

# ALS-associated mutations in TDP-43 increase its stability and promote TDP-43 complexes with FUS/TLS

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**Dominant mutations in two functionally related DNA/RNA-binding proteins, trans-activating response region (TAR) DNA-binding protein with a molecular mass of 43 KDa (TDP-43) and fused in sarcoma/translocation in liposarcoma (FUS/TLS), cause an inherited form of ALS that is accompanied by nuclear and cytoplasmic aggregates containing TDP-43 or FUS/TLS. Using isogenic cell lines expressing wild-type or ALS-linked TDP-43 mutants and fibroblasts from a human patient, pulse-chase radiolabeling of newly synthesized proteins is used to determine, surprisingly, that ALS-linked TDP-43 mutant polypeptides are more stable than wild-type TDP-43. Tandem-affinity purification and quantitative mass spectrometry are used to identify TDP-43 complexes not only with heterogeneous nuclear ribonucleoproteins family proteins, as expected, but also with components of Drosha microprocessor complexes, consistent with roles for TDP-43 in both mRNA processing and microRNA biogenesis. A fraction of TDP-43 is shown to be complexed with FUS/TLS, an interaction substantially enhanced by TDP-43 mutants. Taken together, abnormal stability of mutant TDP-43 and its enhanced binding to normal FUS/TLS imply a convergence of pathogenic pathways from mutant TDP-43 and FUS/TLS in ALS.**

mass spectrometry | protein stability | amyotrophic lateral sclerosis | microRNA | ribonucleoproteins

**P**athological protein aggregation is one of the hallmarks of neurodegenerative diseases (1). In 2006, trans-activating response region (TAR) DNA-binding protein with a molecular mass of 43 KDa (TDP-43) was identified as a major component of ubiquitinated inclusions found in frontotemporal lobar degeneration with ubiquitin aggregates (FTLD-U) and ALS patients (2, 3). Since then, intracellular TDP-43-positive inclusions have been found in an array of neurodegenerative diseases, including Alzheimer's disease (AD), Pick's disease, various forms of Parkinson's diseases (PD), and others (4).

Starting in 2008, multiple studies identified over 30 dominant mutations in TDP-43 in both sporadic and familial ALS patients but not in other neurodegenerative diseases including AD or PD, indicating that these mutations are specific to ALS pathogenesis (4–7). This evidence has shaped an emerging TDP-43 proteinopathy hypothesis in which sequestration of nuclear TDP-43 into pathological inclusions is proposed to contribute to disease pathogenesis (8). Additionally, mutations in a second functionally related gene, fused in sarcoma/translocation in liposarcoma (FUS/TLS), were found to be linked with ALS (9, 10).

In a normal context, both TDP-43 and FUS/TLS are involved in RNA transcription and splicing regulation (4). However, how ALS-linked mutations in TDP-43 and FUS/TLS contribute to cellular toxicity is not understood. TDP-43 is thought to be predominantly a nuclear protein found in nuclear bodies (TDP bodies) that are distinct from other known nuclear structures (11). The normal functions of TDP-43 are not established, although it has been proposed that TDP-43 is involved in transcription repression (12, 13) and splicing regulation (14–16). TDP-43 was first identified as a transacting factor binding to the TAR DNA promoter region of HIV to repress the expression of the TAR gene

(13). Similarly, repression of the mouse SP-10 gene during spermatogenesis by TDP-43 was also proposed (12). Reduction of TDP-43 in cell culture leads to down-regulation of cyclin-dependent kinase 6 and histone deacetylase 6, further supporting TDP-43's role in regulating gene expression (17, 18). There is, however, growing evidence indicating that TDP-43 functions in RNA processing. In particular, presence of TDP-43 affects the exon usage of the cystic fibrosis transmembrane regulator (CFTR), apolipoprotein A-II, and survival of motor neuron (SMN) transcripts (14–16). TDP-43 interacts with heterogeneous nuclear ribonucleoproteins (hnRNP) A2/B1 and hnRNP C in vitro, and these interactions may be required for splicing site selection (19). In addition to a nuclear function, TDP-43 also colocalizes with fragile X mental-retardation protein (FMRP) and Staufien proteins in the neurites of primary neurons, which suggests a role in RNA transport and localization (20).

Whether nuclear or cytoplasmic, the RNA targets and protein interactors of TDP-43 have not yet been systematically identified, and it is not known how ALS-linked mutations in TDP-43 affect its normal function(s). In disease conditions, affected neurons typically lose nuclear TDP-43 staining, possibly before the formation of intracellular aggregates (21). In addition, TDP-43 seems to be ubiquitinated, phosphorylated, and fragmented in pathological conditions (3). Transient expression of TDP-43 fragments in mammalian cell lines and yeast have led to a widely held view that the C-terminal fragment of TDP-43 is extremely toxic (22, 23), although it remains unresolved how C-terminal fragments are generated under physiological or pathological conditions (24, 25).

Two key questions for understanding the TDP-43 proteinopathies are (i) what are the normal functions of TDP-43 and (ii) what are the acquired toxicities (gain of function) and/or perturbed normal functions (loss of function) of TDP-43 in disease conditions. Here, we address these questions by the use of site-directed recombination to produce a series of isogenic cell lines expressing a single copy gene encoding ALS-linked mutations. With these isogenic cell lines as well as fibroblasts from a human patient, we show that the mutant TDP-43 proteins are more stable than the wild-type protein. Additionally, with isotope labeling to produce quantitative mass spectrometry, we define core TDP-43 protein complexes to contain hnRNP family proteins and components of Drosha microprocessor complexes, establishing a direct link of TDP-43 to microRNA biogenesis. Furthermore, a fraction of TDP-43 interacts with FUS/TLS. Most intriguingly, FUS/TLS interacts more prominently with mutant TDP-43, even in the absence of TDP-43 nuclear aggregates. Our results suggest

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that mutations in TDP-43 perturb normal FUS/TLS function, which may be an early event before any mislocalization and aggregation, and possible convergence of pathogenic pathways in ALS by TDP-43 and FUS/TLS.

