

## A90V TDP-43 variant results in the aberrant localization of TDP-43 in vitro

Matthew J. Winton<sup>a,1</sup>, Vivianna M. Van Deerlin<sup>a,b,c,1</sup>, Linda K. Kwong<sup>a</sup>, Wuxing Yuan<sup>a</sup>, Elisabeth McCarty Wood<sup>a</sup>, Chang-En Yu<sup>d,e</sup>, Gerard D. Schellenberg<sup>d,e</sup>, Rosa Rademakers<sup>f</sup>, Richard Caselli<sup>g</sup>, Anna Karydas<sup>h</sup>, John Q. Trojanowski<sup>a,b,c</sup>, Bruce L. Miller<sup>h</sup>, Virginia M.-Y. Lee<sup>a,b,c,\*</sup>

<sup>a</sup> Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

<sup>b</sup> Alzheimer's Disease Core Center, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

<sup>c</sup> Institute on Aging, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

<sup>d</sup> Geriatric Research Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle Division, WA 98108, USA

<sup>e</sup> Department of Medicine, Division of Gerontology and Geriatric Medicine, University of Washington, WA 98195, USA

<sup>f</sup> Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, 4500 San Pablo Road, Jacksonville, FL 32224, USA

<sup>g</sup> Department of Neurology, Mayo Clinic and Arizona Alzheimer's Disease Center, Scottsdale, AZ 85259, USA

<sup>h</sup> Department of Neurology, University of California at San Francisco, CA 94117, USA

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**Abstract** TAR DNA-binding protein-43 (TDP-43) is a highly conserved, ubiquitously expressed nuclear protein that was recently identified as the disease protein in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS). Pathogenic TDP-43 gene (*TARDBP*) mutations have been identified in familial ALS kindreds, and here we report a *TARDBP* variant (A90V) in a FTLD/ALS patient with a family history of dementia. Significantly, A90V is located between the bipartite nuclear localization signal sequence of TDP-43 and the in vitro expression of TDP-43-A90V led to its sequestration with endogenous TDP-43 as insoluble cytoplasmic aggregates. Thus, A90V may be a genetic risk factor for FTLD/ALS because it predisposes nuclear TDP-43 to redistribute to the cytoplasm and form pathological aggregates.

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### 1. Introduction

Frontotemporal lobar degeneration (FTLD) refers to a clinically, genetically and pathologically heterogeneous group of neurodegenerative diseases that account for up to 20% of presenile dementia cases. Clinically, FTLD is characterized by behavioral and/or language dysfunction [1–3], but it also can

co-occur with movement abnormalities such as parkinsonism and motor neuron disease, including the most common type, amyotrophic lateral sclerosis (ALS) [4,5]. Although neurodegenerative tauopathies account for many familial and sporadic cases of FTLD, those cases with ubiquitin-positive tau- and  $\alpha$ -synuclein-negative inclusions (UBIs) make up the most common neuropathological subtype of FTLD, i.e. FTLD-U [6,7]. Recently, the transactive response (TAR) DNA-binding protein 43 (TDP-43) was identified as the major disease protein in UBIs that accumulate in the central nervous system (CNS) of patients with FTLD-U as well as in patients with sporadic and familial ALS [8], but not in the majority of patients with familial ALS (FALS) due to *SOD-1* gene mutations [8–12]. These data provided compelling evidence that FTLD-U and ALS represent a clinicopathological spectrum of the same neurodegenerative disorder, i.e. TDP-43 proteinopathy, and this view is supported by the recent detection of several pathogenic *TARDBP* mutations in a number of FALS kindreds [13–16]. TDP-43, encoded by the *TARDBP* gene on chromosome 1, is a highly conserved, ubiquitously expressed nuclear protein implicated in repression of gene transcription, inhibition of exon splicing and interactions with splicing factors and nuclear bodies [17,18]. Under physiological conditions, TDP-43 is predominately localized to the nucleus; however, pathological TDP-43 forms inclusions in neuronal perikarya and neurites, suggesting that the redistribution and sequestration of TDP-43 in the cytoplasm is a pathogenic mechanism [8]. Moreover, we have identified a specific bipartite nuclear localization signal (NLS) sequence in the amino terminal domain of TDP-43 that is required for nuclear targeting [19]. Over-expression of TDP-43 with mutated NLS sequence ( $\Delta$ NLS mutants) not only re-directed TDP-43 to the cytoplasm, but also reduced its solubility, resulting in the accumulation of cytoplasmic aggregates, high Mr smears and C-terminal fragments of TDP-43 similar to those seen in FTLD-U/ALS cases [19]. Thus, these data imply that perturbation of nuclear and cytoplasmic trafficking of TDP-43 leads to the formation of cytoplasmic aggregates with morphological

