SUPPLEMENTARY METHODS

Cell cultures

NSC34 is a hybrid cell line that retains the ability to proliferate and express several motor neuron characteristics (49) and were kindly provided by Neil Cashman. NSC34 was routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum (FBS), 1 mM glutamine, 1% sodium pyruvate and 1% antibiotics, as described previously (50). Murine Neuro2a (N2a) neuroblastoma cells (A.T.C.C., Manassas, VA, USA) were cultured in minimum essential medium eagle (MEM) supplemented with 10% FBS, 1 mM glutamine and 1% antibiotics, as described previously (51). Cell cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C and grown until they reached 80% confluence for a maximum of 20 passages.

Analysis of phosphorylation and ubiquitination of TDP-43 IBs

The analysis of phosphorylation and ubiquitination of TDP-43 was assessed on NSC34 cells plated in 6-well plates (150,000 cells/well density). After 48 h of transient transfection with vehicle, 10 μg of plasmid, 4 μg/mL of control IBs and 5.7 μg/mL of TDP-43 IBs, the incubation medium (DMEM) was replaced with fresh complete medium and the analysis was performed. After washing with PBS, cells were fixed in 2% (w/v) buffered paraformaldehyde for 10 min at room temperature (20 °C) and permeabilized with a 0.5% (v/v) Triton X-100 solution for 5 min. The colocalization of TDP-43 or phosphorylated TDP-43 with ubiquitin was monitored using 1:350 rabbit polyclonal anti-TDP-43 antibody (Sigma-Aldrich, St. Louis, MO, USA) or 1:500 rabbit anti-TDP-43 phosphorylation sites 409/410 (Cosmo Bio Co., Ltd., Tokyo, Japan) for 60 min at 37 °C, 1:150 mouse monoclonal anti-ubiquitin antibodies (Life Technologies, CA, USA) for 60 min at 37 °C, and then with 1:1000 Alexa Fluor 488-conjugated secondary antibodies (Life Technologies, CA, USA) for 60 min at 37 °C and 594-conjugated secondary antibodies (Life Technologies, CA, USA) for 60 min at 37 °C. The colocalization of TDP-43 with ubiquitin was estimated for the regions of

interest (12-22 cells) in three different experiments using the ImageJ (NIH, Bethesda, MD, USA) and JACOP plugin (rsb.info.nih.gov) software (52).

Dot-Blot analysis

To exclude a potential cross-reactivity of the anti-murine TDP-43 antibodies with TDP-43 IBs containing human TDP-43, we carried out a dot-blot analysis. Briefly, 2.0 µl of 1.0 µg/µl of control IBs and TDP-43 IBs were spotted onto PVDF membrane, and then incubated with 1:1000 dilution of mouse monoclonal anti-TDP-43 antibodies (Novus Biologicals, Ltd, Cambridge, UK) or 1:1000 rabbit polyclonal anti-murine TDP-43 antibodies (LSBio, Seattle, WA), and then with 1:5000 diluted peroxidase-conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology Inc.) or with 1:5000 diluted peroxidase-conjugated anti-rabbit secondary antibodies (Santa Cruz Biotechnology Inc.), respectively.

Subcellular fractionation

NSC34 cells plated in 6-well plates at 360,000 cells/well density were treated with vehicle, 4 and 10 µg plasmid for 48 h, 25 nM siRNA for 72 h, 4 µg/mL of control IBs and 5.7 µg/mL TDP-43 IBs. After treatment, the cells were washed, centrifuged and collected with 250 µL cytosolic buffer consisting of 0.1% sodium citrate (w/v), 0.1 % Triton X-100, 1 mM PMSF and 1:1000 protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). Following 1 h incubation at 4 °C the samples were subjected to three freeze-thaw cycles and centrifuged at 750 g for 10 min. The supernatant (cytoplasmic fraction) was collected and the pellet (nuclear fraction) was resuspended in the nuclear buffer (20 mM Hepes, 250 mM sucrose, 2 mM EGTA and 1 mM EDTA, pH 7.4), sonicated twice for 5 s on ice and then collected. Nuclear (N) and cytoplasmic (C) fractions were quantified using the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) and the distribution of TDP-43 was visualized by Western Blotting.