## Results

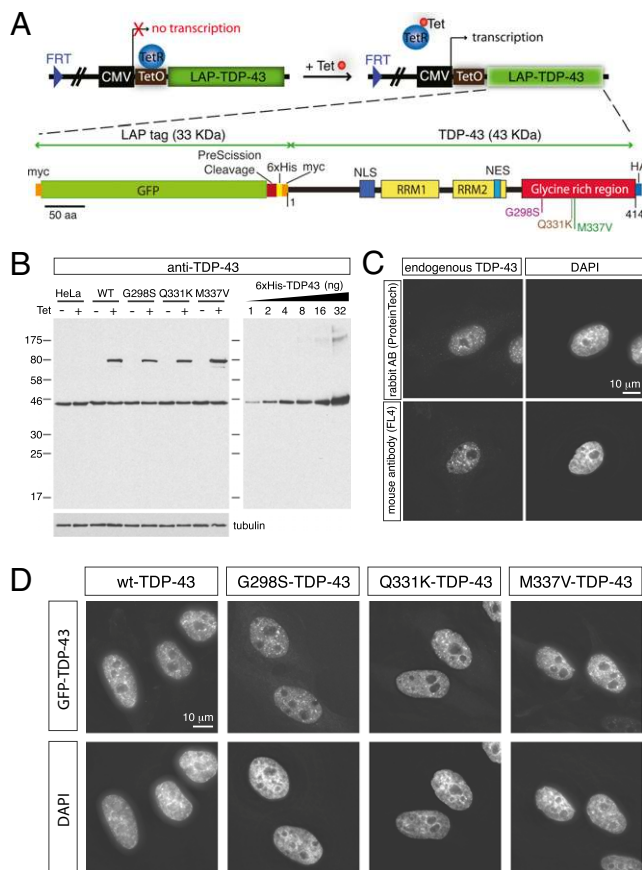
**ALS-Linked TDP-43 Mutations Exhibit Longer Protein Half-Lives.** A hallmark of TDP-43 protein pathology is intracellular inclusions of aggregated TDP-43. Neither the stability of TDP-43 nor its ALS-linked mutations have been determined. Although several prior reports using transient transfection to express abnormally high levels of TDP-43 (or portions of it) have led to the proposal that C-terminal TDP-43 fragments are toxic (22, 26), we sought to establish the half-lives of wild-type and ALS-linked mutations in TDP-43 at physiologically relevant endogenous levels. We first determined the normal abundance of TDP-43. Using immunoblotting of known amounts of recombinant full-length human TDP-43 (Fig. S1) in parallel with total protein extracts from a known number of cells, endogenous TDP-43 was identified to comprise 0.04% of total cell protein (8.3 ng per  $3 \times 10^4$  cells containing 20  $\mu$ g of total cell protein). Thus, endogenous TDP-43 corresponds to  $\sim 4 \times 10^6$  molecules per cell (Fig. 1).

To achieve similar levels of expression from wild-type or mutant-encoding transgenes, we initially used a site-directed (Flp) recombinase-based system to generate isogenic cell lines (27) in which a cytomegalovirus (CMV) promoter was used to drive the expression of tetracycline-inducible wild-type and mutant genes that were integrated at a common locus (28). Each single copy transgene of TDP-43 was multiply tagged, including a localization-affinity purification (LAP) tag (comprised of GFP and hexa-histidine tags) useful for visualization and purification (29) and additional amino and carboxyl-terminal epitope tags [myc (EQKLISSEEDL) and HA (YPYDVDPYA), respectively] (Fig. 1A). Using this approach, isogenic cell lines expressing wild-type or each of three ALS-linked mutations (G298S, Q331K, and M337V) in TDP-43 were obtained (Fig. 1).

On tetracycline addition, the full-length transgene-encoded polypeptide accumulated to a level similar to endogenous TDP-43 (Fig. 1B). Furthermore, no smaller fragments were detected for wild-type TDP-43 or any of the mutants with either myc or HA antibodies (Fig. S24), showing that none of these ALS-linked mutations generated fragments that accumulate in these cells. Moreover, except for a slight, apparent elevation in the cytoplasmic pool in some cells, LAP-tagged TDP-43 (Fig. 1D and Fig. S2) localized indistinguishably from the endogenous protein (Fig. 1C), suggesting that the LAP tag did not interfere with TDP-43 function or cause protein misfolding. At steady state, both endogenous (Fig. 1C) and LAP-tagged TDP-43 (Fig. 1D and Fig. S2) appeared as nuclear proteins and formed similar nuclear foci.

Half-lives of wild-type and TDP-43 mutations were measured in randomly cycling cells through use of short-term incubation with [<sup>35</sup>S]methionine/cysteine to radiolabel newly synthesized proteins, and the stability of the labeled proteins followed with time (30). This analysis revealed, surprisingly, that, in this *in vivo* context, the TDP-43 mutations were degraded two (for TDP-43<sup>Q331K</sup>) to four (for TDP-43<sup>G298S</sup> and TDP-43<sup>M337V</sup>) times more slowly than was wild-type TDP-43, yielding estimated half-lives for the mutants of  $\sim 24$ – $48$  h versus 12 h for wild-type TDP-43 (Fig. 2B).