\*Corresponding author. Address: Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.

E-mail address: [vmylee@mail.med.upenn.edu](mailto:vmylee@mail.med.upenn.edu) (V.M.-Y. Lee).

<sup>1</sup>These authors contributed equally to this study.

similarities to authentic TDP-43 pathology in FTLD-U/ALS [19]. Therefore, to demonstrate physiological relevance of increased cytoplasmic TDP-43 as a potential pathogenic mechanism, we investigated whether genetic variant(s) with defective *TARDBP* NLS sequences are found in FTLD patients with or without ALS and if such variant(s) perturbed TDP-43 distribution in the nucleus and cytoplasm.

## 2. Methods

### 2.1. DNA sequencing

Genomic DNA was extracted from blood of living patients or from postmortem brains using standard methods (Qiagen Inc., Valencia, CA). The *TARDBP* gene was screened for mutations in a cohort of patients from University of California at San Francisco (UCSF). This included 134 patients with clinical FTLD or FTLD/ALS, as well as in autopsy cases with confirmed TDP-43 pathology and neuropathological diagnoses of ALS ( $n = 2$ ) and FTLD-U or FTLD plus MND ( $n = 12$ ). Autopsy cases were conducted at the Center for Neurodegenerative Disease Research at the University of Pennsylvania (UPenn). Cases with mutations in the progranulin gene were excluded. The coding region of *TARDBP*, consisting of exons 2–5 and the first 531 nucleotides (nt) of exon 6, as well as at least 100 nt of the flanking intronic regions of each exon were fully sequenced in both directions. Primers used to amplify part or all of each exon were selected using Primer3 software and are available upon request.

Amplification reactions (50  $\mu$ l) were performed with 200 ng DNA using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 0.8  $\mu$ M (final concentration) each primer. Touchdown amplification protocol used consisted of 95 °C (10 min), followed by 14 cycles of 95 °C (30 s), 60 °C (exons 4–6) or 58 °C (exons 2 and 3) with a reduction of 0.5 °C per cycle (30 s), 72 °C (1 min) and 20 cycles of 95 °C (30 s), 53 °C (30 s), 72 °C (1 min), with a final 72 °C extension (5 min). Amplification products were purified using AMPure (Agencourt Bioscience, Beverly, MA) followed by single pass bidirectional sequencing performed by Agencourt Bioscience or CEQ8000 (Beckman Coulter, Fullerton, CA) with Beckman Coulter sequencing reagents. Results were analyzed using Mutation Surveyor software (SoftGenetics, PA).

### 2.2. SNP genotyping of *TARDBP* variant

Genotyping of control samples for *TARDBP* c.269C > T (p.A90V) was performed using a TaqMan chemistry-based allelic discrimination assay with “Assay by Design” (Applied Biosystems) probes on an Applied Biosystems 7900 followed by analysis with Sequence Detection System 2.2.1 software (Applied Biosystems). A total of 1385 control samples were obtained from the following sources: 276 controls from the Coriell Institute (Neurologically Normal Caucasian control panels, Camden, NJ; mean age 70 y), 48 clinical controls (mean age 76 y) from the Alzheimer Disease Core Center at UPenn, 42 normal postmortem brain samples (mean age 69 y) from the UPenn brain bank, 638 normal controls (mean age 63 y) from the Mayo Clinic Scottsdale and 381 normal controls (mean age 76 y) from the University of Washington. The DNA sample with the variant identified by DNA sequencing served as a positive control.

