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TITLE: *RNA-Based Approach to Decrease TDP-43 Pathology and Neurotoxicity in ALS*

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CONTRACTING ORGANIZATION: Saint Louis University

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14. ABSTRACT The aggregation of the TAR DNA binding protein (TDP-43) is the hallmark feature of ALS. TDP-43 aggregates found in the brain and spinal cord of almost all ALS patients are associated with toxicity and loss of cell viability. Our project will leverage our position as experts in TDP-43 biology and use the tools we have developed to analyze TDP-43 aggregation and inhibit this pathogenic process. We recently found that RNA molecules are potent inhibitors of TDP-43 aggregation, which leads us to ask: <i>Can specific RNA molecules be used to prevent TDP-43 aggregation and decrease neurotoxicity?</i> This question will be tested using our established methods using purified TDP-43, human cellular models and mouse models of ALS. Dr. Timothy Miller, the co-Principal Investigator in this proposal, and his team are experts in using RNA to reduce neurodegeneration in various models and in first-in-kind patient clinical trials. Together, our team will screen and identify new molecules to effectively reduce TDP-43 aggregation and its associated pathology.					
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1. Introduction.

This proposal is responsive to ongoing efforts to treat and cure Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease. ALS is a devastating neurodegenerative disorder for which there is no cure or efficient detection tools. We seek to accelerate the development of new therapeutic molecules to reduce neurodegeneration and toxicity in ALS.

ALS is a late onset neurodegenerative and fatal disease that affects women and men worldwide and leads to muscle atrophy in patients. In a portion of these cases, ALS is linked to cognitive impairment associated with frontotemporal lobar degeneration or frontotemporal dementia. On average, ALS strikes patients in their 60s and some clinicians have reported an increase in the number of 20-30 year old patients diagnosed with ALS. *According to existing evidence, military men and women are twice as likely to be affected by ALS than non-military individuals*, regardless of whether the individual served in combat or not. Among the possible explanations for this increased risk are elevated stress and traumatic brain injury during training or combat. ALS causes the death of individuals, often from respiratory failure, two to five years post-diagnosis. Therefore, it is imperative to develop strategies to decrease the factors contributing to the development of ALS.

TAR DNA-binding protein (TDP-43) aggregation and loss of function is the hallmark of almost all familial and sporadic ALS cases. TDP-43 inclusions accumulate in neurons and glial cells in the brain and spinal cord. These deposits coincide with a dramatic reduction of normal nuclear TDP-43 detection, implying a loss of function upon aggregation. This is viewed as a major factor impeding neuronal function as TDP-43 is known to be essential for development and survival. Whether disease results from aggregate toxicity, from sequestration of functional TDP-43 into aggregates, or from a combination of both mechanisms is yet unclear. Either way, reducing the accumulation of TDP-43 aggregates is a major goal to decrease neurodegeneration. We found that TDP-43 binding to RNA strongly inhibits its aggregation depending on nucleotide sequence composition. These findings strongly suggest that RNA acts as a chaperone maintaining TDP-43 solubility and leads us to ask: *Can specific RNA molecules be used to prevent TDP-43 aggregation and decrease neurotoxicity?* This question is being tested using our established methods using purified TDP-43, human cellular models and mouse models of ALS. Dr. Timothy Miller, the co-Principal Investigator in this proposal, and his team are experts in using RNA to reduce neurodegeneration in various models and in first-in-kind patient clinical trials. Further, Dr. Miller is the director of the Washington University ALS Center. As a physician, Dr. Miller is dedicated to providing quality care and state-of-the-art treatment to ALS patients. Together, our team will screen and identify new molecules to effectively reduce TDP-43 aggregation and its associated pathology.

2. Keywords

Amyotrophic lateral sclerosis (ALS), neurodegeneration, TDP-43 (TAR DNA binding protein), RNA, protein aggregation, RNA binding proteins

3. Accomplishments

What were the major goals of the project?

Based on our earlier observations, we hypothesize that specific RNA molecules may be used to decrease TDP-43 aggregation, which will reduce toxicity and restore protein function. We sought to design specific RNA molecules that significantly prevent TDP-43 aggregation without causing loss of protein function or cell toxicity and to test their efficacy using various models of TDP-43 pathology. Our project is divided into three specific aims as follows:

Aim 1. Establish RNA-based methods to block TDP-43 aggregation. Our goal is to identify specific RNA molecules that efficiently inhibit TDP-43 aggregation, increase solubility and restore protein function. These experiments will test purified TDP-43 cultured human cell-based models.

Aim 2. Determine whether RNA inhibits intracellular TDP-43 aggregate seeding and propagation. For this aim we are testing the ability of RNA to inhibit intracellular TDP-43 aggregate seeding by pre-formed fibrils. This will be performed using our established reporter cell line and seeding protocols to quantify aggregate spread.