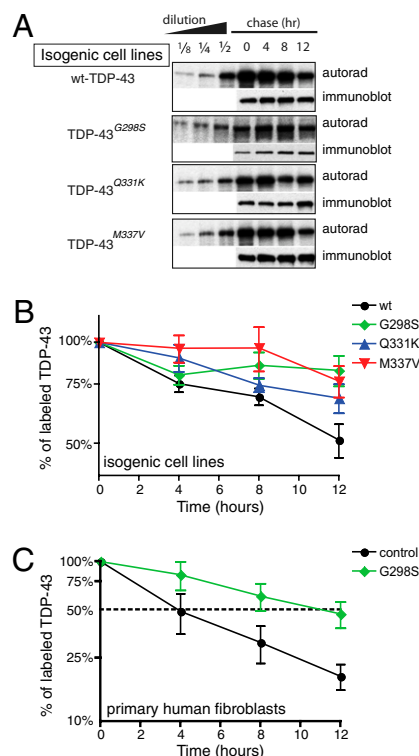
To extend this test to a more disease-relevant setting for TDP-43 half-life, we used primary fibroblasts collected from a human patient containing a dominant G298S mutation in TDP-43 in which one copy of TDP-43 carries a G to A substitution, which, in turn, leads to glycine to serine substitution (31). Analysis of pulse radiolabeling of these cells revealed that TDP-43 in wild-type fibroblasts exhibited a 4-h half-life, whereas, in cells heterozygous for one copy of the G298S mutation in TDP-43, the half-life of TDP-43 was 11 h (Fig. 2C), showing



**Fig. 1.** Characterization of isogenic cell lines expressing a single copy of wild-type and ALS-linked TDP-43 mutations. (A) Schematic representation of site-directed recombinase-based system to generate isogenic stable cell lines, in which CMV promoter was used to drive the expression of tetracycline (Tet)-inducible wild-type and mutant genes that were integrated at a common locus [Flp Recognition Target (FRT) site]. Normally, Tet repressor (TetR) binds to Tet operator (TetO), repressing transcription. On addition, binding of Tet to TetR induces a conformation change and releases TetR from TetO, allowing transcription to start. Lower shows the LAP tag of TDP-43. The LAP tag is composed of GFP followed by PreScission protease cleavage sequences and 6 $\times$  histidine tag. TDP-43 is tagged at the N terminus with myc peptide (EQKLISSEEDL) and at the C terminus with HA peptide (YPYDVDPYA). Three different mutations, G298S, Q331K, and M337V, were used in this study. (B) Expression of transgene in isogenic stable cell lines. The transgenes are under TetR control. The transgenes express on incubating with tetracycline (–, without tetracycline; +, with tetracycline); 20  $\mu$ g total cell extract was loaded for each lane. Recombinant TDP-43 of known amount is loaded for the quantification, and tubulin is used as loading control. Exposure shown was taken on the same blot for quantification. (C) Immunofluorescence images of TDP-43 in HeLa cells. Both rabbit polyclonal antibody (ProteinTech) and mouse monoclonal antibody (FL4) showed similar staining pattern for nuclear TDP bodies. (D) Fluorescent images of isogenic cell lines. Upper is GFP signal, and Lower is DAPI-stained to mark the nucleus. All LAP-tagged TDP-43 form nuclear speckles that are similar to immunofluorescence images of endogenous TDP-43 (C). (Scale bar, 10  $\mu$ m.)

a 2.8-fold slower turnover rate for wild-type plus mutant TDP-43 in these cells.

**TDP-43 Associates with hnRNP Complexes and microRNA Processing Machinery.** It has been shown that TDP-43 interacts with hnRNP A2/B1 and hnRNP C *in vitro* using a blot-overlay assay (19). In addition, using yeast two-hybrid screening, TDP-43 was identified as a putative direct interactor with the Xrn2 (5'  $\rightarrow$  3' exonuclease) involved in RNA degradation (32). Additionally, 261 putative



**Fig. 2.** ALS-linked TDP-43 mutations exhibit longer half-lives. (A) Representative autoradiogram and immunoblots of the pulse-chase assay using isogenic cell lines expressing wild type (wt), G298S, Q331K, and M337V mutations in TDP-43. LAP-tagged wt-TDP-43 and its mutants were immunoprecipitated and run on SDS-PAGE for autoradiography and immunoblotting. Serial dilutions of 0-h point (start of chase) were used to monitor the linearity of the autoradiograph signal. The autoradiograph signals were normalized to the immunoblotting signals and plotted using 0 h as 100%. (B) Half-lives of LAP-tagged TDP-43 and its ALS-linked mutations ( $n = 5$ ). Error bar represents SEM. (C) Half-lives of TDP-43 in primary human fibroblasts. Wild-type TDP-43 exhibits 4-h half-life, whereas TDP-43 in heterozygote harboring a copy of G298S mutant exhibits 11-h half-life ( $n = 5$ ). Error bar represents SEM.

TDP-43 interacting proteins have been proposed from an approach using a single-step immunoprecipitation from transiently transfected cells (33), albeit many of these are probably abundant, contaminant proteins rather than actual interactors. Indeed, eukaryotic translation elongation and initiation factors and ribosomal proteins, factors known to be common contaminants for their binding to affinity matrices (34), were among these proposed TDP-43 interactors (33).

To identify specific interactors, even those in low abundance, while eliminating abundant contaminant proteins, we combined (i) tandem-affinity purification (TAP) with two sequential affinity purification and elution schemes (29, 35) and (ii) quantitative mass-spectrometry analysis using stable isotope labeling by amino acids in cell culture (SILAC) (36). A typical SILAC-TAP experiment is outlined in Fig. 3A. Cell lines stably expressing the LAP double-affinity tag containing wild-type TDP-43 were grown in isotopically heavy medium containing  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ -arginine, and  $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ -lysine, whereas the parental line (i.e., no transgene) was grown in light medium containing normal arginine and lysine. Immunoblotting with an antibody recognizing both endogenous and transgene-encoded TDP-43 revealed that tagged TDP-43 was associated with endogenous TDP-43 throughout the purification (Fig. 3B). From the ratio of 1.6:1 molecules of transgene-encoded and endogenous TDP-43 in the initial extract, the first GFP immunoprecipitation step produced a 4:1 ratio. A comparable ratio

carried through the first elution and subsequent second affinity steps (Fig. 3B), showing that (i) tagged TDP-43 forms a co-complex with native TDP-43 and (ii) endogenous TDP-43 is present in complexes with more than one TDP-43 molecule.

