Monomerization of TDP-43 in ALS

Literature Seminar #1

B4 Youhi Hwang

2024/12/5(Thu)

Contents

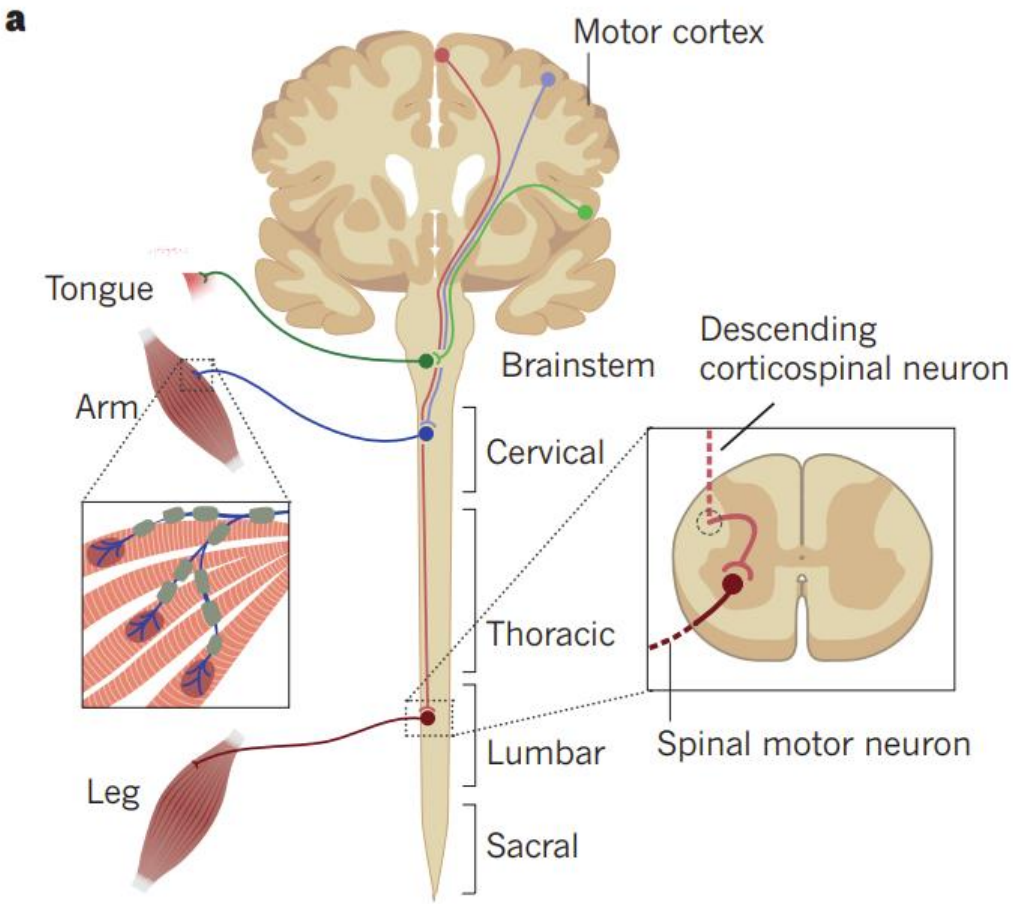
Contents

Amyotrophic Lateral Sclerosis (ALS)

ALS early symptoms

From a website of Active Home Health & Hospice

Components of the nervous system that are affected by ALS

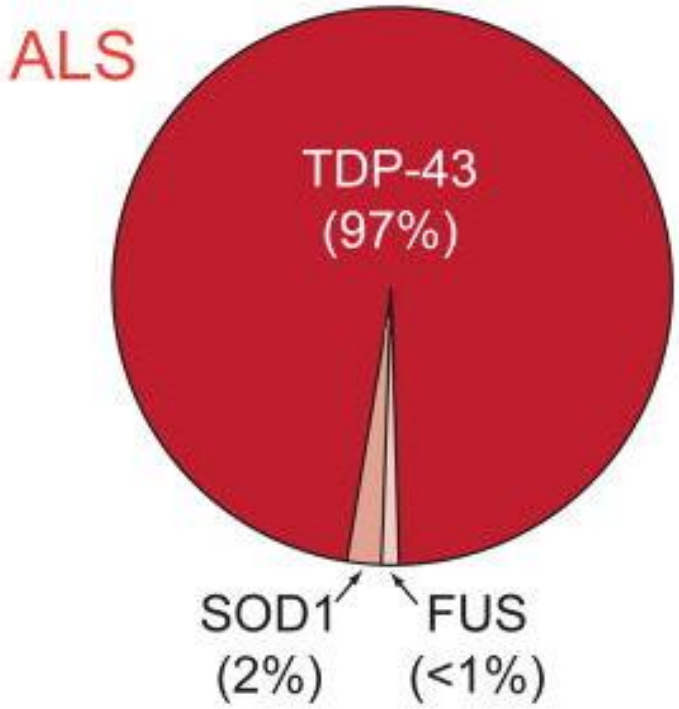


Taylor, J., Brown, R. & Cleveland, D. *Nature* **539**, 197–206 (2016).

TDP-43

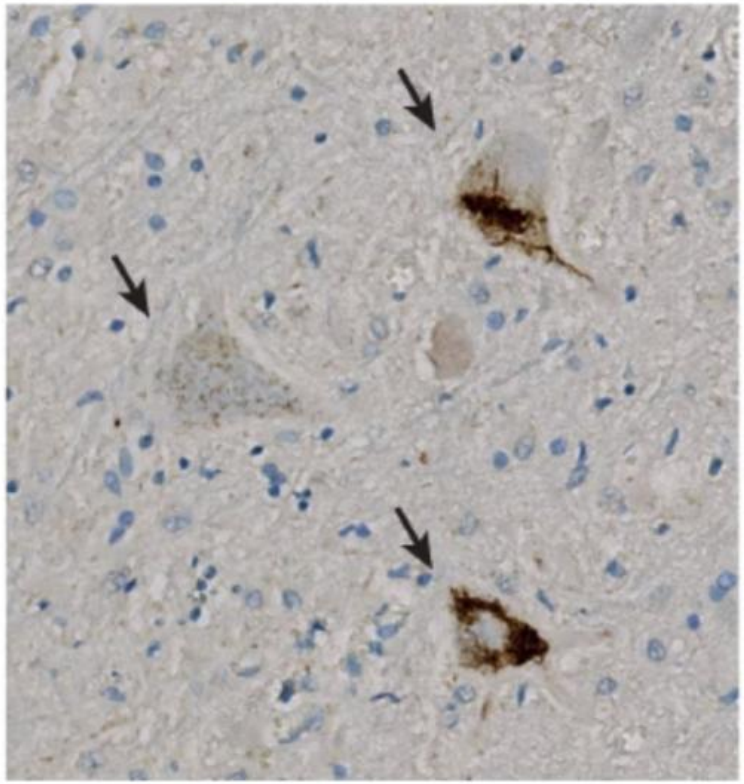
TDP-43 :ubiquitously expressed and predominantly nuclear RNA binding protein shuttling between the nucleus and the cytoplasm, which is encoded by the *TARDBP* gene

Pathological inclusions in ALS



Ling, S. C. , et al. *Neuron* , **2013,** 79, 417

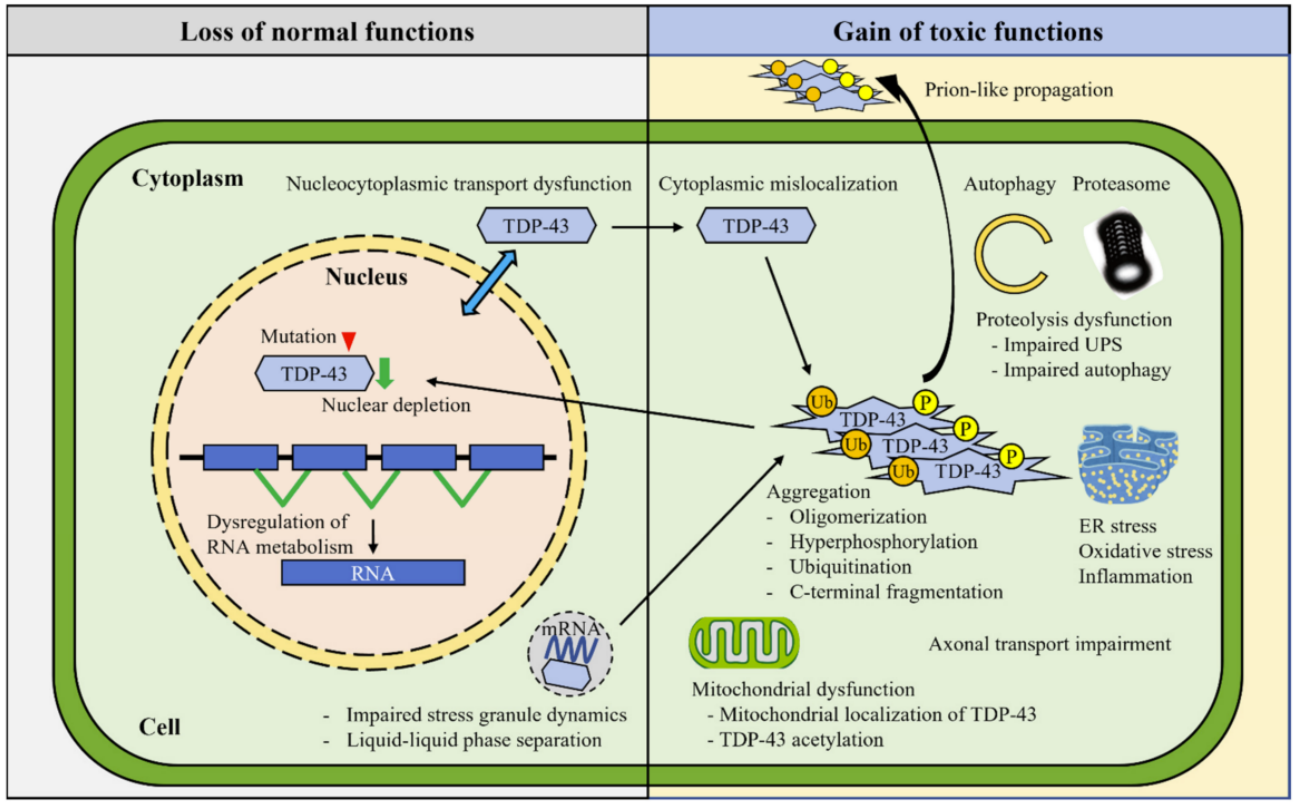
TDP-43 redistribution to cytoplasmic inclusions (arrows) in spinal motor neurons in ALS



Taylor, J., Brown, R. & Cleveland, D *Nature* **539**, 197–206 (2016).

TDP-43

Molecular mechanisms underlying TDP-43 pathogenesis



Tamaki, Y.; Urushitani, M. Int. J. Mol. Sci. 2022, 23, 12508.