In a further set of experiments, the cytoplasmic fractions of cells treated with 4 µg/mL of control IBs and 5.7 µg/mL of TDP-43 IBs were centrifuged at 22,000 g for 30 min and the pellet (P) and supernatant (SN) fractions were collected and analysed by Western Blotting.

Western Blotting

Immunoblot analysis of TDP-43 levels in nuclear and cytoplasmic fractions of NSC34 cell lysates were carried out on a 12% (w/v) SDS/PAGE, blotted onto a polyvinylidene difluoride (PVDF) Immobilio-P Transfer Membrane (Millipore Corporation, Bedford, MA, USA), incubated with 1:1000 diluted mouse monoclonal anti-TDP-43 antibodies (Novus Biologicals, Ltd, Littleton, CO, USA) and with 1:5000 diluted peroxidase-conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or with 1:1000 diluted rabbit polyclonal anti GAPDH antibodies (Santa Cruz Biotechnology Inc.) and with 1:5000 diluted peroxidase-conjugated anti-rabbit secondary antibodies (Santa Cruz Biotechnology Inc.) or with 1:500 goat polyclonal antibodies anti Ac-Histone H3 (Lys 9/14) and with 1:5000 diluted peroxidase-conjugated anti-goat secondary antibodies (Santa Cruz Biotechnology Inc.). Immunoblot analysis was also performed in cytoplasmic fractions of NSC34 cell lysates with 1:500 rabbit anti-TDP-43 phosphorylation sites 409/410 antibodies (Cosmo Bio Co., Ltd., Tokyo, Japan) or 1:1000 diluted rabbit polyclonal anti GAPDH antibodies (Santa Cruz Biotechnology Inc.) and with 1:5000 diluted peroxidase-conjugated anti-rabbit secondary antibodies (Santa Cruz Biotechnology Inc.). The immunolabelled bands were detected using a Super Signal West Dura (Thermo Fisher Scientific, Waltham, MA, USA). The densitometric analysis was carried out using ImageJ software (NIH, Bethesda, MD, USA).

SDS-PAGE of TDP-43 IBs

Human TDP-43 was overexpressed in *E. coli.* The resulting cells were collected and lysed as previously described (46). The resulting inclusion bodies (TDP-43 IBs) were purified as previously described (46). The expression of human TDP-43 in *E. coli* and its presence in the supernatant (SN)

or in the pellet (P) fractions after cell lysis and purification of IBs were checked by SDS-PAGE, using 12% (w/v) polyacrylamide gels. P and SN aliquots were collected and mixed with 4× sample buffer with 20% 2-mercaptoethanol. SDS-PAGE analysis was performed in accordance with Laemmli (53) using a 12% polyacrylamide gels. Proteins were visualized by Coomassie Blue staining (0.1% Coomassie Blue, 10% acetic acid, 40% methanol).