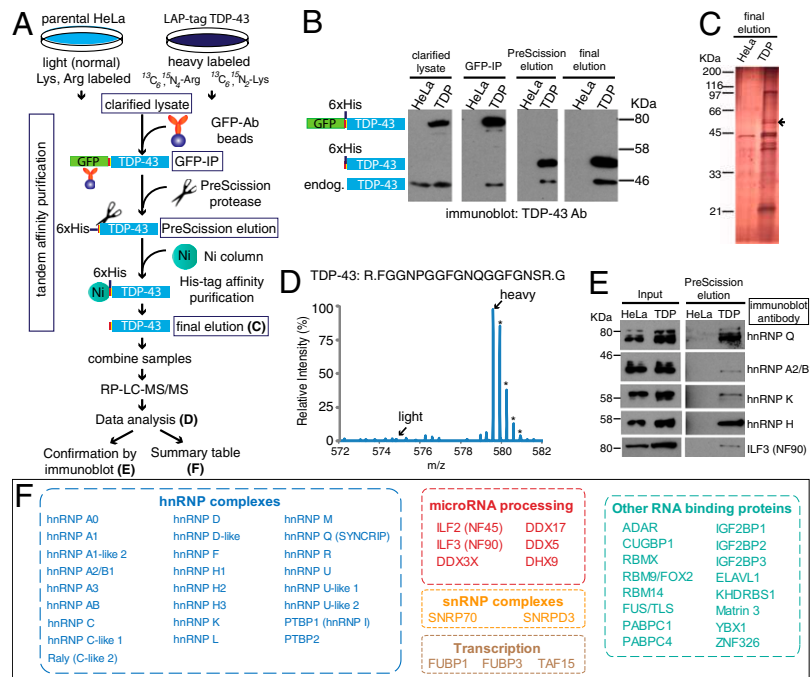
Silver staining revealed that the final eluates after tandem-affinity chromatography contained several polypeptides in addition to tagged TDP-43 (Fig. 3C). To identify these TDP-43-related proteins, quantitative mass spectrometry was used. The spectrum for a representative peptide is shown as Fig. 3D (see also Fig. S3). Each peptide yielded a characteristic spectrum of monoisotopic distributions of mass to charge species ( $m/z$ ) as expected from the natural abundances of isotopes of  $^{13}\text{C}$  and  $^{15}\text{N}$ . Stringent selection criteria for TDP-43-associating proteins were defined as: (i) enrichment of all peptide signals to at least 8-fold or higher compared with the TAP control (purification from the parental HeLa cell line), (ii) all proteins must be identified with more than one unique peptide detected, and (iii) only proteins identified in at least two independent runs were retained.

All of the proteins whose peptides were found to be TDP-43-associated in the SILAC mass-spectrometric analyses, the TDP-43 interactome, are summarized in Fig. 3F. TDP-43 was identified to be associated with the majority of the known hnRNP proteins (A0, A1, A2/B1, C, D, F, H1, H2, H3, I, K, L, M, Q, R, and U), consistent with TDP-43 as an integral component of hnRNP complexes. Several of the protein partners found by SILAC mass spectrometry were subsequently confirmed by immunoblotting, including the hnRNP components, hnRNP A2/B1, hnRNP Q, hnRNP K, and hnRNP H. In addition, TDP-43 was associated with multiple RNA-binding proteins previously implicated in other human diseases. These included CUG-BP1 (involved in myotonic dystrophy) (37) and perhaps of highest interest, FUS/TLS, another ALS-linked DNA/RNA-binding protein (see below, *Preferential ALS-Linked Mutant TDP43 Association with FUS/TLS*).

Our analysis also identified a second major complex with which TDP-43 was associated: the Drosha microprocessing complex, whose action is essential for microRNA biogenesis. Drosha components identified within the TDP-43 complexes included interleukin-enhancer binding factor 2/nuclear factor 45 kDa (ILF2/NF45), interleukin-enhancer binding factor 3/nuclear factor 90 kDa (ILF3/NF90), DEAD (Asp-Glu-Ala-Asp) box polypeptide 17, also known as p72 (DDX17), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 5, also known as p68 (DDX5). All of these latter components were previously found by Gregory et al. (38) after affinity purification of epitope-tagged Drosha. ILF3/NF90 was confirmed to be TDP-43-associated by immunoblotting of the PreScission elution of purified TDP-43 (Fig. 3E). Thus, our SILAC mass-spectrometric data show TDP-43 association with complexes that mediate microRNA biogenesis in addition to proposed functions in transcription repression and splicing regulation.

#### Preferential ALS-Linked Mutant TDP43 Association with FUS/TLS.

Discovery of FUS/TLS, another ALS-linked gene product, in TDP-43 complexes raised the possibility of a common link underlying disease pathogenesis. We first tested an association of endogenous FUS/TLS and TDP-43 by an additional immunoprecipitation for TDP-43. Approximately 20% of TDP-43 was successfully precipitated along with <1% of endogenous FUS (Fig. 4A). Reciprocal FUS immunoprecipitation followed by immunoblotting for TDP-43 confirmed an interaction, albeit only with a very small proportion of FUS/TLS. Ten percent of endogenous FUS/TLS was successfully precipitated, but, as judged by the input and amount of protein pull down, a much smaller percentage (<1%) of TDP-43 was coprecipitated. To test if the FUS/TLS interaction with TDP-43 was affected by ALS-linked mutation in TDP-43, we used GFP-affinity purification with PreScission elution of extracts from our isogenic cell lines expressing LAP-tagged wild-type or mutant TDP-43. Strikingly,