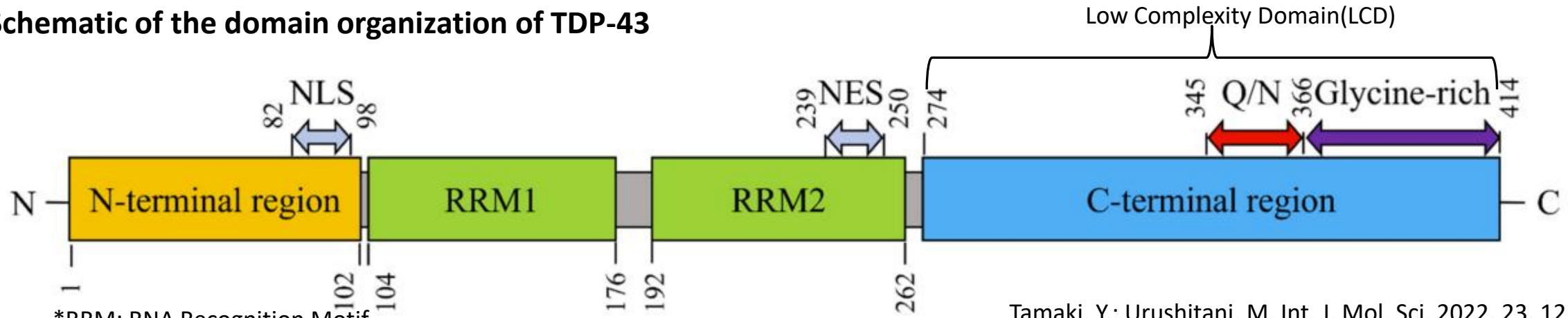
- TDP-43 plays a critical role in multiple aspects of RNA metabolism, such as mRNA splicing.

- Pathological hallmarks in patients with ALS :cytoplasmic mislocalization, aggregation, hyperphosphorylation of TDP-43

- The exact molecular mechanism of TDP-43–mediated neurodegeneration remains unclear.

TDP-43

Schematic of the domain organization of TDP-43



*RRM: RNA Recognition Motif

Tamaki, Y.; Urushitani, M. Int. J. Mol. Sci. 2022, 23, 12508.

・Cleaved C-terminal fragments of TDP-43 accumulate in the brain and the spinal cord of patients with ALS.

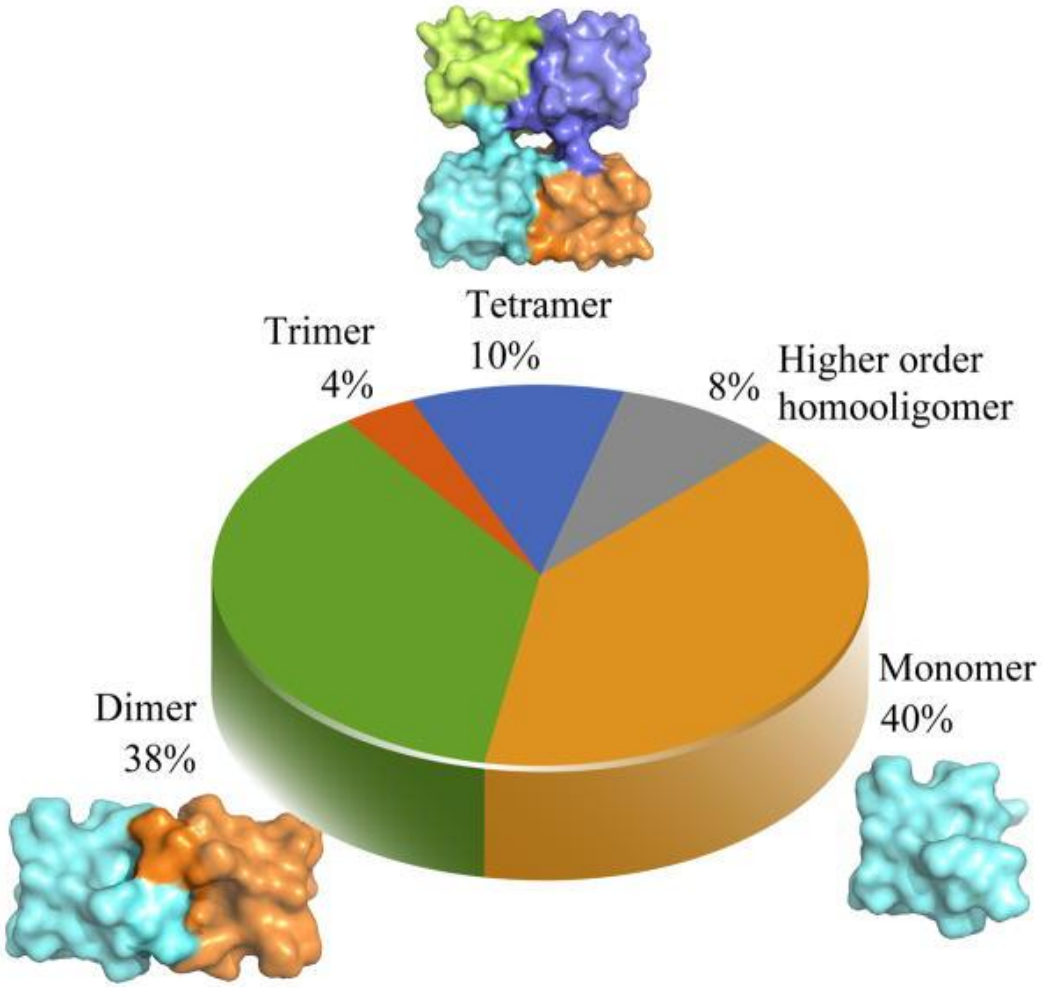
・Almost all ALS-linked mutations are located in the C-terminal domain(CTD).

→The CTD is a critical component of TDP-43 pathology in ALS.

・The functions of the N-terminus of TDP-43 remain largely unknown, but recent studies have highlighted the functional significance of **TDP-43 NTD**.

Contents

Many proteins form dimeric/multimeric complexes



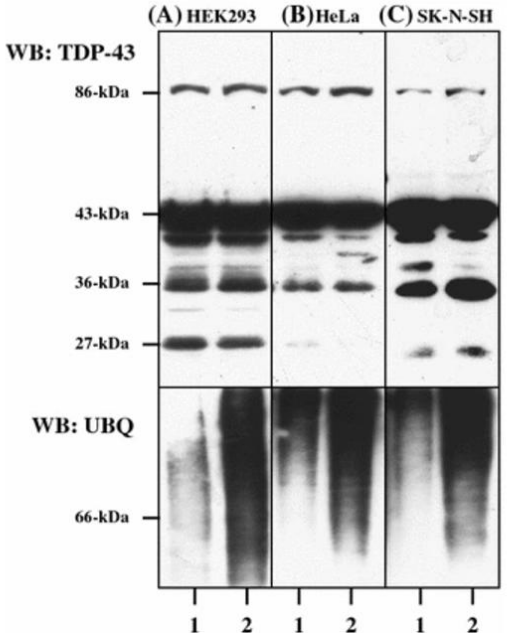
Kosuke Hashimoto et al 2011 Phys. Biol. 8 035007

Distribution of homooligomeric states in a non-redundant set of Protein Data Bank (PDB) structures.

- Proteins in biological systems often bind other biomolecules.
- Dimerization/multimerization can confer several different structural and functional advantages to proteins.

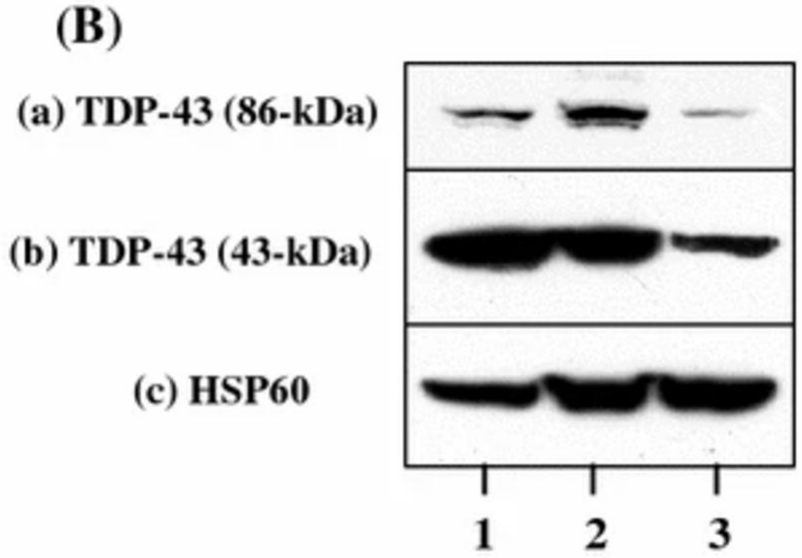
The dimerization of TDP-43

The constitutive expression of an 86-kDa TDP-43 in human cell lines in culture



(exposed for 24 h to the vehicle (DMSO) (*lane 1*) or 1-μM MG-132 (*lane 2*))

The effects of transient expression of TDP-43 siRNA



The lanes (*1–3*) represent non-transfected cells, the cells transfected with the vector of a scrambled sequence, and the cells transfected with the vector of TDP-43 siRNA, respectively.

Shiina, Y., *et al. Cell Mol. Neurobiol* .**30**, 641–652 (2010).

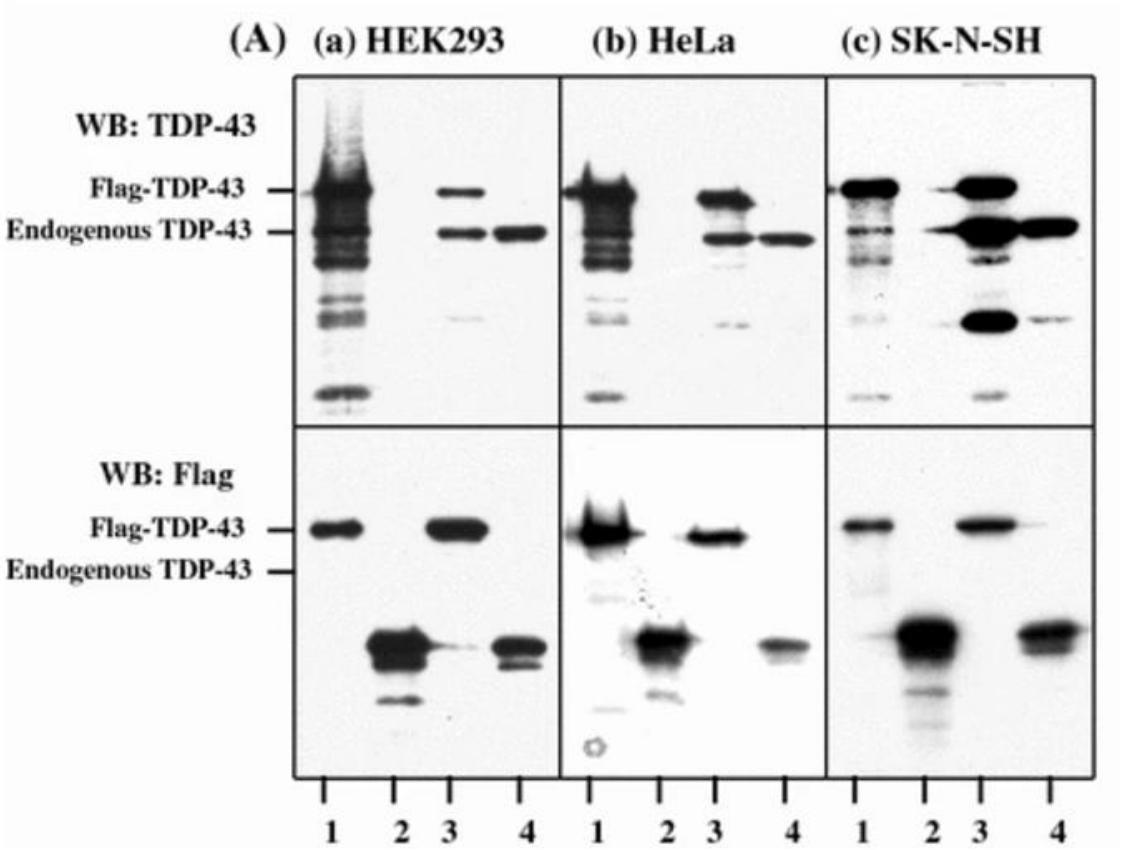
- A discernible amount of the 86-kDa TDP-43-immunoreactive band is expressed in all the cells examined.
- Transient expression of the siRNA vector targeted specifically to TDP-43 substantially reduced the expression of both 43- and 86-kDa TDP-43.