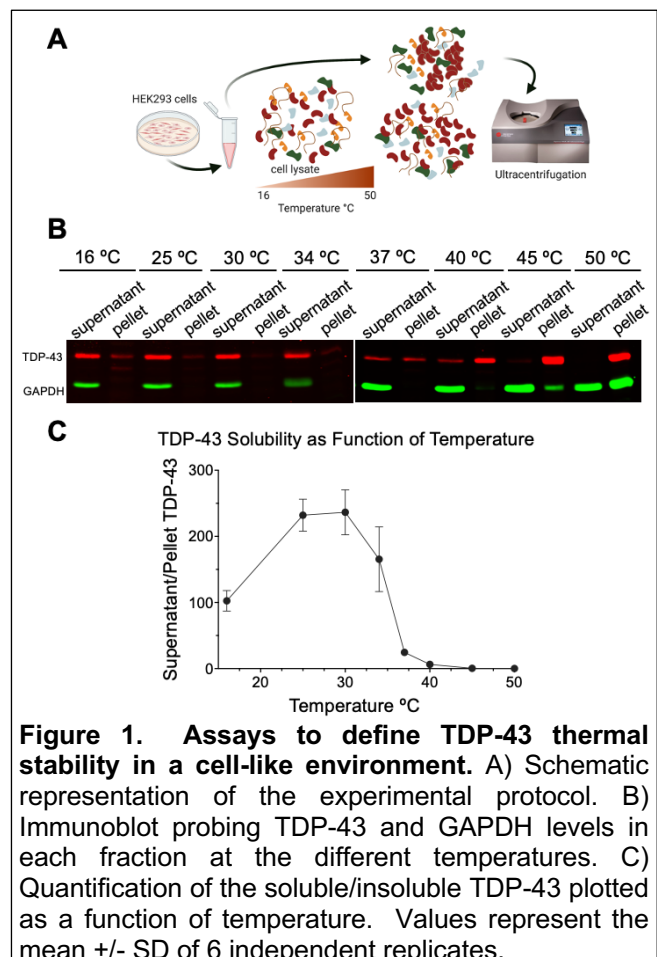
Aim 3. Use RNA to decrease TDP-43 pathology and neurotoxicity in a mouse model of ALS. We are testing the potential use of RNA-based protocols in reducing TDP-43 pathology in vivo using a previously generated mouse model that accumulates TDP-43 pathology and develops strong neurotoxicity. For these studies we are investigating whether RNA decreases TDP-43 deposition and neurodegeneration.

What was accomplished under these goals?

Aim 1. Establish RNA-based methods to block TDP-43 aggregation:

Our work published during the 2nd year of this award demonstrated that specific RNA molecules increase the liquid properties of TDP-43 (1) and prevent the aggregation of the purified protein (2). We found that GU-rich RNA molecules inhibit the conversion of TDP-43 biomolecular condensates to fibrils, or maturation into irreversible aggregates. We speculate that RNA binding prevents the conversion of TDP-43 droplets into solid-like complexes and that RNA may be able to prevent TDP-43 misfolding. Our work also suggests that RNA oligonucleotides may be harnessed to effectively block TDP-43 pathology as early as during the initial aggregation stage.

Our recent observations made during the most recent period of the award are summarized below:



We established a thermal profiling assay to analyze TDP-43 solubility in a cellular context. Because we observed a significant, dose-dependent effect of GU-rich RNA in maintaining TDP-43 solubility in cells, we established an assay to further study this interaction in a controlled cell-like environment. For these assays, we prepared cell lysate from human embryonic kidney cells in culture (HEK293) and incubated fractions at increasing temperatures to generate a ‘melting’ curve for TDP-43 in a cellular context (**Fig. 1A**). Each fraction was then fractionated into soluble and insoluble fractions to measure the ratio of soluble protein in each condition. The different samples were analyzed by immunoblotting to compare the amount of protein in supernatant (soluble) and pellet (insoluble) fractions at each temperature (**Fig. 1B**). These values were represented as the TDP-43 soluble to insoluble ratio as a function of temperature (**Fig. 1C**). As anticipated, we observed a decrease in solubility at higher temperatures because of increased misfolding. We then focused on a temperature that showed TDP-43 in both the soluble and insoluble fractions to test the effect of RNA on TDP-43 misfolding. We found that GU-repeat RNA greatly increased soluble TDP-43 in a dose dependent manner, while a non-specific RNA sequence does not affect TDP-43 solubility. These results provide strong evidence that GU-rich RNA stabilizes the soluble conformation of TDP-43 through specific binding in a cellular environment.