**Fig. 3.** Quantitative proteomic analysis of TDP-43 using SILAC coupled with TAP. (A) Schematic representation of TAP purification and quantitative analysis using SILAC. (B) Samples from key steps (clarified lysate, immunoprecipitation for GFP (GFP-IP), PreScission elution, and final elution) in purification were probed with TDP-43 antibody, showing that tagged TDP-43 associates and copurifies with endogenous TDP-43. (C) Silver stain of the samples from the final elution of TAP step. Arrow indicates tagged TDP-43. (D) Representative mass spectrum for TDP-43 peptide. The corresponding light isotope-containing peptide is below detection limit, whereas the heavy Lys/Arg-containing peptide is detected. Asterisks indicate the natural occurrences of  $^{13}\text{C}/^{15}\text{N}$  in the peptide, as revealed by high resolution mass spectrometry. (E) Confirmation of putative TDP-43-associated proteins by immunoblotting, including hnRNP Q, hnRNP A2/B1, hnRNP K, hnRNP H, and interleukin-enhancer binding factor 3/nuclear factor 90 kDa (ILF3/NF90). (F) Summary for putative TDP-43-associated proteins. TDP-associated proteins were identified with peptides containing only heavy Lys/Arg and by multiple different peptides from at least two independent runs. The associated proteins were grouped according to the known assigned functions for the proteins.

although the distribution of FUS/TLS seemed normal in the presence of mutant TDP-43 (Fig. S4), the association of FUS/TLS with TDP-43 was sharply enhanced for both TDP43<sup>Q331K</sup> and TDP43<sup>M337V</sup> ALS-linked mutations (Fig. 4B).

To further confirm the association between TDP-43 and FUS/TLS and to eliminate the possibility that a TDP-43 and TLS/FUS interaction was an artifact arising during cell lysis (39), we used an in vivo, in situ proximity ligation assay (Fig. 4C). In this assay, cell membranes are gently lysed, primary antibodies against wild-type or mutant TDP-43 (in this case, we used a myc antibody recognizing LAP-tagged TDP-43) and FUS/TLS were added, much as is done in conventional immunofluorescence. Instead of fluorescence-conjugated secondary antibodies, however, two different oligonucleotides were linked to the secondary antibodies (one for each of the two different primary antibodies). If the distance between the two different oligonucleotides is less than 50 nm, they can be hybridized and used as primers for rolling-circle amplification. Subsequently, fluorescent-labeled oligonucleotides were then hybridized with the amplification products, and the signals were observed with a fluorescence microscope. A combination of anti-myc antibody and rabbit IgG molecules was used as negative control, whereas a combination of anti-myc and anti-TDP-43 antibodies was used as positive control. Although FUS/TLS was seen to be associated with wild-type TDP-43 in only a small proportion (10%) of cells (consistent with the immunoprecipitation evidence above) (Fig. 4D), intranuclear proximity signals to FUS/TLS were observed in ~40% cells expressing either of two ALS-linked TDP-43 (TDP-43<sup>Q331K</sup> and TDP-43<sup>M337V</sup>).

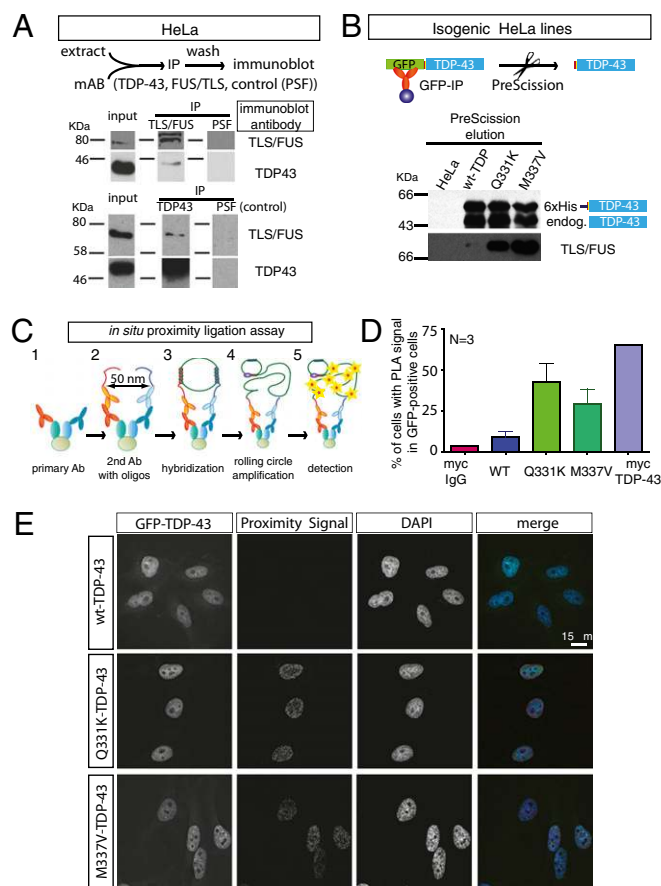
## Discussion

A key question in understanding how ALS-linked dominant mutations in TDP-43 cause cellular toxicity is if, and if so how,

these point mutations alter the normal function of TDP-43. In contrast to Cu/Zn superoxide dismutase 1 (SOD1) where ALS-linked mutations destabilize the mutant protein (30), using isogenic stable cell lines expressing a single copy of each transgene (wild type, TDP43<sup>G298S</sup>, TDP43<sup>Q331K</sup>, and TDP43<sup>M337V</sup>), we showed that all three of these ALS-linked mutations exhibit longer protein half-lives compared with wild-type protein, suggesting that abnormal stability may be a common feature for ALS-linked TDP-43 mutations. Furthermore, we showed that one of the ALS-linked TDP-43 mutants (G298S) generates higher TDP-43 stability in primary fibroblasts from a human patient (Fig. 2C), where only one copy of the *TARDBP* gene is mutated and under the authentic promoter. This highly unexpected discovery suggests that an inherently increased half-life may be, or at least may contribute to, the underlying mechanism for the accumulation of TDP-43 aggregations found in ALS patients.