→suggesting that the 86-kDa protein is composed of TDP-43

The dimerization of TDP-43

Coimmunoprecipitation with Flag-tagged proteins

Fig. 4



Flag-tagged FL TDP-43 (*lanes 1* and *3*) or GFP (*lanes 2* and *4*) The lanes (*1–4*) represent (*1* and *2*) the immunoprecipitates and (*3* and *4*) the corresponding input controls.

The proteins binding to tagged TDP-43 always included the endogenous FL TDP-43 protein.

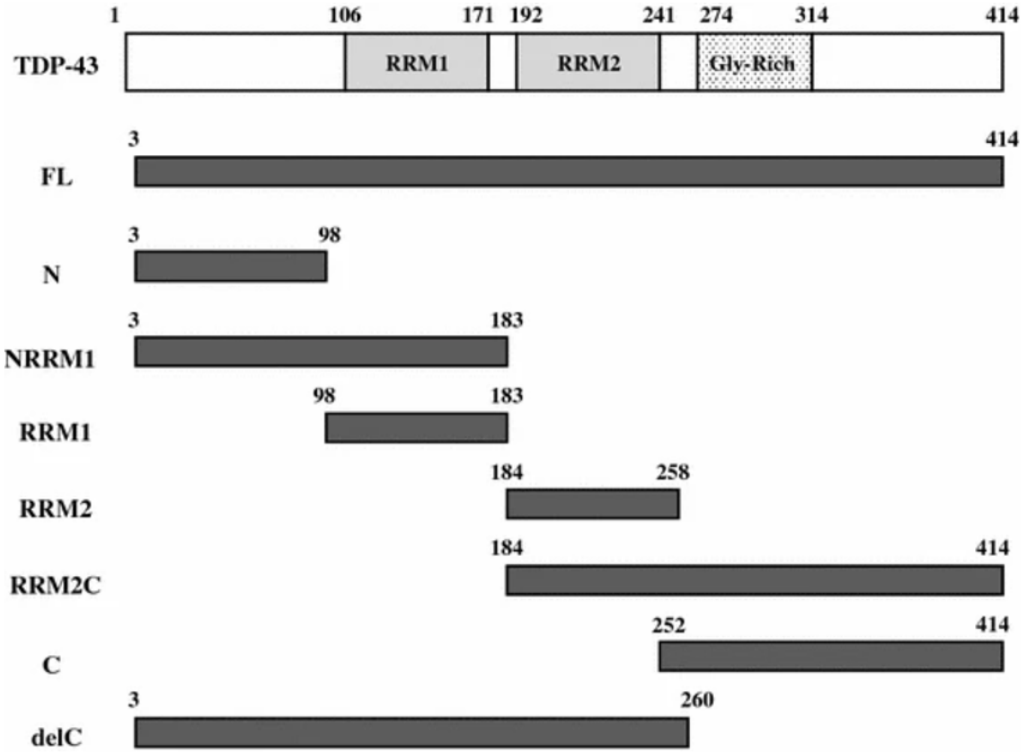
→suggesting that TDP-43 intrinsically forms the dimer.

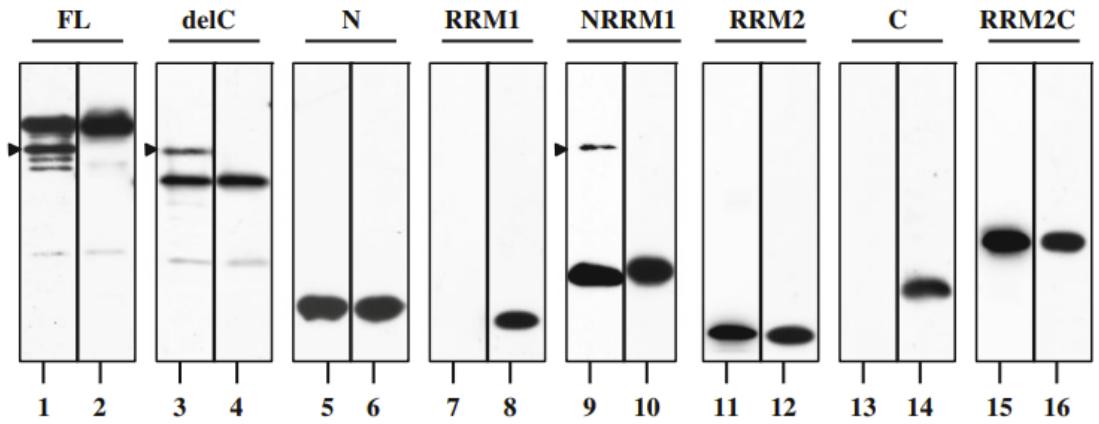
Shiina, Y., *et al. Cell Mol. Neurobiol.* **30**, 641–652 (2010).

The dimerization of TDP-43

The domains involved in the intermolecular interaction of TDP-43 proteins

Fig. 1



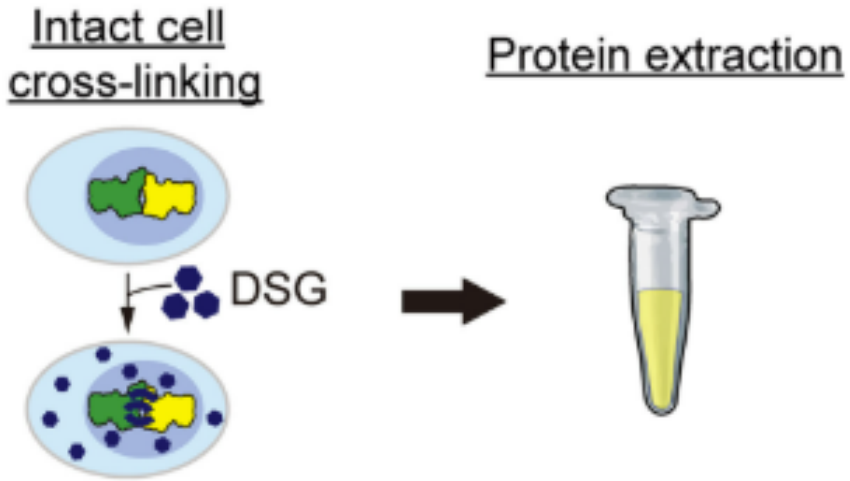


Western blot with anti-TDP-43 antibody (*lanes 1*, *3*, *5*, *7*, *9*, *11*, *13*, and *15*) or anti-Flag M2 antibody (*lanes 2*, *4*, *6*, *8*, *10*, *12*, *14*, and *16*). Extracted from HEK293 cells.

Shiina, Y., *et al. Cell Mol. Neurobiol.* **30**, 641–652 (2010).

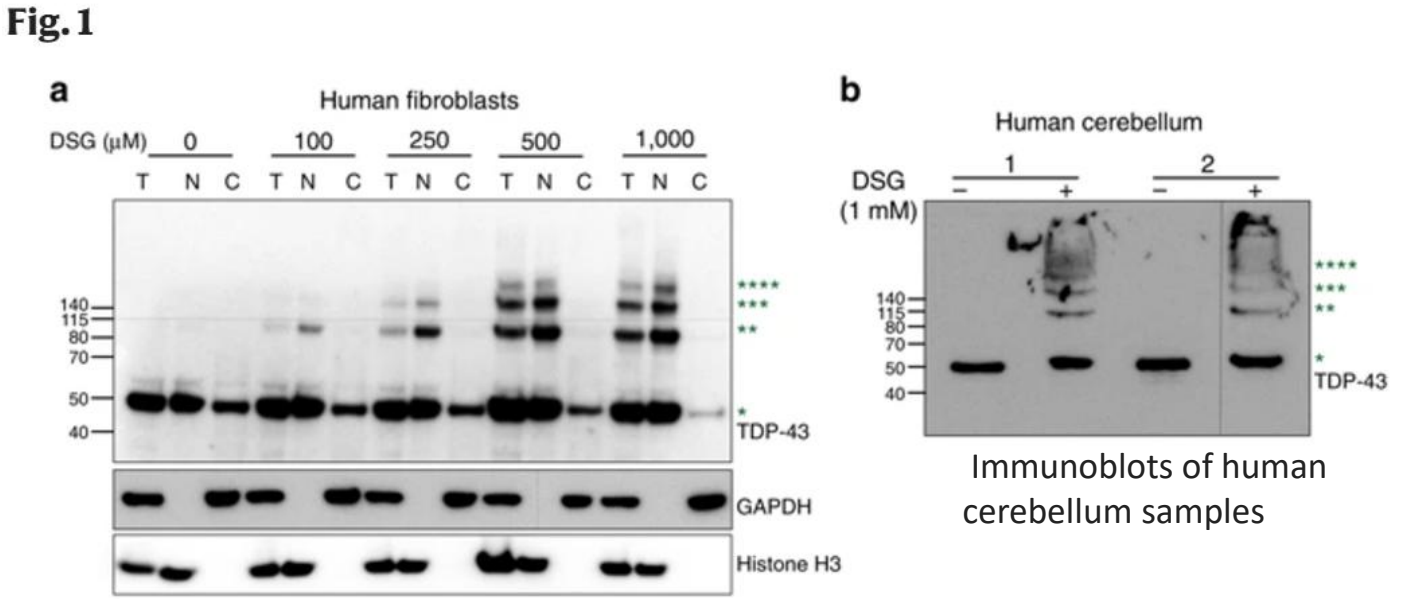
- N-terminal half domain is sufficient for the intermolecular interaction of TDP-43, possibly by constructing a conformationally ordered interacting domain.
- **TDP-43 seems to natively dimerize or exist in a monomer-dimer equilibrium.**

The multimerization of TDP-43



DSG cross-linking assay

Kotaro Oiwa *et al.* , *Sci.Adv.***9**,eadf6895(2023).