SUPPLEMENTARY FIGURES AND LEGENDS

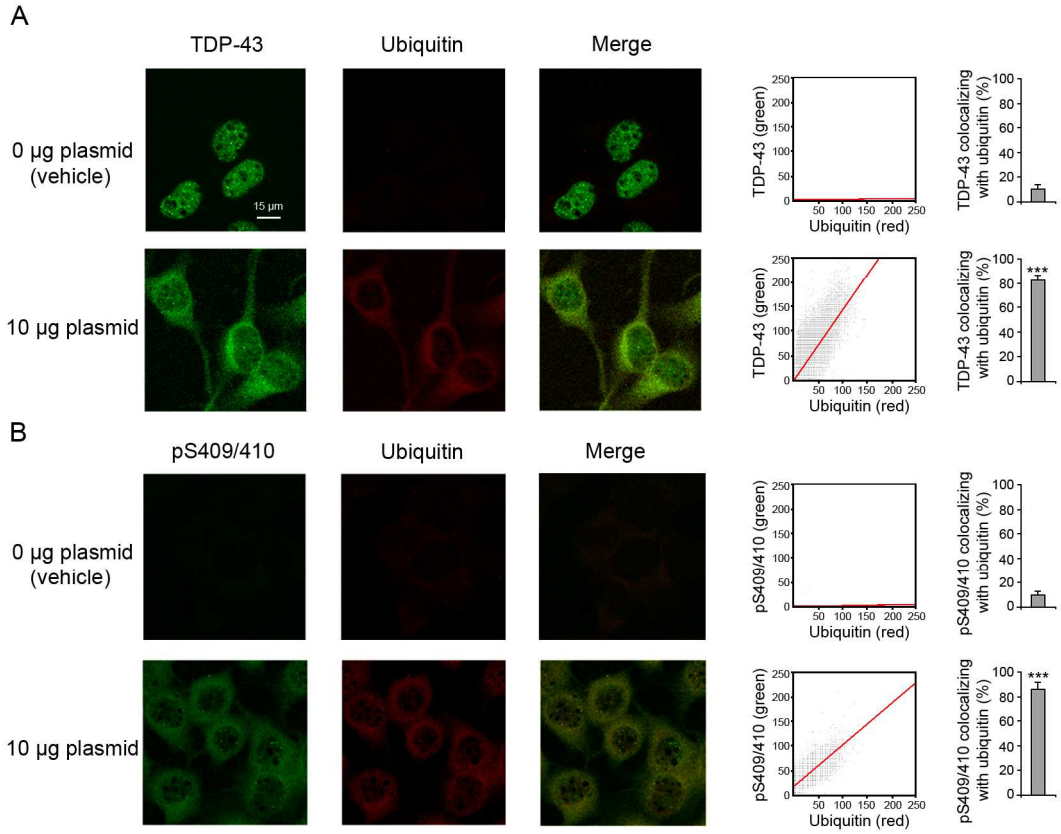

Figure S1. (*A*) Representative confocal scanning microscope images showing the colocalization of cytoplasmic TDP-43 with ubiquitin-positive aggregates in NSC34 cells transfected with vehicle or 10 µg of pCI-neo plasmid expressing human TDP-43. The green fluorescence indicates TDP-43 detected with antibodies that recognize both the endogenous (murine) and exogenous (human) protein. The red fluorescence indicates ubiquitin. (*B*) Representative confocal scanning microscope images showing the colocalization of TDP-43 phosphorylated at Ser409/410 and ubiquitin-positive aggregates in NSC34 cells transfected with vehicle or 10 µg of pCI-neo plasmid expressing human TDP-43. Green and red fluorescence indicate TDP-43 phosphorylated at Ser409/410 and ubiquitin, respectively. The cytofluorograms at the right of each row of images show the cytoplasmic green fluorescence intensity (as pixel intensity, y axis) *versus* cytoplasmic red fluorescence intensity (x axis). The histograms show the percentage of colocalization on regions of interest (12–13 cells) using the ImageJ (NIH, Bethesda, MD, USA) and JACOP plugin (rsb.info.nih.gov) software. Error bars are SEM. The triple (***) asterisk refers to p values lower than 0.001 relative to cells transfected with vehicle.

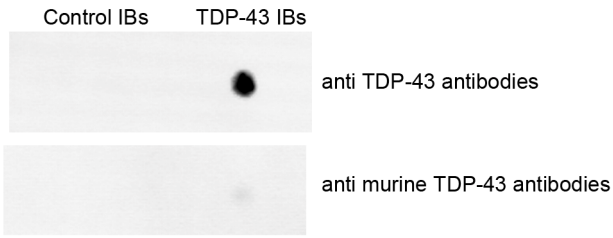

Figure S2. Dot-blot analysis of bacterial control IBs devoid of TDP-43 (left) and bacterial IBs containing human TDP-43 (right) with mouse monoclonal anti-TDP-43 antibodies that recognise both murine and human TDP-43 (upper panel) and rabbit polyclonal anti- murine TDP-43 antibodies that recognise only murine TDP-43 (bottom panel). The specificity of the antibodies raised against murine TDP-43 is shown by the lack of cross-reactivity to TDP-43 IBs containing human TDP-43 (bottom panel).