Perhaps even more importantly, we have shown that a significantly higher proportion of endogenous, wild-type FUS/TLS is associated with both of two ALS-linked mutations tested (TDP43<sup>Q331K</sup> and TDP43<sup>M337V</sup>). This interaction is exclusively intranuclear but without apparent nuclear aggregation. Taken together, our findings imply that the increased association between mutant TDP-43 and FUS/TLS may be driven, in part, by the increasing stability of mutant TDP-43 (Fig. S5). Conceivably, this aberrant association caused by the dominant mutations in TDP-43 could lead to potential perturbations of the normal functions of both TDP-43 and FUS/TLS, suggesting a possible convergence of pathogenic pathways in ALS by TDP-43 and FUS/TLS.

Interestingly, familial PD-linked A53T substitution of  $\alpha$ -synuclein also shows increased stability, which, in turn, probably contributes to the age-dependent accumulation of mutant  $\alpha$ -synuclein in transgenic



**Fig. 4.** ALS-linked TDP-43 mutations associate with FUS/TLS. (A) Reciprocal immunoprecipitation shows that TDP-43 interacts with FUS/TLS using HeLa cells. A monoclonal antibody against splicing factor proline/glutamine-rich or polypyrimidine tract binding protein-associated splicing factor (SFPQ/PSF) was used for control. (B) PreScission elution fractions from GFP pull-down samples showed that FUS/TLS associates more prominently with Q331K and M337V mutations in TDP-43. (C) Schematic representation of in situ proximity ligation assay. (D) Quantification of in situ proximity ligation assay (PLA) results ( $n = 3$ ; cell numbers > 100 per experiment). Error bar represents SEM. A combination of anti-myc antibody and rabbit IgG molecules was used as negative control, whereas a combination of anti-myc and anti-TDP-43 antibodies was used as positive control. The monitored signal used a combination of anti-myc and anti-TLS/FUS antibody. (E) Representative results for in situ proximity ligation assay. (Scale bar, 15  $\mu\text{m}$ .)

mice expressing mutant  $\alpha$ -synuclein (40). Prolonged stability could give rise to at least two additional nonmutually exclusive effects on TDP-43: (i) permitting additional or aberrant posttranslational modifications, such as phosphorylation and ubiquitinylation, which were reported in disease conditions (3, 41) and/or (ii) permitting aberrant interactions with other proteins, such as with FUS/TLS reported in this study (Fig. 4). It will now be important to determine whether ALS-linked TDP-43 mutations show age-dependent accumulation and mutant-specific interactions in genetically engineered animals, such as transgenic mice (42). Additionally, because accumulation is the net balance between synthesis and degradation, it will be essential to determine how TDP-43 is degraded and whether the ALS-linked mutations cause any defect in the degradation process. Although recent evidence indicates that TDP-43 may be degraded through either autophagosome and/or proteasome pathways (43, 44), it is not clear whether one of the pathways is the major default path. Understanding the degradation process may aid the design of future therapeutic interventions, because in-

tracellular TDP-43 inclusions are hallmarks of various neurodegeneration diseases.

The major advantage of using quantitative mass-spectrometry analysis is that the common and abundant contaminant proteins can be easily eliminated and low abundant but specific interactors can be readily identified (45). Using the SILAC-TAP approach, we identified the stable core component for TDP-43 complexes. The majority of the TDP-43-associating proteins are hnRNP family proteins, indicating that TDP-43 is an integral part of hnRNP complexes, which associate with nascent transcripts and influence their fate (46). The biochemical findings are consistent with TDP-43's role in RNA transcription and processing and complement a recent ultrastructural study showing that TDP-43 is enriched in perichromatin fibrils, nuclear sites of transcription, and cotranscriptional splicing (47). Beside hnRNPs, several additional RNA-binding proteins were identified within TDP-43 complexes, including RBM9 (or FOX2) and CUG-BP1 (Fig. 3F). Mice overexpressing CUG-BP1 in muscles reproduce the pathological features found in myotonic dystrophy patients accompanied by disrupted normal splicing patterns (37). Similarly, RBM9/FOX2 influences splicing-site usages by positioning near the exon-intron junctions in embryonic stem cells (48). These findings are consistent with a proposed role of TDP-43 in splicing regulation.

Several TDP-43 interacting proteins were found to be components of Drosha microRNA processing complexes, including ILF2/NF45, ILF3/NF90, DDX5, DDX17, and DDX3X (38). The overlapping components between epitope-tagged Drosha (38) and TDP-43 (in this study) strongly suggest that TDP-43 is involved in microRNA biogenesis. Indeed, NF45/NF90 has been shown to complex with pre-miRNAs, and this association reduces the generation of mature miRNAs (49). In addition, adenosine deaminases (ADARs), found to bind to TDP-43 in our proteomic study, are known to influence microRNA processing through their editing activities (50). Defects in glutamine (Q) to arginine (R) substitution of glutamate AMPA receptors by ADAR-mediated RNA editing have been linked to sporadic ALS (51), suggesting yet another potential pathogenic mechanism for TDP-43. Indeed, a recent study showed reduced ADAR immunoreactivity in ALS is accompanied by presence of phosphorylated (pathological) TDP-43 in ALS patients (52). Taken together, TDP-43 may be involved in both RNA transcription and processing through its interaction with hnRNP complexes and microRNA biogenesis by its association with micro-processing complexes (Fig. S5). Because gene expression is coordinated and coupled through interconnected multicomponent machineries (53), it is tempting to speculate that TDP-43 may coordinate and regulate mRNA processing with microRNA biogenesis pathway and through this linkage, regulate expression of various transcripts (54, 55).