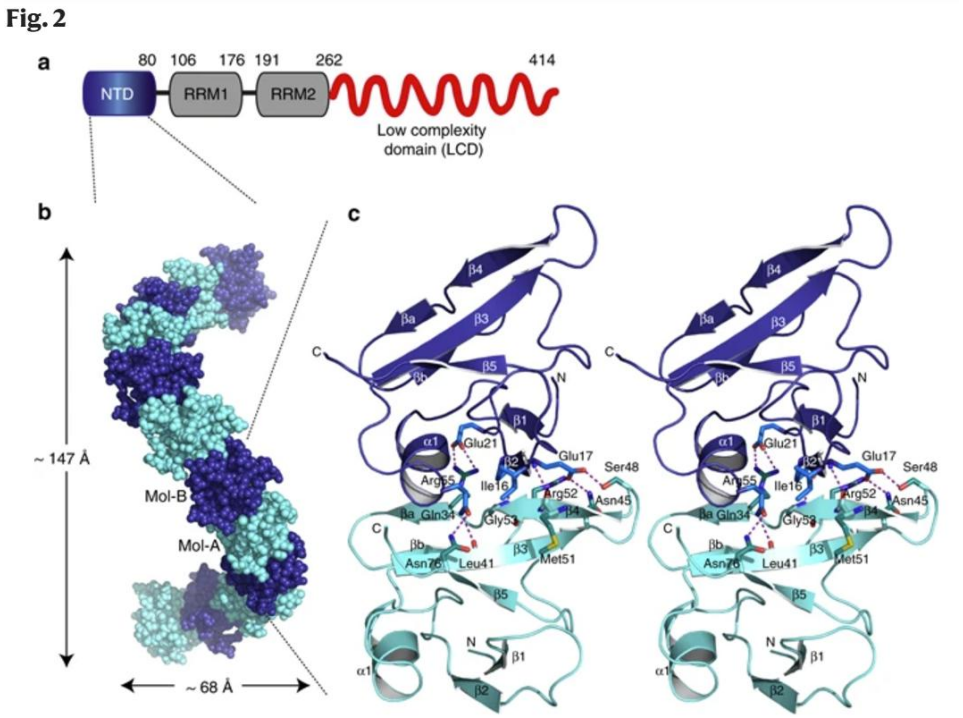
Immunoblots of human fibroblast fractions
*C : cytoplasm N : nuclear

Immunoblots of human cerebellum samples

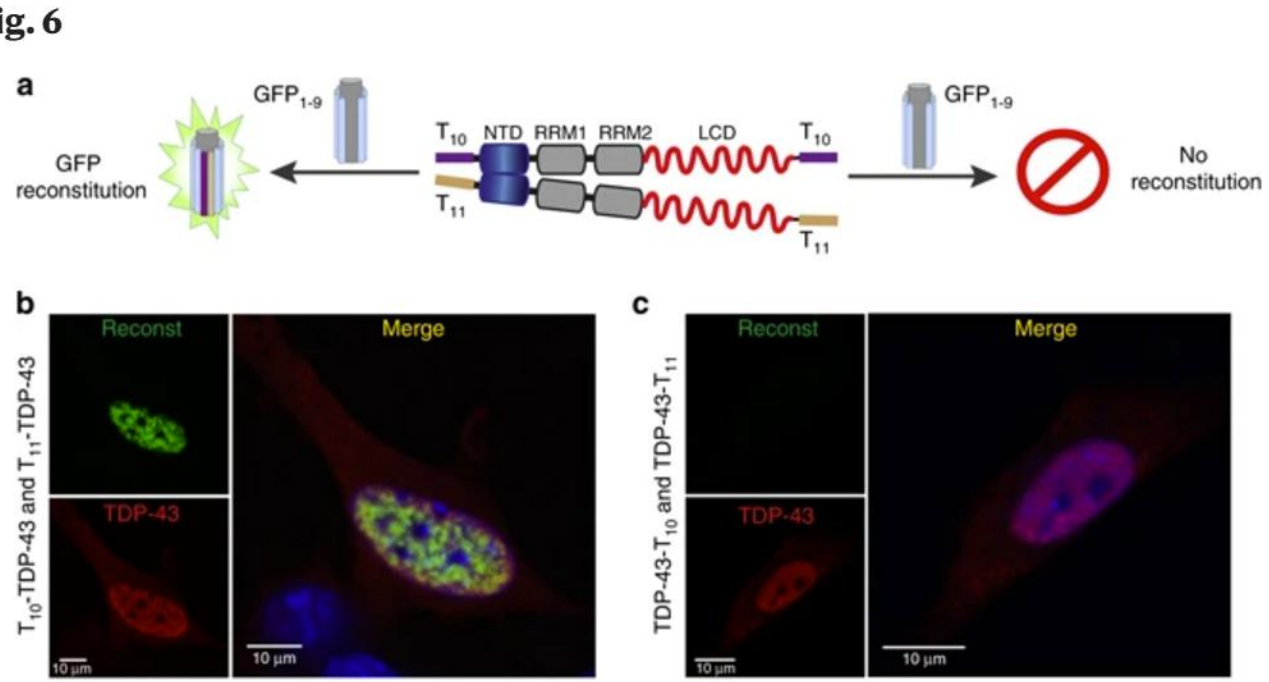
Afroz, *et al. Nat. Commun.* **8**, 45 (2017).

- TDP-43 forms physiological multimers in human tissues.
- Multimeric TDP-43 species are detected predominantly in the nucleus.

TDP-43 multimerization impedes pathologic aggregation



Crystals of TDP-43 N-terminal domain(NTD) showing helical filaments with single molecules arranged in head-to-tail fashion

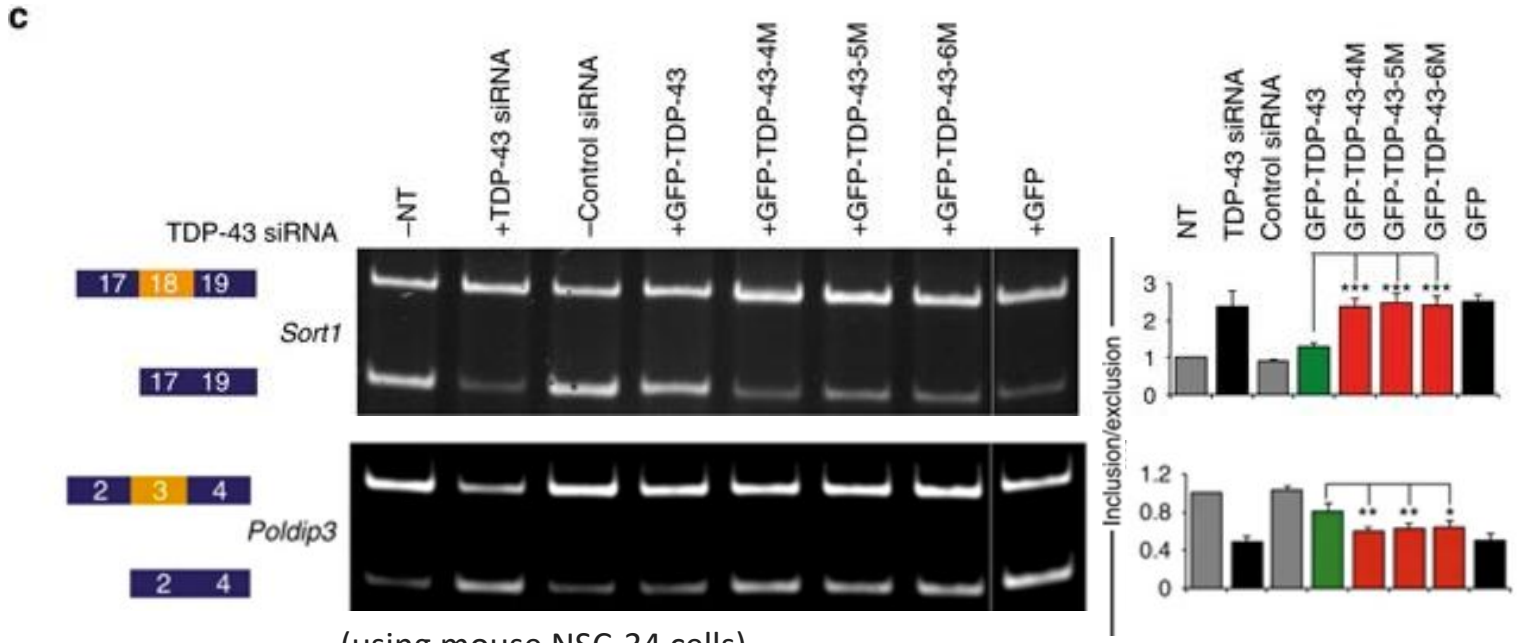


Tripartite GFP complementation assay(in mouse C17.2 cells)

Afroz, *et al. Nat. Commun.* **8**, 45 (2017).

- NTD-mediated TDP-43 multimerization impedes inter-molecular LCD interactions by distantly locating them from each other and antagonizes pathologic protein aggregation.

TDP-43 multimerization is crucial for RNA splicing regulation



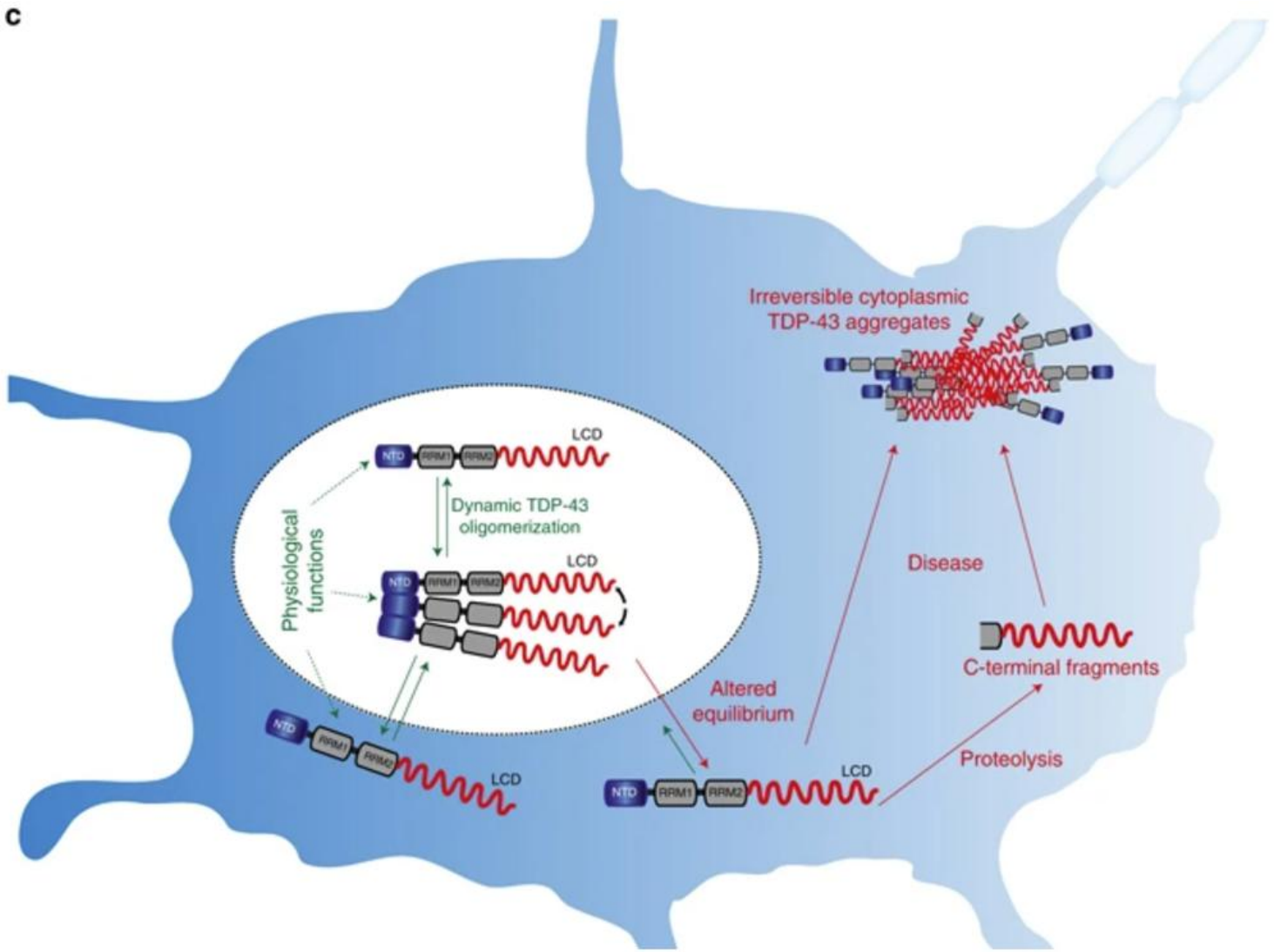
(using mouse NSC-34 cells)

Afroz, *et al. Nat Commun* **8**, 45 (2017).