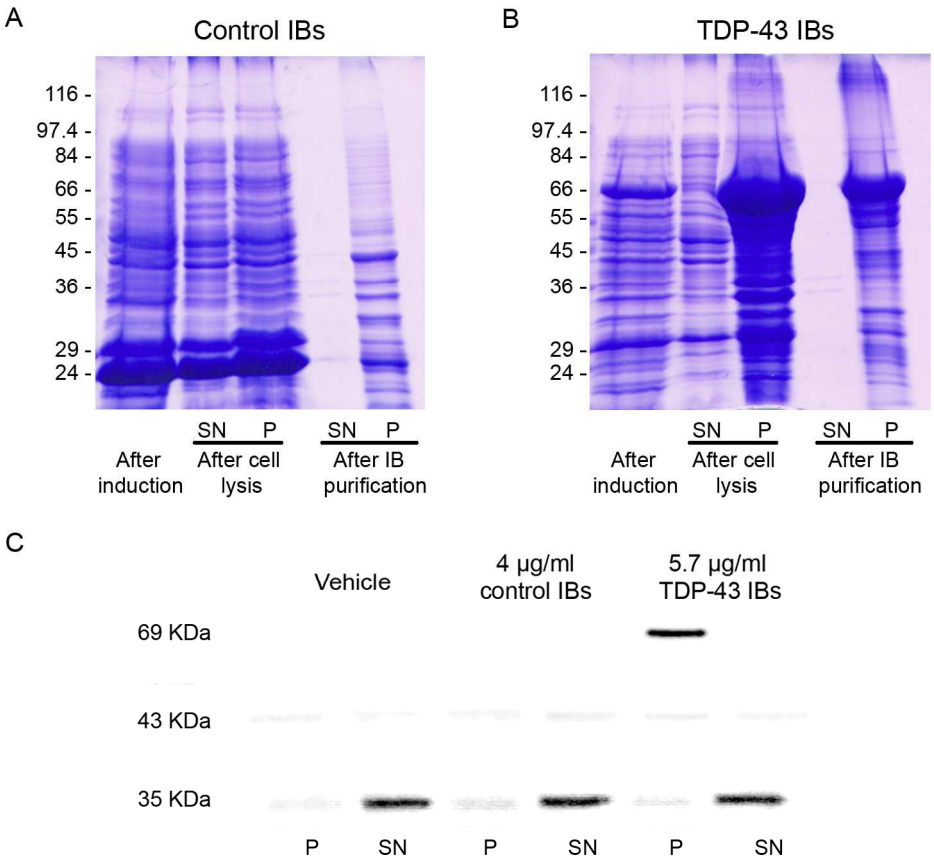

Figure S3. (*A*,*B*) SDS-PAGE analysis of proteins from *E. coli* cells containing control IBs (left) and TDP-43 IBs (right) after induction of the GST/TDP-43 fused protein with 1 mM IPTG for 16 h at 37 °C. The lanes refer to total proteins after IPTG induction (lane 1), supernatant (SN) and pellet (P) fractions after cell lysis (lanes 2 and 3, respectively), and of SN and P after IB purification (lanes 4 and 5, respectively). The bands at 69 kDa indicate the fused GST/TDP-43 protein, that is absent in the control IBs samples. (*C*) Western blot analysis of the fused GST/TDP-43 distribution (69 kDa) in the P and SN of the cytoplasmic fraction in NSC34 cells transfected with vehicle, 4 µg/mL control IBs and 5.7 µg/mL TDP-43 IBs. The detection of GAPDH (35 kDa) was carried out as a loading control (10 µg total protein).