Lastly, FUS/TLS, another ALS-linked protein (9, 10), was found associated with TDP-43. Although less than 1% of wild-type FUS/TLS interacts with wild-type TDP-43, the association is strongly enhanced by ALS-linked mutations (Fig. 4). It is tempting to speculate that dominant mutations in TDP-43 may perturb normal FUS/TLS function, thus providing possible convergence of pathogenic pathways in ALS by mutant TDP-43 and FUS/TLS. Now needed are efforts to determine whether the increasing association between mutant TDP-43 and FUS/TLS shown here affects the RNA targets for TDP-43, FUS/TLS, or both.

## Materials and Methods

**In Situ Proximity Ligation Assay.** In situ proximity ligation assays were done following manufacture's protocol (Olink Bioscience, Sweden). Anti-myc (clone 4A6, BD Bioscience), which detected LAP-tag TDP-43, was paired with anti-TDP43 (ProteinTech), anti-TLS/FUS (Aviva) and purified rabbit IgG (Sigma-Aldrich) for the primary antibodies. Anti-myc and anti-TDP43 pair serves as a positive control, and anti-myc and purified rabbit IgG as a negative control. Detailed methods for plasmids and recombinant protein puri-

fication, cell culture and creations of isogenic stable cell lines, pulse-chase assay, tandem-affinity purification and quantitative mass-spectrometry analysis using SILAC, immunoprecipitation, immunofluorescence, immunoblotting, and antibodies are described in *SI Materials and Methods*.

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# Supporting Information

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## SI Materials and Methods

**Plasmids and Recombinant Protein Purification.** A localization and affinity purification tag (LAP) contains GFP followed by PreScission protease cleavage sites, and 6× histidine was inserted into pcDNA5/TO/FRT/myc (1) to generate pcDNA5/TO/FRT/LAP. Human trans-activating response region (TAR) DNA-binding protein with a molecular mass of 43 kDa (TDP-43) cDNA, which was epitope-tagged with an N-terminal myc peptide (EQKLISSEEDL) and C-terminal HA-peptide (YPYDVPDYA; C. Shaw, King's College London, London, UK), was amplified by PCR and inserted into pcDNA5/TO/FRT/LAP using BamH-I and Not-I sites. ALS-linked mutations were generated by QuikChange mutagenesis (Stratagene) and confirmed by sequencing the entire ORF. For making recombinant protein, full-length (1–414 amino acids) or the first 251 amino acids (NT1–251) or the 164 carboxy terminal amino acids (CT251–414) fragment of human TDP-43 were amplified by PCR and cloned into pQE80 plasmids (Qiagen) with BamH-I and Xho-I sites to generate an N-terminal 6× histidine fusion tag. The recombinant proteins were purified using Ni-NTA following the manufacturer's protocol (Qiagen).

**Cell Culture and Creations of Isogenic Stable Cell Lines.** In brief, a single Flp recognition target (FRT) site was stably integrated in the HeLa cell genome (Flp-In TRex-HeLa cells) (2), and the TDP-43 gene was subsequently inserted into the FRT locus by flp recombinase-mediated recombination. In contrast to the traditional way of making stable cell lines by random insertion of plasmids into the host genome, this methodology produces cell lines that differ only in the ectopic expression of the target genes in otherwise the same genomes and hence, isogenic. Isogenic cell lines were grown at 37 °C and 5% CO<sub>2</sub> in DMEM supplemented with 10% tetracycline-free FBS and penicillin/streptomycin. To establish isogenic stable cell lines expressing a single copy of wild-type or ALS-linked mutations in TDP-43, a 10-fold amount of pOG44, the plasmid expressing flp recombinase, was mixed with a 1-fold amount of pcDNA5-FRT-TO containing LAP-tagged TDP-43 with FuGene 6 (Roche) following the manufacturer's protocol. After selection in 250 µg/mL hygromycin, colonies were pooled and expanded. To express LAP-tagged TDP-43, cells were treated with 1 µg/mL tetracycline for 16–24 h. Primary human fibroblasts were maintained at 37 °C and 5% CO<sub>2</sub> in DMEM supplemented with 20% FBS, 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate, and penicillin/streptomycin.

**Pulse-Chase Assay.** Isogenic stable cell lines expressing LAP-tagged wild-type TDP43, G298S, Q331K, and M337V mutations were seeded at  $5 \times 10^5$  cells in 60-mm dishes 2 d prior, and 1 µg/mL tetracycline was added to the cells for 18 h before labeling and was included throughout the experiments. Cells were washed with PBS and incubated with methionine- and cysteine-free DMEM (Invitrogen) supplemented with 5% dialyzed FBS (Invitrogen) for 30 min before labeling with 500 µCi of S<sup>35</sup>-methionine/cysteine (Perkin-Elmer) for another 30 min. Cells were washed with PBS and then replaced with DMEM supplemented with 10% FBS, 2 mM methionine, and 2 mM cysteine to begin the chase period. Cells were collected at 0, 4, 8, and 12 h after the chase period began. Cells were lysed with RIPA buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1%, Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM DTT supplemented with complete protease inhibitors and PhosSTOP (Roche)], and the resulting extracts were spun at 16,000 × g for 10 min at 4 °C; 20 µL

of GFP-binder resin (3) were added to the resulting supernatant and incubated for 2 h at 4 °C. The IP samples were washed three times with RIPA buffer and resuspended in SDS-PAGE sample buffer. Two identical gels were loaded for autoradiography and immunoblot for quantification. The same pulse-chase procedure was done for the human fibroblasts, except that the fibroblasts were seeded at a higher density ( $1 \times 10^6$  cells) because of the slower growth. To ensure that both wild-type and G298S mutants of TDP-43 can be immunoprecipitated, we pooled two monoclonal antibodies that we generated (FL4 and FL9) for immunoprecipitation (Fig. S1).