- Downregulation of TDP-43 resulted in missplicing of specific RNA targets, which was rescued by co-transfection with wild type TDP-43.
- The multimerization-deficient mutants failed to rescue RNA missplicing.

→NTD-driven TDP-43 multimerization is essential for its function in RNA splicing.

Role of TDP-43 multimerization in physiological conditions & in disease



Afroz, *et al. Nat Commun* **8**, 45 (2017).

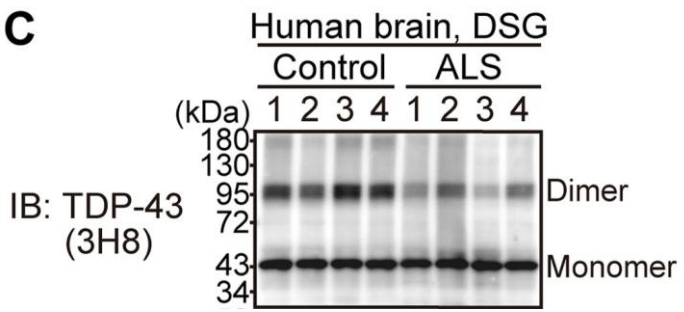
- Within the nucleus, TDP-43 multimerizes via the NTD.

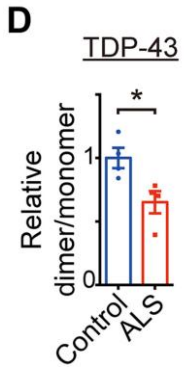
- TDP-43 multimerization prevents irreversible aggregation by spatially separating the LCDs.

- Disturbance in the equilibrium between the oligomeric and monomeric TDP-43 in the cytoplasm may result in proteolytic cleavage of exposed monomeric TDP-43 and generation of C-terminal fragments lacking the NTD, which may initiate formation of irreversible pathologic aggregates via the LCD.

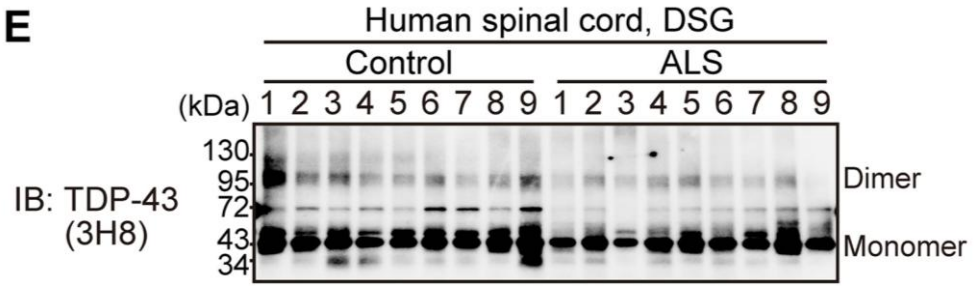
Contents

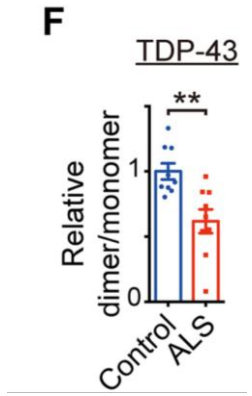
TDP-43 dimerization/multimerization in patients with ALS



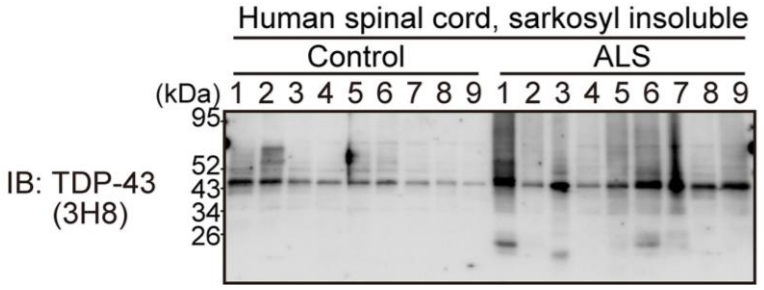


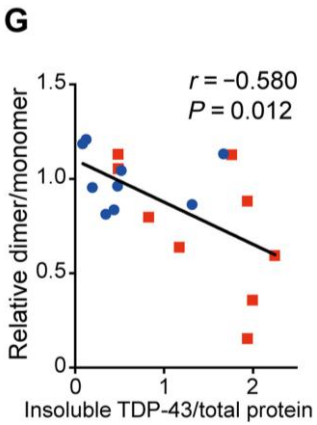
Representative immunoblots (IB) of DSG–cross-linked postmortem brain samples & Quantification of the dimer/monomer ratio of TDP-43





Representative immunoblots (IB) of DSG–cross-linked samples from postmortem spinal cord tissues & Quantification of the dimer/monomer ratio of TDP-43





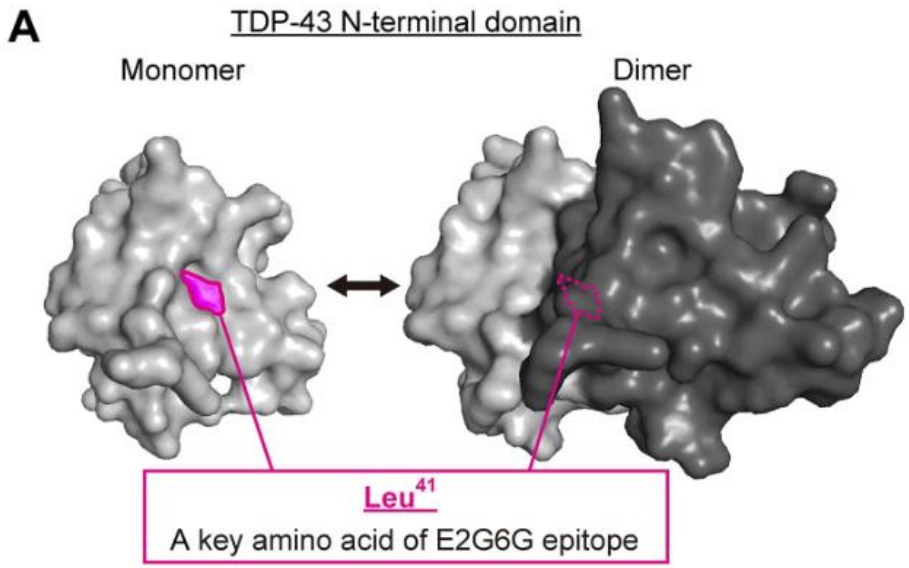
Representative immunoblots (IB) of sarkosyl-insoluble samples from postmortem spinal cord tissues & correlation dot plots of the dimer/monomer ratio (relative to the mean level of the control samples) of TDP-43 and relative levels of sarkosyl-insoluble TDP-43

・The dimer/monomer ratio of TDP-43 was reduced in the affected brain & spinal cord tissues of ALS cases.
・The TDP-43 dimer/monomer ratio was negatively correlated with the insoluble TDP-43 levels in the spinal cord sample

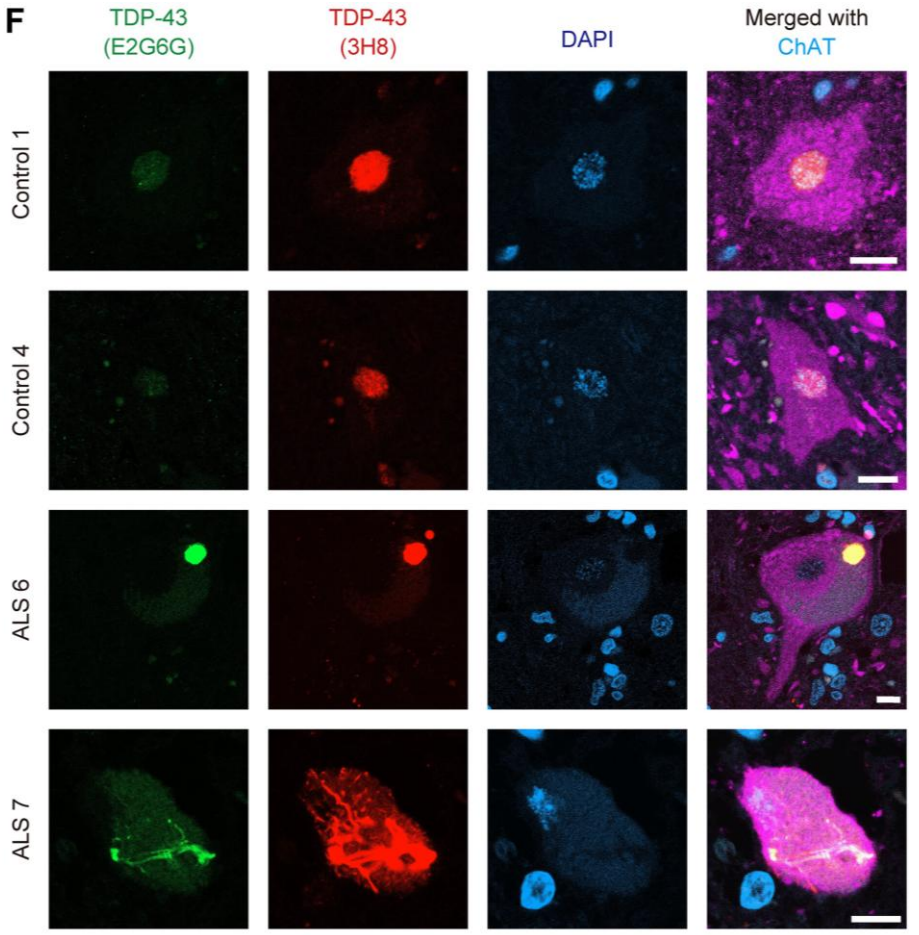
→TDP-43 dimerization/multimerization is impaired in the CNS tissue of patients with ALS
→TDP-43 dimerization/multimerization is more impaired in lesions with more severe TDP-43 pathology.

Kotaro Oiwa *et al.* , *Sci.Adv.***9**,eadf6895(2023).

TDP-43 monomer constitutes pathological inclusions in ALS motor neurons



(**A**) Three-dimensional models of the monomeric and dimeric NTD of TDP-43 as predicted by AlphaFold2. The critical amino acid of the anti–TDP-43 monoclonal antibody (E2G6G) epitope, Leu^{41}, is indicated.