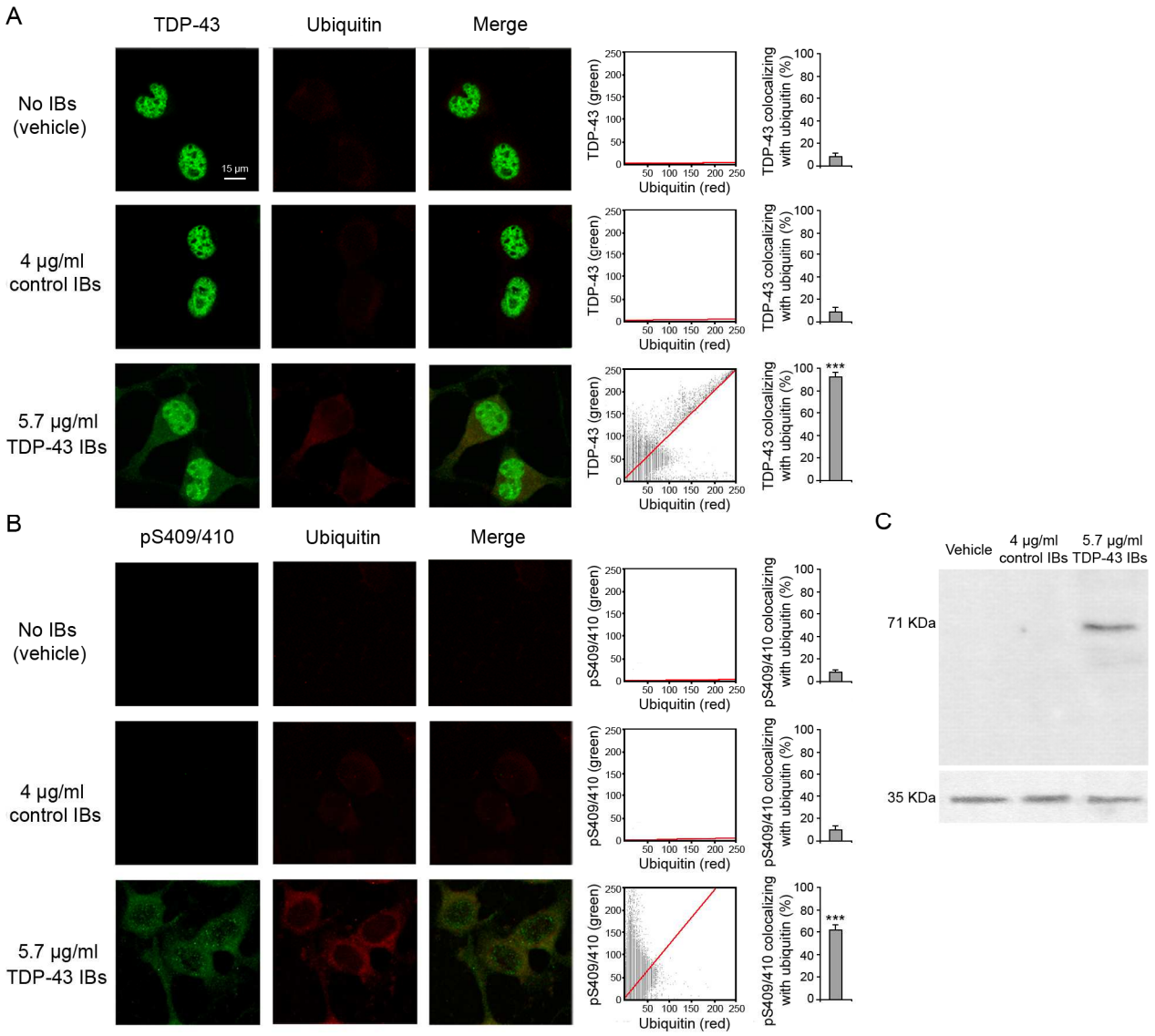

Figure S4. (*A*) Representative confocal scanning microscope images showing the colocalization of cytoplasmic TDP-43 with ubiquitin-positive aggregates in NSC34 cells transfected with vehicle, 4 µg/mL of control IBs or 5.7 µg/mL of TDP-43 IBs. Green and red fluorescence have the same meaning as in supplemental Fig. S1A. (*B*) Representative confocal scanning microscope images showing the colocalization of TDP-43 phosphorylated at Ser409/410 and ubiquitin-positive aggregates in NSC34 cells transfected with vehicle, 4 µg/mL control IBs or 5.7 µg/mL TDP-43 IBs. Green and red fluorescence have the same meaning as in supplemental Fig. S1B. The cytofluorograms and histograms have the same meaning as in supplemental Fig. S1. (C) Western blot analysis of the phosphorylated TDP-43 (pS409/410) levels in the cytoplasm of NSC34 cells transfected with vehicle, 4 µg/mL of control IBs and 5.7 µg/mL of TDP-43 IBs. The band at 71 kDa arises from the exogenous TDP-43 phosphorylated at Ser409/410 fused to GST. Detection of GAPDH (35 kDa) was carried out as a loading control for cytoplasmic fractions (30 µg total protein).