**Tandem-Affinity Purification and Quantitative Mass-Spectrometry Analysis Using Stable Isotope Labeling by Amino Acids in Cell Culture.** For quantitative mass-spectrometry analysis, cells were grown in stable isotope labeling by amino acids in cell culture (SILAC) DMEM (Thermo Scientific) supplemented with 10% dialyzed FBS and penicillin/streptomycin with 0.4 mM L-arginine and 0.8 mM L-lysine. For parental Flp-In TRex-HeLa cell line, normal (light) L-arginine (69 µg/mL) and L-lysine (117 µg/mL) were added to the growth medium; for stable cell lines expressing LAP-tag TDP-43, heavy L-Arg-<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>4</sub>.HCl (88 µg/mL) and L-Lys-<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub>.HCl (152 µg/mL) were added. Cells were passaged in SILAC media for at least 5–6 doubling times to ensure complete incorporation of isotopic amino acids (4). Cells were harvested, and cell extracts were prepared as described previously with the following modifications (5). Cells were lysed in lysis buffer [50 mM HEPES, pH 7.5, 150 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 1 mM DTT, 50 µM latrunculin B, 50 µM cytochalasin D, 20 U/mL SUPERase-In (Ambion) supplemented with complete protease inhibitor and PhosSTOP (Roche)] and sonicated to obtain total cell extracts. Clarified lysates or high-speed supernatants (HSS) were prepared by spinning the total cell extract at 100,000 × g for 20 min at 4 °C. The protein concentrations of HSS were measured using the bicinchoninic acid (BCA) assay (Thermo Scientific) and normalized for both light and heavy samples. The typical concentration for HSS was 30–60 mg/mL, and 20 mg of total proteins were used for tandem-affinity purification. GFP immunoprecipitation was carried out with 1/20 volume (to HSS) of GFP binder at 4 °C for 2 h (3). PreScission protease was added to liberate TDP-43 and its associated proteins from the GFP-binder beads. The eluates were then incubated with Ni-NTA (Qiagen) to capture the 6× histidyl moiety of tagged TDP-43. After extensive washes, TDP-43 and its associating proteins were eluted with 50 mM Tris, pH 7.5, 150 mM KCl, and 100 mM EDTA with or without 8 M urea. The sample was prepared as described previously with the following modifications (6). The MS/MS data were collected by an LTQ Orbitrap Discovery and subsequently searched on Sorcerer-SEQUEST using a semitryptic monoisotopic database generated for the human IPI database, version 3.47. A 20-ppm parent mass tolerance and variable modification for lysine and arginine were included in the search. The searched data were then analyzed by TPP.

**Immunoprecipitation, Immunofluorescence, Immunoblotting, and Antibodies.** Cells ( $2 \times 10^7$ ) were lysed with 1 mL lysis buffer [50 mM HEPES, pH 7.5, 150 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 1 mM DTT, 5 µM latrunculin B, 5 µM cytochalasin D, 20 U/mL SUPERase-In (Ambion) supplemented with complete protease inhibitor and PhosSTOP (Roche)] and spun at 16,000 × g for 10 min at 4 °C. The resulting

supernatant was precleared with anti-mouse IgG-agarose (Sigma-Aldrich) for 1 h at 4 °C and then incubated for 2 h at 4 °C with anti-TDP-43 (FL4 and FL9, mouse monoclonal antibodies), anti-fused in sarcoma/translocated in liposarcoma (FUS/TLS; clone 4H11; Santa Cruz), and anti-SFPQ/PSF (splicing factor proline/glutamine-rich, or polypyrimidine tract binding protein associated splicing factor, clone B92; Sigma-Aldrich), which were preincubated with anti-mouse IgG-agarose for 1 h. The beads were washed three times with wash buffer (50 mM HEPES, pH 7.5, 150 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1% Nonidet P-40, 10% glycerol) and resuspended in SDS-PAGE sample buffer.

For immunofluorescence analysis of cultured cells, isogenic cell lines expressing wild-type and mutant TDP-43 cells were grown on hydrochloric acid-washed poly-L-lysine (Sigma-Aldrich)-coated 12-mm glass coverslips. Transgene expression was induced by adding 1 µg/mL tetracycline 18 h before fixation. Cells were fixed with 3.7% formaldehyde and 4% sucrose in 1× PBS for 15 min at room temperature. After washing with 1× PBS, cells were permeabilized and blocked with immunofluorescence (IF)-wash buffer (5% newborn calf serum, 0.1% Triton X-100 in 1× Tris buffered saline [TBS]) for 30 min. Primary antibodies were diluted with IF-wash buffer, anti-TDP-43 (1:200; ProteinTech),

anti-myc (clone 4A6, 1:750; BD bioscience), anti-HA (1:200; Covance), and anti-TLS/FUS (clone 4H11, 1:50; Santa Cruz), incubated for 1 h, and subsequently, detected with FITC- or TexasRed-conjugated secondary antibodies (1:200; Jackson ImmunoResearch). Nuclei were counterstained with DAPI, and coverslips were mounted with ProLong anti-fade agent (Invitrogen). Images of fixed cells were acquired using a 60× or 100× oil objective on a DeltaVision-modified inverted microscope (IX70; Olympus) using SoftWoRx software (Applied Precision) and were deconvolved.

Standard protocol for SDS-PAGE and immunoblotting was described previously (7). The following primary antibodies were used at the indicated dilutions: mouse anti-TDP-43 (clone FL9, 1:1,000), rabbit anti-TDP-43 (10782, 1:2,000; ProteinTech), mouse anti-HA (1:2,000; Covance), mouse anti-myc (clone 4A6, 1:2,000; BD Bioscience), mouse anti-hnRNP A2/B1 (clone DP3B3, 1:1,000; Sigma-Aldrich), mouse anti-hnRNP Q (clone 18E4, 1:2,500; Sigma-Aldrich), rabbit anti-hnRNP K (1:2,000; Bethyl), and rabbit anti-hnRNP H1 (1:2,000; Bethyl). The immunoreactivity was revealed using an appropriate peroxidase-conjugated anti-mouse IgG (1:10,000; GE Healthcare), anti-rabbit (1:10,000; GE Healthcare), and the chemiluminescent system (Thermo Scientific).

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