(**F**) Representative images of spinal motor neurons from controls and patients with sporadic ALS, with similar results within the groups, immunostained with E2G6G, anti–panTDP-43 (3H8), and anti–choline acetyltransferase (ChAT).

*DAPI: marker of nucleus
ChAT: marker of motor neuron

Kotaro Oiwa *et al.* , *Sci.Adv.***9**,eadf6895(2023).

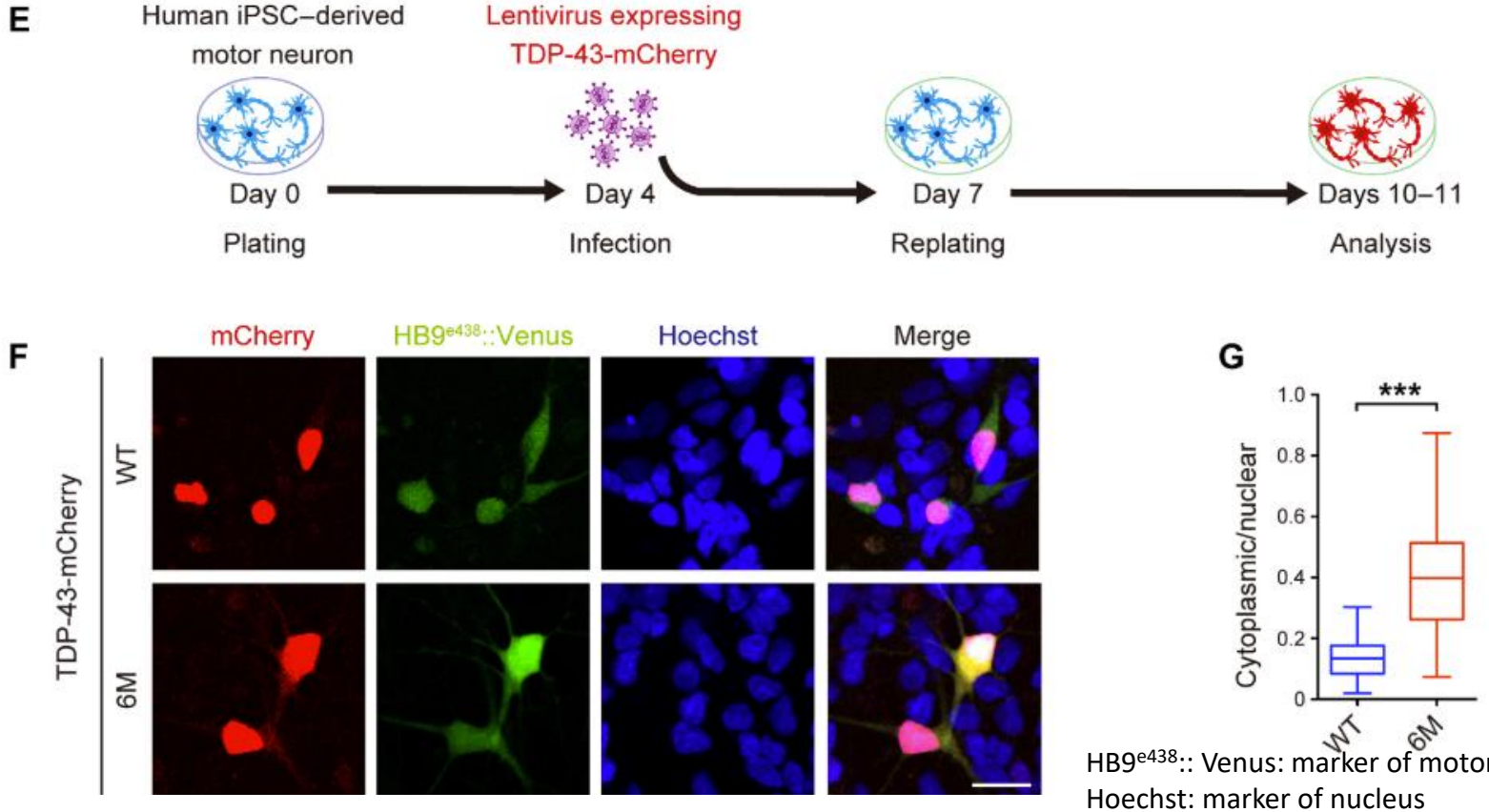
- E2G6G strongly stained the pathological aggregates of TDP-43 in the motor neurons from ALS cases.

→TDP-43 monomer constitutes the pathological inclusions in ALS motor neurons.

Contents

Monomerization of TDP-43 induces its cytoplasmic mislocalization

※NDD TDP-43 = N-terminal dimerization–deficient TDP-43



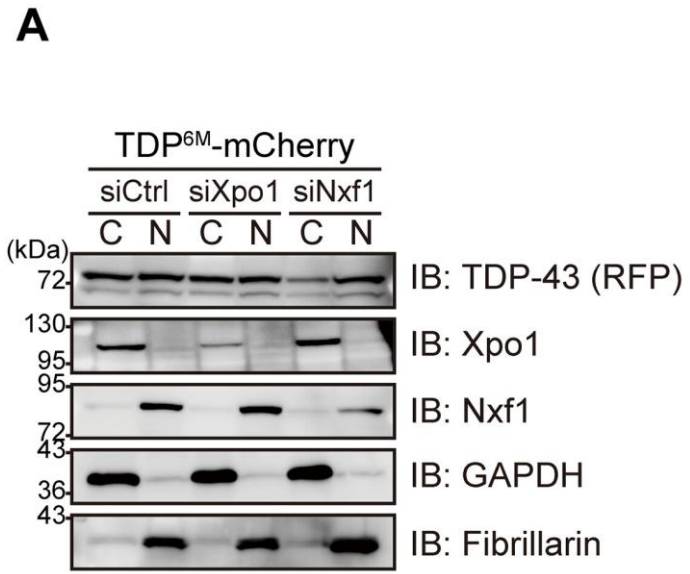
(**E**) Experimental protocol
(**F**) representative images of human iPSC–derived motor neurons infected with lentivirus expressing HB9^{e438}::TDP-43^{WT}- or HB9^{e438}::TDP-43^{6M}-mCherry-IRES-Venus.
(**G**) Box and whisker plots of the cytoplasmic/nuclear ratios of TDP-43-mCherry fluorescence quantified from (F)

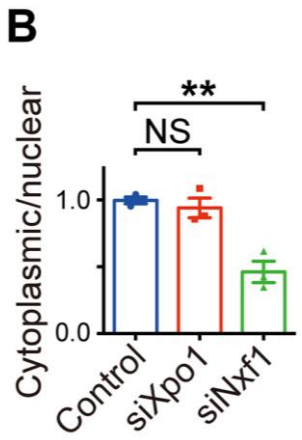
HB9^{e438}:: Venus: marker of motor neuron
Hoechst: marker of nucleus

Kotaro Oiwa *et al.* , *Sci.Adv.***9**,eadf6895(2023).

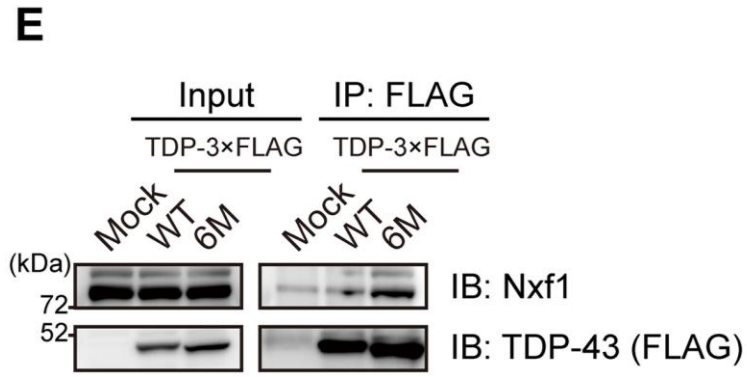
・The mislocalization of the NDD TDP-43 mutant into the cytoplasm was confirmed in human induced pluripotent stem cell (iPSC)–derived motor neurons
→Indicating that the monomerization of TDP-43 promotes its cytoplasmic mislocalization in human motor neurons.

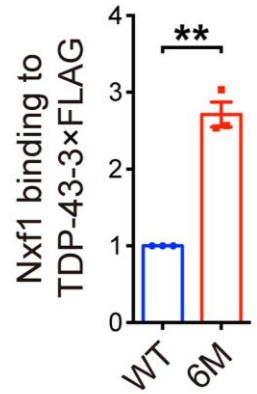
Nxf1 mediates cytoplasmic transport of TDP-43 monomers





(**A**) Neuro2a cells transiently expressing TDP-43^{6M}-mCherry were treated with control siRNA or siRNAs targeting Xpo1 or Nxf1, followed by nucleocytoplasmic fractionation. GAPDH and fibrillarin were used as the cytoplasmic and nuclear markers, respectively.
(**B**) Relative cytoplasmic/nuclear ratios of TDP-43-mCherry in (A)
*C : cytoplasm N : nuclear
Xpo1 : exportin-1 Nxf1: nuclear RNA export factor-1





(**E**) TDP-43-3 × FLAG WT or 6M mutant was transiently transfected in the Neuro2a cells. An anti-FLAG antibody was used for immunoprecipitation of the lysates. Bound endogenous Nxf1 was detected by immunoblotting with an anti-Nxf1 antibody.
(**F**) Relative Nxf1 levels bound to TDP-43-3 × FLAG

Kotaro Oiwa *et al.* , *Sci.Adv.***9**,eadf6895(2023).

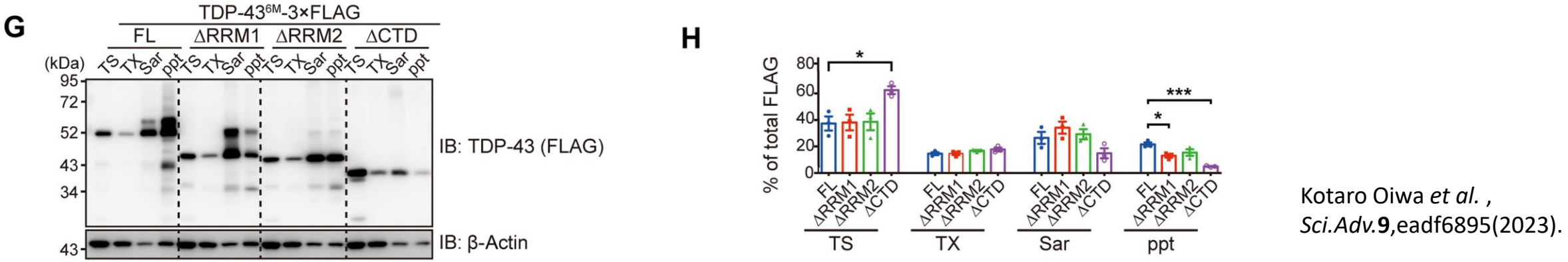
- The suppression of Nxf1 substantially prevented the mislocalization of TDP-43^{6M}.
- The affinity of TDP-43^{6M} for Nxf1 was higher than that of TDP-43^{WT} .

→suggesting that Nxf1 recognizes TDP-43 monomers rather than dimers and mediates their nuclear export.

Contents

TDP-43 monomerization induces insoluble aggregates

(**E**) Representative immunoblots of Neuro2a cells transiently expressing TDP-43-3 × FLAG WT and mutants. Proteins sequentially extracted with tris-HCl (TS), 1% Triton X-100 (TX), and 1% sarkosyl (Sar), as well as a sarkosyl-insoluble fraction (ppt), were analyzed by immunoblotting. (**F**) Quantification of the percentage of each fraction relative to the total amount of TDP-43-3 × FLAG in (E).