### 2.3. TDP-43 constructs

cDNA encoding human TDP-43 (Accession no. NM007375) in the plasmid pENTR-221 was obtained from Invitrogen (Carlsbad, CA). The addition of a myc epitope tag to the 5'-end of TDP-43 was achieved by PCR, using the primers; 5'-AAGCTTGATGGAA-CAAAAACCTCATCTCGGAAGAGGATCTGTCTGAATATATTC-GGGTAACC-3' and 5'-TCTAGAGCTACATCCCCAGCCAGA-AGACTTAGA-3'. The PCR product was cloned into the pGEM-T vector (Promega, Madison, WI). Following sequence analysis, the PCR product was then subcloned into the plasmid pcDNA 3.1 (Invitrogen) using restriction sites HindIII and XbaI, creating pcDNA 3.1/myc-TDP-43-WT. Site-directed mutations (see below) were generated using the plasmid pcDNA 3.1/myc-TDP-43-WT as a template.

### 2.4. Site-directed mutagenesis of TDP-43

Site-directed mutagenesis (Quikchange kit; Stratagene, La Jolla, CA) was used to create  $\Delta$ NLS1 [19] and A90V missense mutation in the longest human TDP-43 isoform. Sequence of the mutagenized oligonucleotides are as follows: A90V: 5'-G GAT GAG ACA GAT GTT TCA TCA GCA GTG AAA GTG-3'. All constructs were subjected to sequence analysis.

### 2.5. Cell culture and transfection

QBI-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and 1% L-glutamate. QBI-293 cells were transfected using the Amaxa Nucleofector system (Amaxa Inc., Gaithersburg, MD) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### 2.6. Antibodies

Antibodies used here included: rabbit anti-TDP-43 antibody raised to recombinant TDP-43 (Protein Tech Group, Chicago, IL); anti-myc mAb (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) and an anti- $\alpha$ -tubulin mAb (Sigma, St. Louis, MO).

### 2.7. Immunofluorescence studies

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.02% or 0.2% Triton X-100 (Sigma) in PBS for 10 min, blocked with 5% powdered milk in PBS for 2-h and incubated overnight with primary antibody at 4 °C. Primary antibodies were visualized with secondary antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (Vector Laboratories, Burlingame, CA) and nuclei were detected using DAPI. All cells were analyzed using a Nikon TE-2000-E (Nikon, Tokyo, Japan) and images were captured using a CoolSnap-HQ camera (Photometrics, Tuscon, AZ). All micrographs show individual cells representative of the total cell population. For all experiments, at least four separate experiments were performed in duplicate.

### 2.8. Solubility and biochemical analysis

To examine the solubility profile of TDP-43, sequential extractions were performed. Cells were washed twice with PBS, lysed in cold RIPA buffer, and sonicated. Cell lysates were cleared by centrifugation at 40000 rpm for 30 min at 4 °C to generate the RIPA soluble samples. To prevent carry over the resulting pellets were washed twice (i.e. re-sonicated and re-centrifuged). Only the supernatants from the first centrifugation were analyzed and all supernatants produced from the second and third wash step were discarded. RIPA insoluble pellets were then extracted with urea buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris, pH 8.5), sonicated and centrifuged at 40000 rpm for 30 min at 22 °C. Protease inhibitors were added to all buffers prior to use (1 mM PMSF and a cocktail of protease inhibitors). Protein concentration was determined by bicinchoninic acid method (Pierce, Rockford, IL) and all proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Following transfer, nitrocellulose membranes were blocked in 5% powdered milk and incubated in the primary antibody overnight at 4 °C. Primary antibodies were detected with horseradish peroxidase-conjugated anti-IgG antibody (Jackson ImmunoResearch, Westgate, PA) and blots were developed with Renaissance Enhanced Luminal Reagents (NEN Life Science Products, Boston, MA). Digital images were acquired using a Fuji Film Intelligent Darkbox II (Fuji Systems, Stamford, CT).