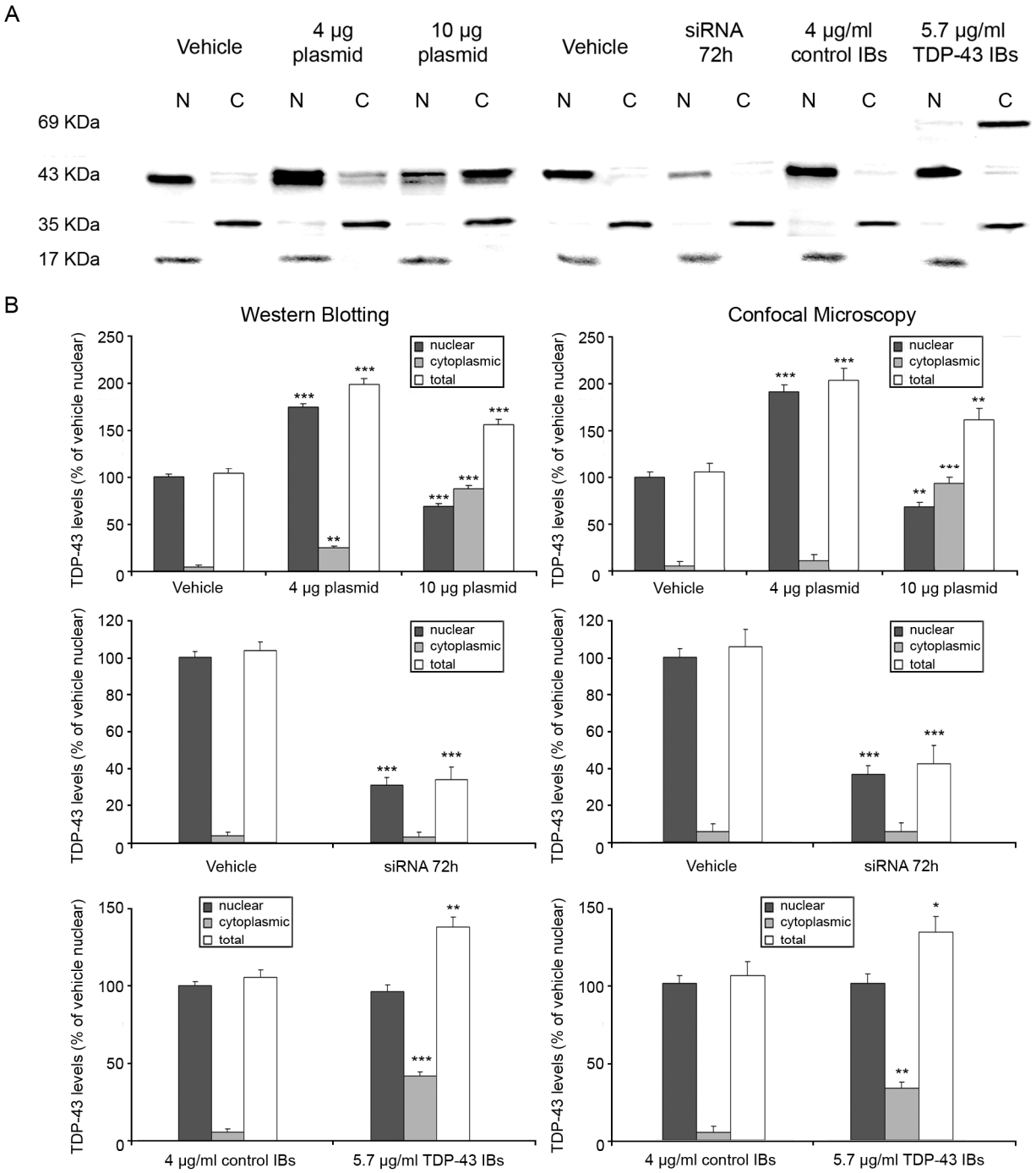

Figure S5. (*A*) Western blot analysis of the TDP-43 levels in the nucleus (N) and cytoplasm (C), in NSC34 cells transfected with vehicle, 4 and 10 µg of plasmid for 48 h, 25 nM siRNA for 72 h or with 4 µg/mL of control IBs and 5.7 µg/mL of TDP-43 IBs. TDP-43 is revealed as a band at 43 kDa. In the latter sample the band at 69 kDa arises from the exogenous TDP-43 fused to GST. Detection of GAPDH (35 kDa) and Ac-Histone H3 (17 kDa) was carried out as a loading control for cytoplasmic and nuclear fractions, respectively (30 µg total protein). (*B*) Semi-quantitative analysis of nuclear (dark gray), cytoplasmic (pale gray) and total (blank) TDP-43 levels as percentages relative to nuclear levels after transfection with vehicle, obtained both from Western blotting (left graphs) and confocal microscopy (right graphs). Experimental errors are SEM. The single (*), double (**) and triple (***) asterisks refer to p values lower than 0.05, 0.01 and 0.001, respectively, relative to nuclear or cytoplasmic TDP-43 levels of cells transfected with vehicle.