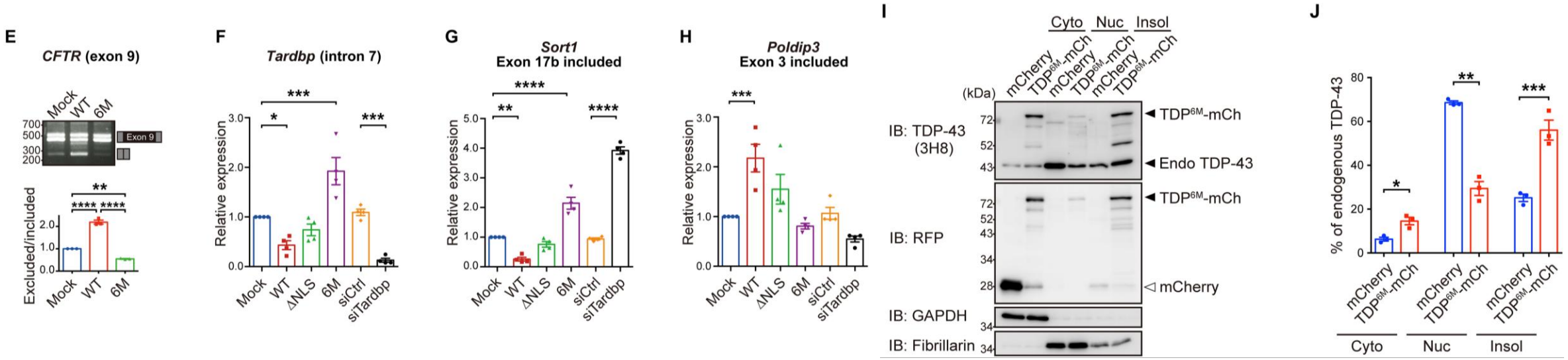
Kotaro Oiwa *et al.* , *Sci.Adv.***9**,eadf6895(2023).

(**G**) Representative immunoblots of the sequentially extracted lysates from Neuro2a cells transiently expressing TDP-43^{6M}-3×FLAG full-length (FL) and deletion mutants. (**H**) Quantification of the percentage of each fraction to the total amount of TDP-43-3×FLAG in (G).

- All NDD-TDP-43 variants except TDP-43$^{\Delta NLS}$ were significantly enriched in the sarkosyl-insoluble fraction than TDP-43^{WT} .
- TDP-43^{6M} revealed that the CTD was the key domain for aggregation.

→ indicating that NDD-TDP-43 forms insoluble aggregates through CTD.

TDP-43 monomer inhibits the physiological functions of endogenous TDP-43



(**E**) Exon skipping assay for *CFTR* exon 9 (top). Quantification of the exclusion/inclusion ratio of *CFTR* exon 9 (relative to mock-transfected cells) was performed from the band intensities (bottom). (**F & G**) Quantification of RNA levels of *Tardbp* intron 7 (F) & *Sort1* (G) with indicated exon inclusion. Neuro2a cells were transiently transfected with TDP-43, control siRNAs, or siRNAs targeting TDP-43. (**I**) Representative immunoblots showing endogenous TDP-43 and mCherry levels in cytoplasmic (Cyto), nuclear (Nuc), and 1% Triton X-100 insoluble fractions (Insol) of Neuro2a cells transiently expressing mCherry or TDP-43^{6M}-mCherry. (**J**) Quantification of the percentage of endogenous TDP-43 in each fraction to the total endogenous TDP-43 in (I).

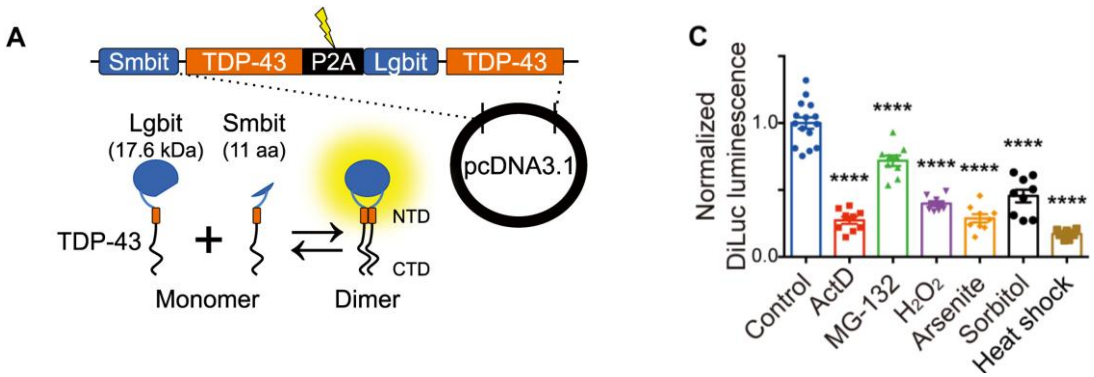
Kotaro Oiwa *et al.* , *Sci.Adv.***9**,eadf6895(2023).

- TDP-43^{6M} lost its ability to skip exon 9 of the *CFTR* gene and to regulate the splicing of *Tardbp, Sort1, Poldip3.*
- TDP-43^{6M} showed a phenotype similar to that obtained after siRNA-mediated TDP-43 knockdown.
- The overexpression of TDP-43^{6M} reduced the amount of endogenous TDP-43 in the nucleus and induced its accumulation in the insoluble fraction.

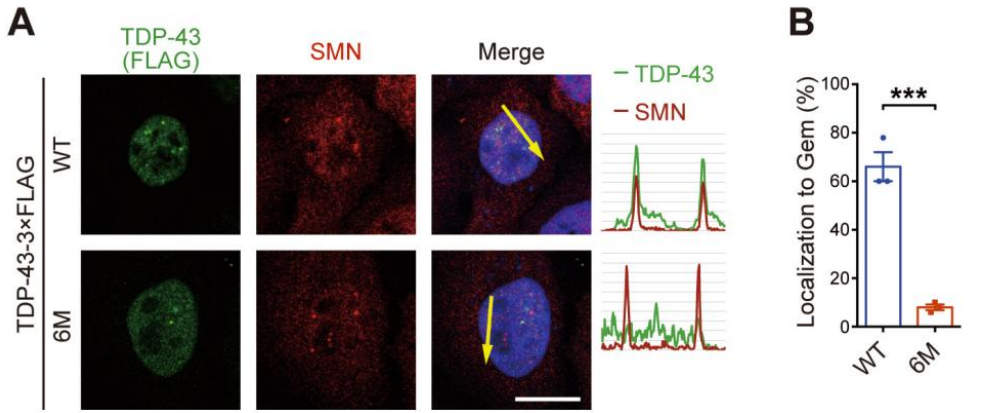
→suggesting that monomeric TDP-43 aggregates induce cytotoxicity by losing the function as splicing regulator & sequestrating the endogenous TDP-43 into the aggregates.

Contents

Spliceosomal integrity is essential for TDP-43 dimerization/multimerization



(**A**) Schematic illustration of TDP-DiLuc, a NanoBiT-based complementation assay to monitor the TDP-43 dimerization/multimerization. (**C**) Quantification of the TDP-43 dimerization under stress conditions.



(**A**) Representative confocal images of HeLa cells transiently expressing TDP-43-3 × FLAG WT or 6M stained with anti-SMN and anti-FLAG antibodies, along with line scan analysis for colocalization of TDP-43 and SMN. Scale bar, 10 μm. (**B**) The percentage of the TDP-43-positive Gems per total Gems was quantified in 100 Gems from three independent experiments.

・Dimerization/multimerization of TDP-43 was impaired to different degrees by all of the examined stresses.

・TDP-43^{6M} lost its localization to the Gems.

Hypothesis : Abnormalities in the Gems under stress conditions might be responsible for TDP-43 monomerization.

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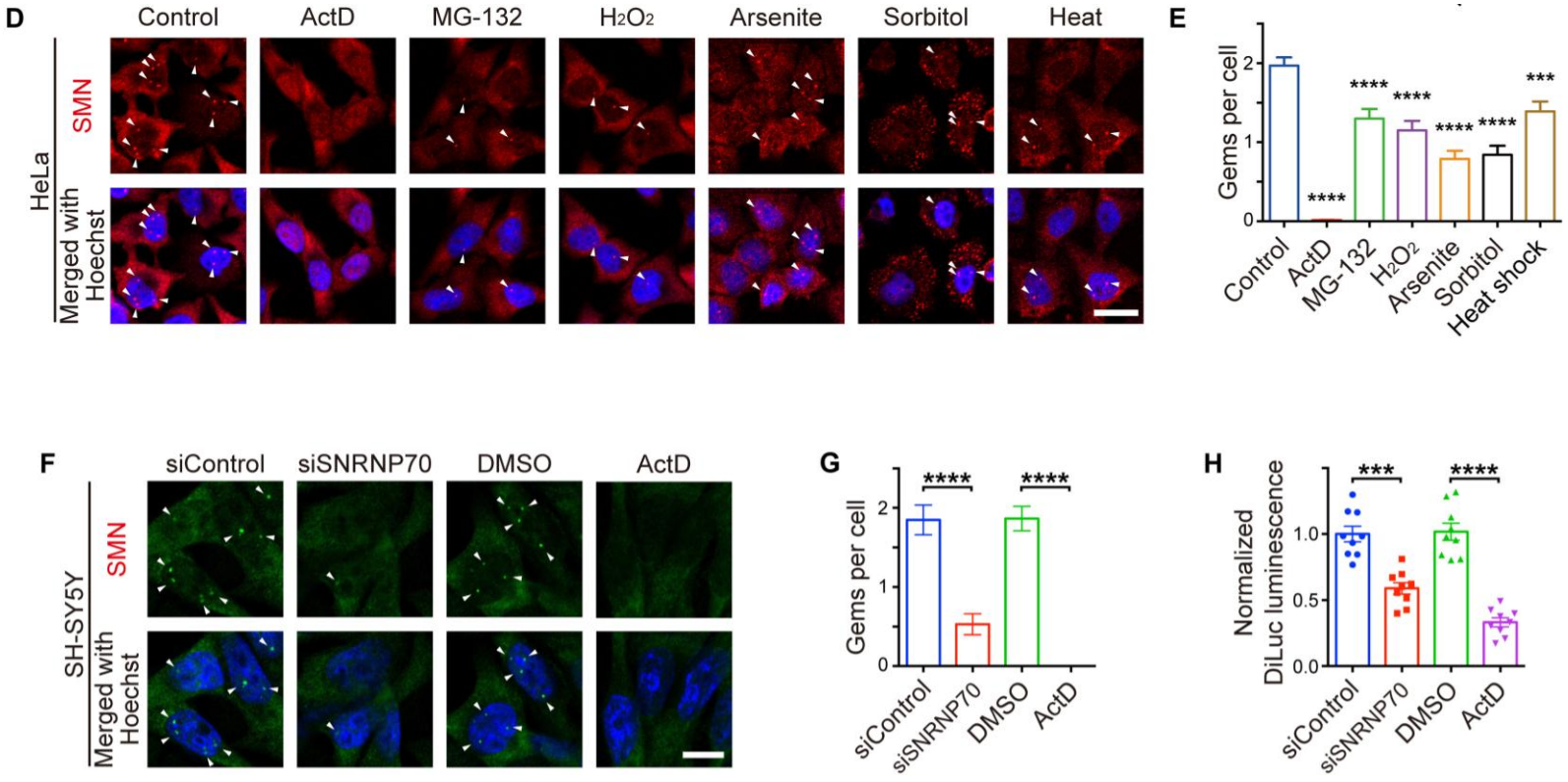
Spliceosomal integrity is essential for TDP-43 dimerization/multimerization



(**D**) Representative confocal images of HeLa cells treated with the indicated reagents followed by staining with anti-SMN. Arrowheads indicate SMN-positive Gems. (**E**) Quantification of Gems per cell in (D). (**F**) Representative confocal images of SH-SY5Y cells transiently transfected with control siRNA or siRNA targeting SNRNP70. Cells treated with ActD (5 μg/ml; 3 hours) served as the positive control. Arrowheads indicate SMN-positive Gems. (**G**) Quantification of Gems per cell in (F). (**H**) Quantification of the relative DiLuc luminescence in SH-SY5Y cells transiently transfected with TDP-DiLuc and control siRNA or siRNA targeting SNRNP70. Cells treated with ActD (5 μg/ml; 3 hours) served as the positive control.