## 3. Results and discussion

Genetic evaluation of *TARDBP* was completed on 148 patients with a clinical and/or autopsy proven diagnosis of FTLD or FTLD/ALS and identified a single living patient with a c.269C > T (A90V) substitution in exon 3. This 72-year-old Caucasian with a family history of dementia (Supplementary Fig. 1) presented clinically with symptoms consisting of slowly progressive FTLD/ALS (age of onset 57 y). Additional family members (deceased, affected or living unaffected) were unavail-

able for testing and no autopsies have been performed in the proband's family to study the neuropathological changes linked to the A90V *TARDBP* variant. Therefore, association of this variant with disease in the family could not be confirmed at this time. Testing of 1385 age-matched controls for the c.269C > T substitution by SNP genotyping identified the variant in a single control case (one out of 1385 cases;

0.07%), 76-year-old at inclusion of the study indicating that this amino acid substitution may be a polymorphism in the *TARDBP* gene. However, based on the functional data presented below we speculate that A90V may increase the risk of FTL/ALS.

As the A90V variant is located between the bipartite NLS sequence of TDP-43, we examined its functional consequences in

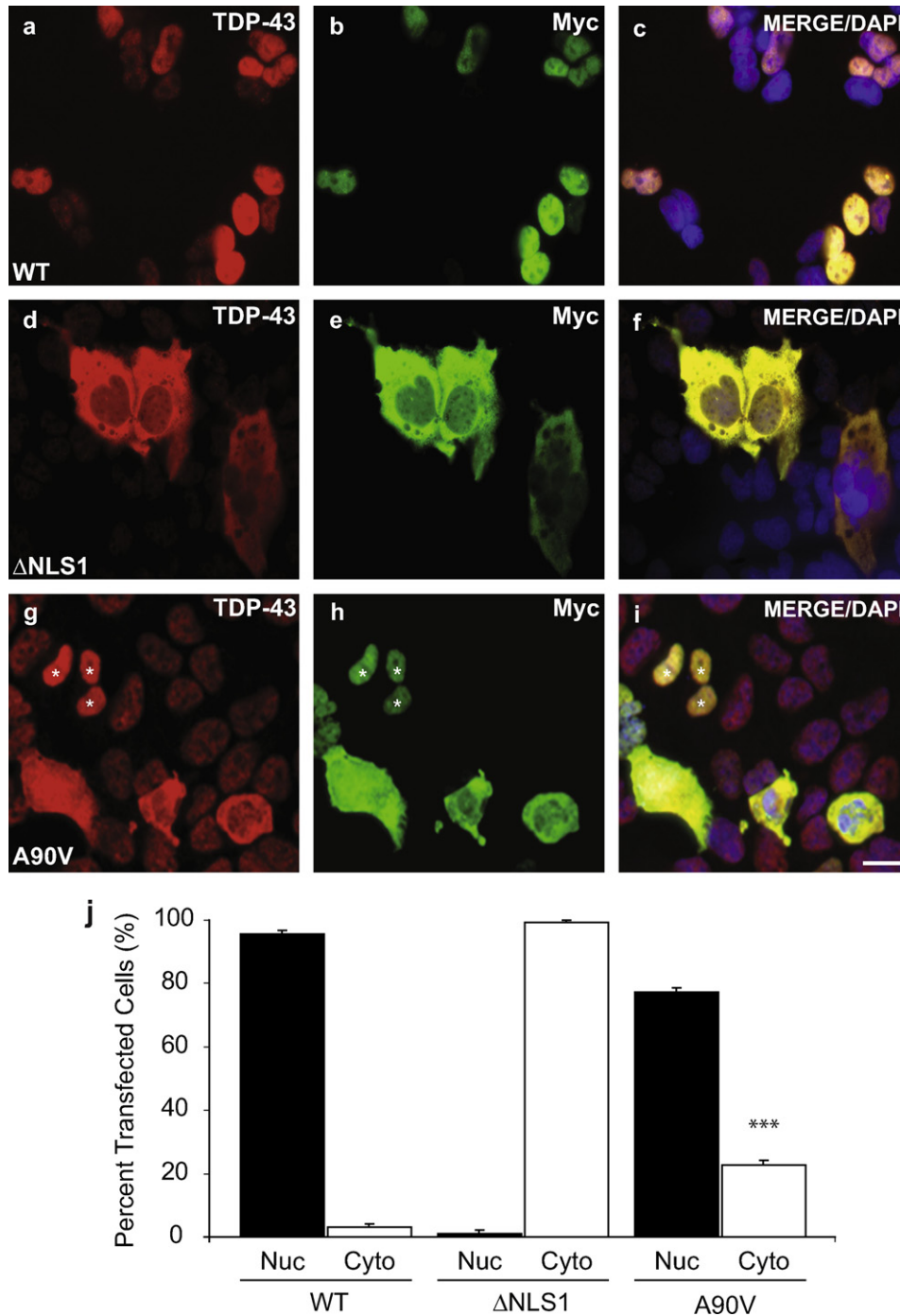


Fig. 1. The A90V TDP-43 variant partially disrupts nuclear localization (a–i). Two-color immunofluorescence of QBI-293 cells transfected with myc-TDP-43-WT (WT), myc-TDP-43-ΔNLS1 (ΔNLS1) or myc-TDP-43-A90V (A90V). Seventy-two hours following transfection, cells were stained with (a, d, g) anti-TDP-43 (red) and (b, e, h) anti-myc (green) antibodies or merged (c, f, i). Nuclei were labeled with DAPI stain (blue). Scale bar; 20 μm. (j). Quantification of the cellular localization, nuclear (nuc; black bars) vs. cytoplasmic (cyto; white bars), of TDP-43 in QBI-293 cells transfected with myc-TDP-43-WT (WT), myc-TDP-43-ΔNLS1 (ΔNLS1) or myc-TDP-43-A90V (A90V). Asterisks (\*\*\*) represents significant differences compared to myc-TDP-43-WT transfected cells ( $P \leq 0.05$ ). Error bars represent S.E.M.



