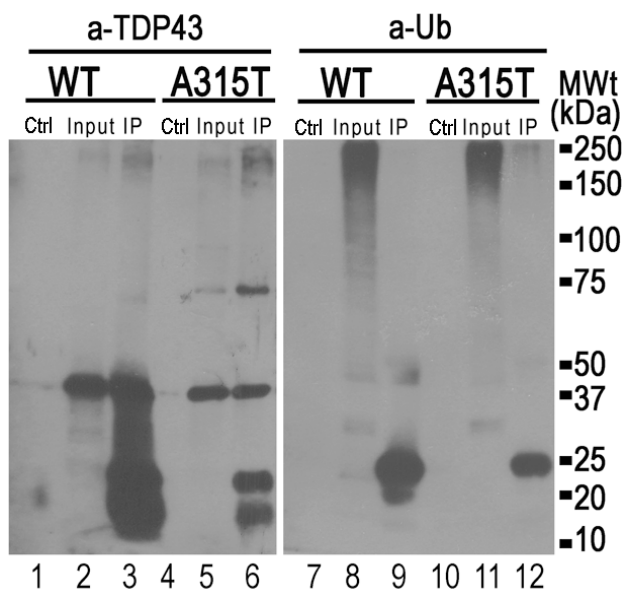
Supplementary Figure S6. The still images of the initial (**a**) and final (**b**) molecular conformations of TDP-43 synthetic peptides by molecular dynamics simulation. The amino-termini of both peptides are at the lower right corner, with the carboxyl termini at the upper left corner. Simulation was carried out with the wild type (WT) and A315T mutant peptides from identical initial conformation and same random number seed for the initialization of atomic velocity. The backbones of the wild type and A315T mutant peptides are shown in green and blue ribbons respectively with corresponding Ala315 or Thr315 residues marked in red. The A315T mutant shows a more extended structure at its carboxyl terminus overall and in the vicinity of the mutation site.
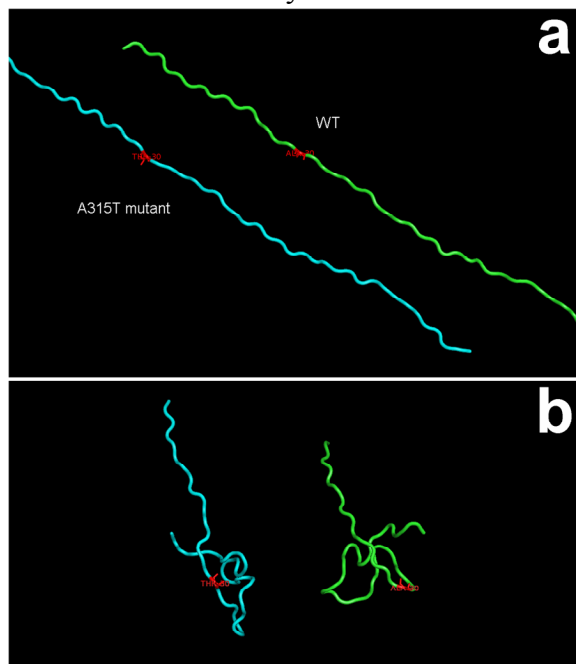
Supplementary Figure S7. A315T mutant does not affect TDP-43 mediated CFTR exon 9 skipping. A CFTR exon 9 minigene was co-transfected into HEK293 cells together with either control vector (Ctrl) or plasmids expressing HA-tagged Wt, A315T or T202 TDP-43. CFTR exon 9 alternative splicing was examined by RT-PCR. (**a**) CFTR exon 9 splicing pattern in different groups with RNA loading control shown at the bottom GAPDH panel. (**b**) The expression of transfected the Wt, A315T or T202 TDP-43 proteins as detected by Western blotting using anti-HA antibody. (**c**) Quantification of splicing regulatory activity on CFTR exon 9 skipping of the Wt or mutant TDP-43. A315T mutant TDP-43 shows similar activity to Wt TDP-43 in stimulating CFTR exon 9 skipping, whereas T202 mutant promotes CFTR exon 9 inclusion. **, $p < 0.01$ (Student's T-test).
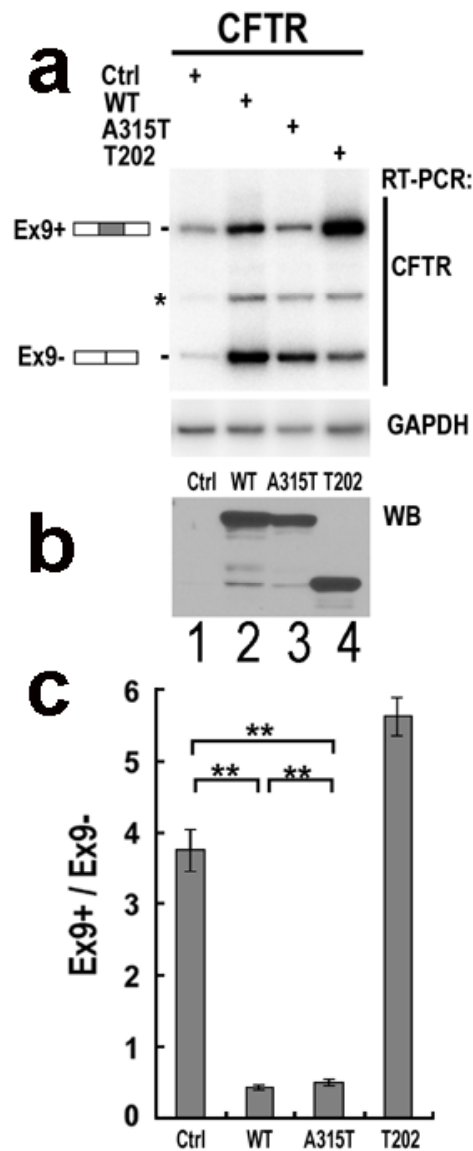
References for Supplementary Information:

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Supplementary movie. Molecular dynamics simulation suggests that the 46mer TDP-43 peptides adopt multiple conformations including collapsed globular conformation at the N-terminal half and extended β-sheet conformation at the C-terminal region, in agreement with the Protscale analyses (Fig. 6). Molecular dynamics simulations were carried out on Wt and A315T mutant TDP-43 synthetic peptide (Q286-Q331) using TINKER, a software tool for molecular design[3]. The force field used in this molecular dynamics simulation was CHARMM-19, in combination with a statistical potential of mean force DOPE (discrete optimized protein energy)[4]. Non-bonded forces (electrostatic and van der Waals) were truncated at 5Å. After the TDP peptide was energy minimized and equilibrated for 100-ps with 1-fs time step, ten (peptide dimer) or thirty (monomer) independent 100-ps trajectories were produced using different random initial conditions. The canonical ensemble simulations were kept at a constant temperature of 300K. The amino-termini of both peptides are at the lower right corner, with the carboxyl termini at the upper left corner. The backbones of the wild type and A315T mutant peptides are shown in green and blue ribbons respectively with corresponding Ala315 or Thr315 residues marked in red.