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- All stresses decreased the number of Gems represented.
- Disrupting the spliceosome by the siRNA-mediated knockdown of SNRNP70 decreased the number of Gems and TDP-43 dimerization/multimerization.

→suggesting that loss of spliceosome integrity by some stress,such as transcriptional inhibition, reduces Gems, which damages TDP43 dimerization/multimerization.

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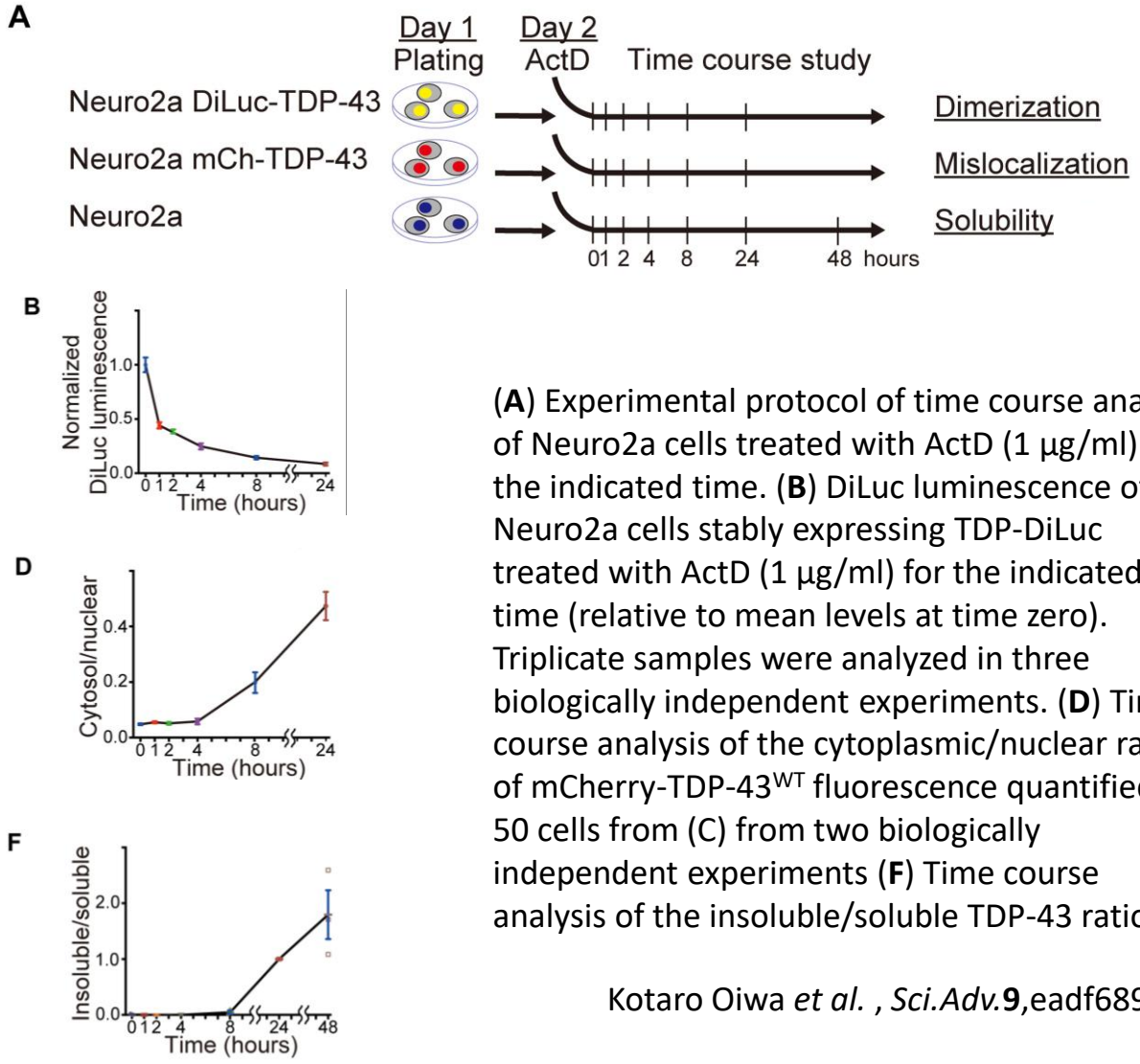
TDP-43 monomerization precedes its pathological changes



(**A**) Experimental protocol of time course analysis of Neuro2a cells treated with ActD (1 μg/ml) for the indicated time. (**B**) DiLuc luminescence of the Neuro2a cells stably expressing TDP-DiLuc treated with ActD (1 μg/ml) for the indicated time (relative to mean levels at time zero). Triplicate samples were analyzed in three biologically independent experiments. (**D**) Time course analysis of the cytoplasmic/nuclear ratios of mCherry-TDP-43^{WT} fluorescence quantified in 50 cells from (C) from two biologically independent experiments (**F**) Time course analysis of the insoluble/soluble TDP-43 ratios.

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- TDP-43 monomerization preceded TDP-43 pathological changes, such as cytoplasmic mislocalization & reduced solubility

→suggesting that the monomerization of TDP-43 NTD precedes the loss of TDP-43 from the nucleus and the formation of aggergates

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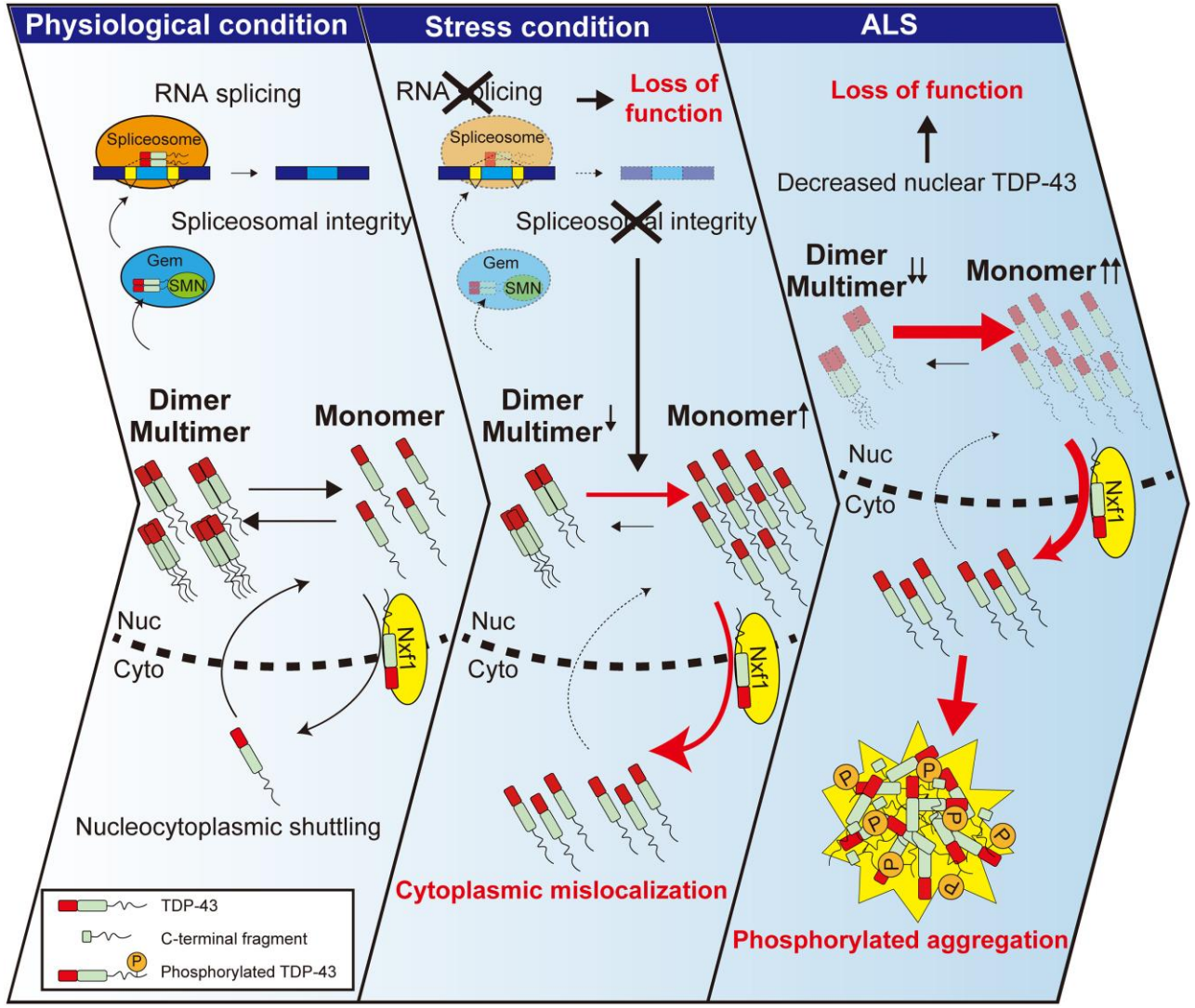
Summary



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○TDP-43 exists in a monomer-dimer/multimer equilibrium in cells.
○The NTD of TDP-43 mediates the formation of physiological homodimers, which are essential for its physiological splicing function.
○The RNA export factor Nxf1 facilitates the nuclear export of TDP-43 monomers.

× Various types of stresses impair TDP-43 dimerization/multimerization and increase amounts of TDP-43 monomers, likely by inducing spliceosomal defects

・Monomeric TDP-43 lost its ability to regulate mRNA splicing.

×Excess of TDP-43 monomers mislocalized in the cytoplasm aggregates with endogenous TDP-43.

Perspectives for diagnosis & treatment

- Focusing on the functional dimerization/multimerization of TDP-43 may be important to develop early diagnostic markers of TDP-43 proteinopathy including ALS.

- Keeping Spliceosomal Integrity(Further research is required)
- Decomposing TDP-43 monomers or aggregates in cytoplasm
- Reducing the ability of TDP-43 to aggregate
- Returning TDP-43 monomers from cytoplasm into nuclear
- Reducing Nxf1 activity of binding to TDP-43 monomers and exporting them from nuclear into cytoplasm