vitro. Myc-TDP-43-A90V was transiently expressed in QBI-293 cells and two-color immunofluorescence was performed on cells 72-h post-transfection. Both myc-TDP-43-WT and myc-TDP-43- $\Delta$ NLS1 were used as controls and as reported previously [19], myc-TDP-43-WT was localized exclusively to the nucleus (Fig. 1a–c) and myc-TDP-43- $\Delta$ NLS1 was detected primarily in the cytoplasm (Fig. 1d–f). However, myc-TDP-43-A90V was detected both in the nucleus and in the cytoplasm of a subset of cells, suggesting only a partial disruption of TDP-43 nuclear localization by the alanine to valine substitution (Fig. 1g–i). Quantitative analysis of transfected cells showed that 78% and 22% of myc-TDP-43-A90V expressing cells showed nuclear and cytoplasmic localization of TDP-43, respectively (Fig. 1j). Further, since the A90V variant is located in between the bipartite NLS sequence, and not directly in the functional region, we hypothesize that this variant may only cause a mild disruption in the nuclear localization of TDP-43 and as a result the mislocalization of TDP-43 only occurs in a sub-set of transfected cells.

To determine if myc-TDP-43-A90V accumulated as an insoluble pool and sequestered endogenous TDP-43, RIPA and urea fractions of transfected cells were analyzed by immunoblot. Since myc-tagged TDP-43 protein migrated slower than endogenous TDP-43 in SDS-PAGE, each could be analyzed separately. At all time points examined, both endogenous and myc-TDP-43-WT were recovered exclusively in the RIPA fractions (Fig. 2a and b). Consistent with the expected  $\Delta$ NLS sequence disruption described previously [19], myc-TDP-43-A90V was detected in the urea fraction as well as in the RIPA fraction 24 h post-transfection and by 72 h both endogenous and the myc-TDP-43-A90V proteins were recovered in the insoluble urea fraction (compare Fig. 2a with b). Thus, the myc-TDP-43-A90V genetic variant showed partial NLS sequence disruption and caused a similar but milder phenotype like that seen in the myc-TDP-43- $\Delta$ NLS mutants [19] including

sequestration of endogenous TDP-43 by myc-TDP-43-A90V in the urea fraction (Fig. 2a and b).

The identification of a human genetic variant in the region between the bipartite NLS sequence, in concert with results from in vitro cell biology experiments gives credence to the notion that functional disruption of the highly conserved NLS sequence could increase the risk for FTL/ALS. Unfortunately, no biological samples were available from other members of the proband's family for genetic analysis; however, there is a very strong documented family history of dementia. Furthermore, the clinical presentation of both FTL/ALS in the proband is consistent with the current hypothesis that FTL/ALS represent two extremes of a clinicopathological spectrum of disease, TDP-43 proteinopathies [8].