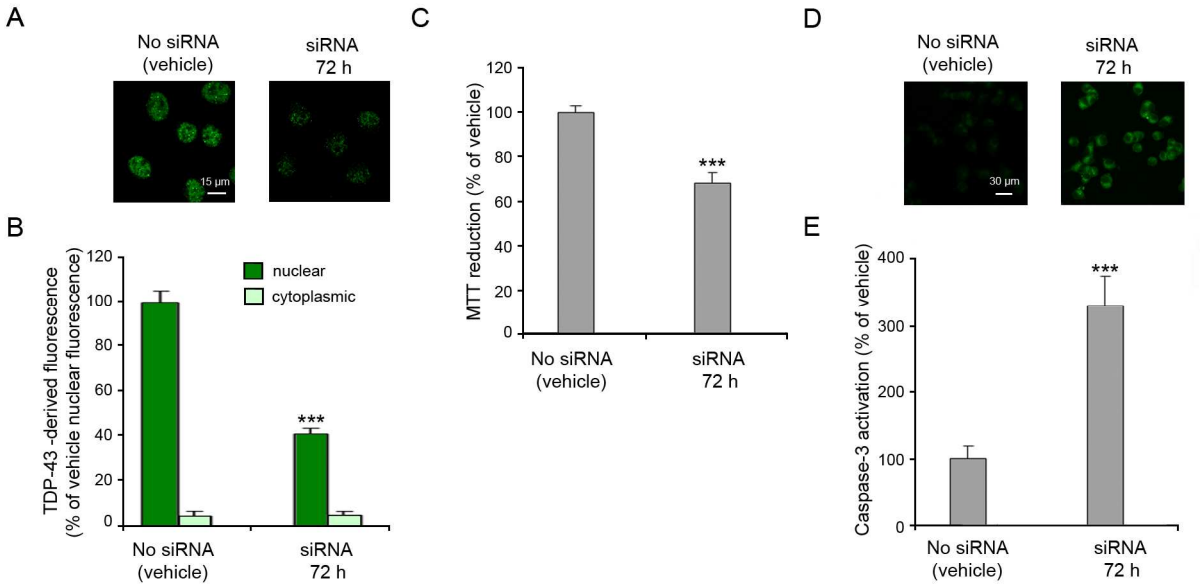

Figure S6. (*A*) Representative confocal scanning microscope images of N2a cells transfected with vehicle and 25 nM siRNA and analysed 72 h after transfection. The green fluorescence indicates TDP-43 detected with immunofluorescence. (*B*) Semi-quantitative analysis of nuclear (green) and cytoplasmic (pale green) TDP-43-derived fluorescence upon transfection with vehicle and siRNA. The triple (***) asterisk refers to p values lower than 0.001, relative to nuclear TDP-43 derived fluorescence of cells transfected with vehicle. (*C*) MTT reduction of N2a cells transfected with siRNA and analysed 72 h after transfection. (*D*) Representative confocal microscope images showing caspase-3 activation in N2a cells, assessed using the fluorescent probe FAM-FLICA™ caspases 3 & 7. The images were acquired at 72h, following transfection with vehicle or 25 nM siRNA. (*E*) Semi-quantitative analysis of the green fluorescence arising from caspase-3 activation. Experimental errors are SEM. The triple (***) asterisk refers to p values lower than 0.001, relative to cells transfected with vehicle.