We have reported previously that over-expression of TDP-43- $\Delta$ NLS mutants in vitro recapitulated the biochemical phenotype found in human neurodegenerative TDP-43 proteinopathies [19]. In concert with this finding a similar, but milder, phenotype was also observed with A90V over-expression. However, as our manuscript was in preparation, the A90V TDP-43 variant was identified in a 53-year-old control (1/372 cases screened) [14] as well as an unspecified control (1/360) [15]. Thus, as of today, this variant has been found in one affected and three control subjects. Therefore, we cannot exclude the possibility that A90V represents a rare benign polymorphism. However, our in vitro data and the strong family history of dementia suggest that the A90V variant maybe a potential genetic risk factor for FTL/ALS. Similar to other known disease risk factor genes, characteristics and lifestyle of the person, and environmental and additional genetic factors can contribute to the development of the disease. Reduced penetrance and variation in expressivity may also play a role in the disease-associated risk of the A90V variant. This is consistent with our in vitro data as the mislocalization of TDP-43 was observed in only a subset of transfected cells. Thus, the presence of the A90V variant may increase the likelihood of developing FTL/ALS.

The recent discovery of multiple pathogenic *TARDBP* mutations in several FALS kindreds [13–16] further supports the importance of TDP-43 in the etiology and pathogenesis of ALS; however, unlike the A90V TDP-43 substitution, these recently identified *TARDBP* mutations are localized to the C-terminus of TDP-43 and thus, are likely to cause the disease through different mechanisms. Current literature suggests that the presence of disease segregating mutations in TDP-43 are rare, and to date, minimal neuropathological data from affected family members has been published. Therefore, further genetic analysis needs to be completed in more familial cases of FTL/ALS before we can conclusively comment on the genetic and mechanistic consequences of TDP-43 mutations, as well as the TDP-43 A90V variant.

Furthermore, although TDP-43 inclusions are found in the nucleus and cytoplasm of diseased cells in association with nuclear clearing of TDP-43, it remains uncertain how *TARDBP* mutations or TDP-43 pathology causes neurodegeneration in FTL-U and ALS. Rapid progress is being made regarding the normal and pathological biology of TDP-43 and this will accelerate efforts to develop new and more effective therapies for neurodegenerative TDP-43 proteinopathies.

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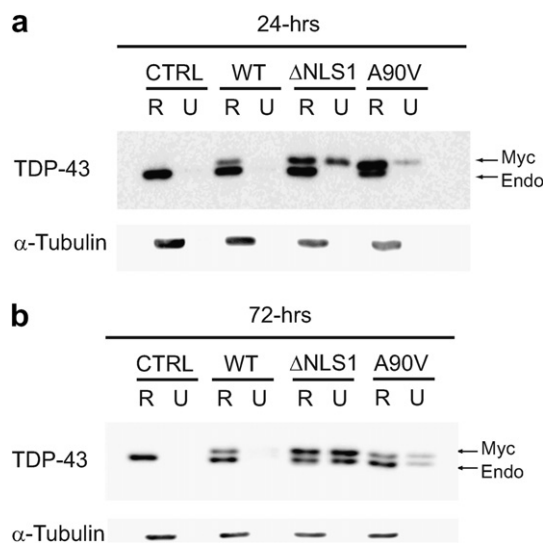


Fig. 2. Endogenous TDP-43 is sequestered in the insoluble fraction with the expression of myc-TDP-43-A90V. Immunoblots of RIPA (R) and urea (U) extracted fractions (a) 24-h or (b) 72-h post-transfection with empty vector (CTRL), myc-TDP-43-WT (WT), myc-TDP-43- $\Delta$ NLS1 ( $\Delta$ NLS1) or myc-TDP-43-A90V (A90V) with anti-TDP-43 antibody. Alpha-tubulin was used as a loading control.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2008.05.024](https://doi.org/10.1016/j.febslet.2008.05.024).

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