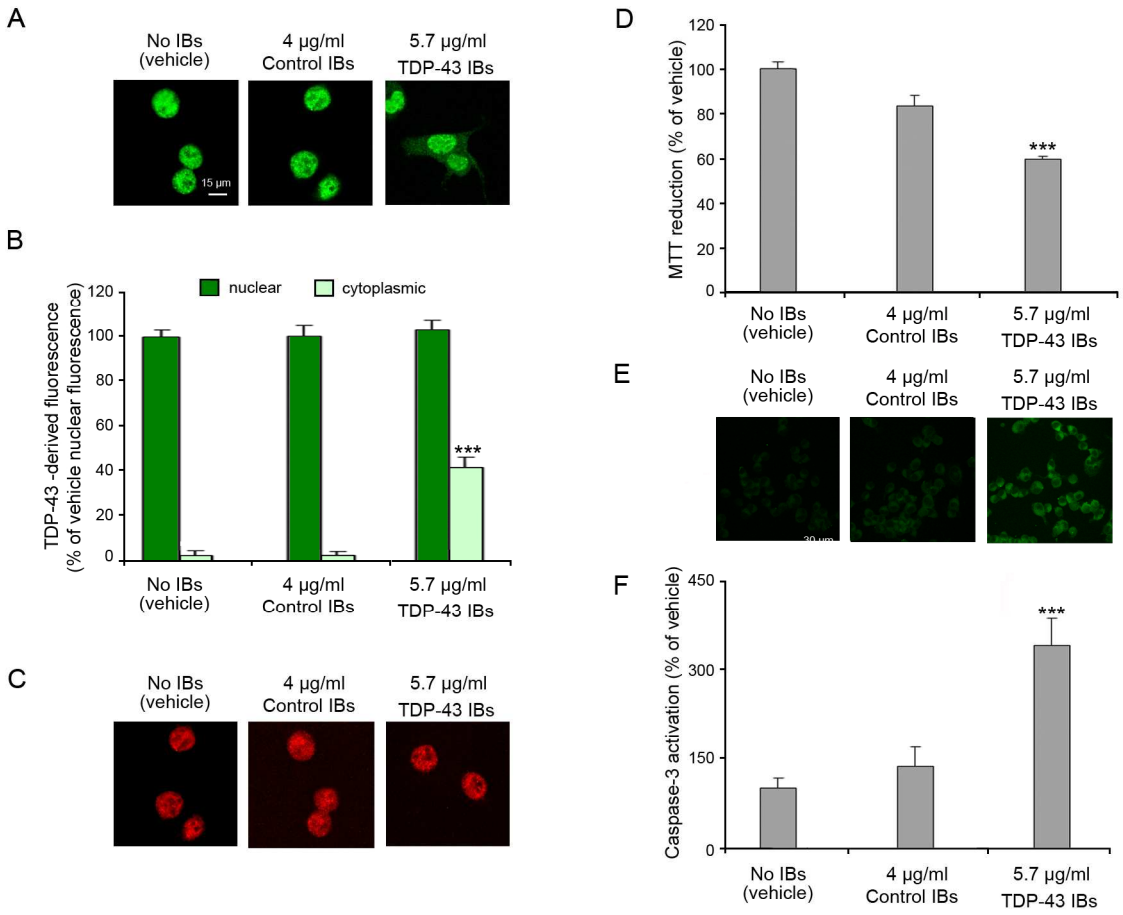

Figure S7. (*A*) Representative confocal scanning microscope images of N2a cells transfected with vehicle, 4 µg/mL of control IBs and 5.7 µg/mL of TDP-43 IBs and analysed after transfection. The green fluorescence indicates total TDP-43 (human and murine). (*B*) Semi-quantitative analysis of nuclear (green) and cytoplasmic (pale green) TDP-43-derived fluorescence upon transfection. All data are percentages relative to nuclear fluorescence after transfection with vehicle. The triple (***) asterisk refers to p values lower than 0.001, relative to nuclear or cytoplasmic TDP-43 of cells transfected with vehicle. (*C*) Confocal scanning microscope images of N2a cells transfected with vehicle, 4 µg/mL of control IBs and 5.7 µg/mL of TDP-43 IBs and analysed after transfection with anti-murine TDP-43 antibodies. The red fluorescence indicates murine endogenous TDP-43. (*D*) MTT reduction of N2a cells transfected with 4 µg/mL of control IBs and 5.7 µg/mL of TDP-43 IBs and analysed after 24 h. The indicated IBs concentrations refer to TDP-43 IBs, which exceed by 30% the corresponding values of control IBs (see the text for the explanation). (*E*) Confocal microscope images of N2a cells showing caspase-3 activation, assessed using the fluorescent probe FAM-FLICA™ caspases 3 & 7, 24 h following transfection. Other details as in panel D. (*F*) Semi-quantitative analysis of the green fluorescence arising from caspase-3 activation. Experimental errors are SEM. The triple (***) asterisk refers to p values lower than 0.001, relative to cells transfected with vehicle.