RNA-mediated assembly of TDP-43 in cells retains the protein in the nucleus. We previously found that loss of RNA binding increases TDP-43 mislocalization in the cytoplasm (3). More recently others showed that specific RNA binding to GU-rich RNA promotes nuclear TDP-43 retention (4). We now discovered that RNA mediated assembly of TDP-43 complexes, mediated through multivalent interactions is critical to retain TDP-43 in the nucleus in a size-dependent manner. This is important for multiple reasons: 1) nuclear localization of TDP-43 is important for its function in the regulation of RNA processing, whereby TDP-43 mislocalization into the cytoplasm impairs alters the expression of genes controlled by the protein; 2) increased TDP-43 levels in the

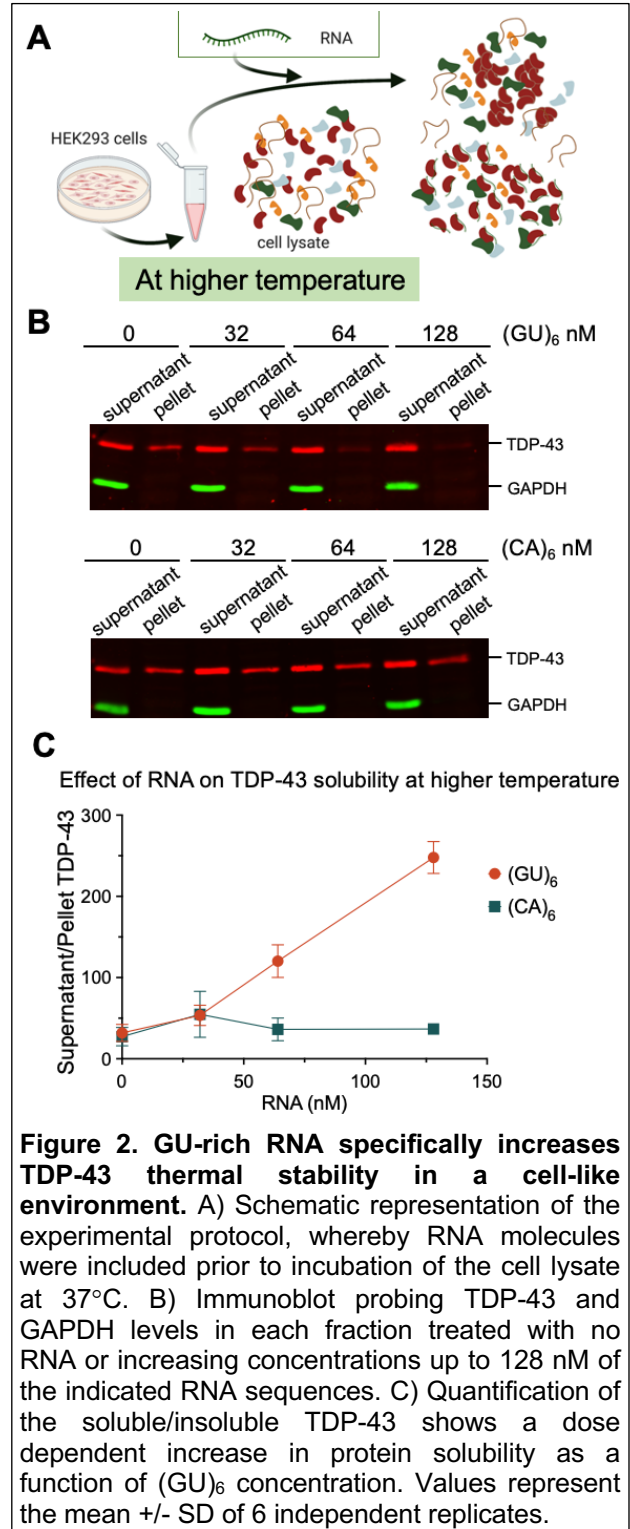


Figure 2. GU-rich RNA specifically increases TDP-43 thermal stability in a cell-like environment. A) Schematic representation of the experimental protocol, whereby RNA molecules were included prior to incubation of the cell lysate at 37°C. B) Immunoblot probing TDP-43 and GAPDH levels in each fraction treated with no RNA or increasing concentrations up to 128 nM of the indicated RNA sequences. C) Quantification of the soluble/insoluble TDP-43 shows a dose dependent increase in protein solubility as a function of (GU)₆ concentration. Values represent the mean $\pm$ SD of 6 independent replicates.

cytoplasm promote protein aggregation. These findings have been submitted as a preprint and the manuscript is under review (5) (see attached). Our work suggests that multiple mechanisms may control TDP-43 cellular distribution by modulating RNA binding as well as interactions mediated by the N- and C-terminal domains. These regulatory factors may be used in future projects to design and develop therapeutic avenues to retain TDP-43 nuclear localization and restore protein function in disease conditions.

RNA treatment of neurons decreases TDP-43-mediated cytotoxicity depending on RNA sequence and chemical modifications. As part of our collaboration with Dr. Sami Barmada (University of Michigan) we tested the effect of RNA oligonucleotide treatment on the survival of rodent primary cortical neurons. These neurons were transfected to express EGFP control or EGFP-tagged wild-type (WT) TDP-43. then followed for 240h by automated microscopy. Neuron death in each group was determined by unbiased image analysis, and relative survival in each condition was visualized and compared using Cox hazards analysis. In cells treated with non-targeting (NT) RNA oligonucleotides, TDP43(WT)-EGFP expression resulted in a significant increase in the risk of cell death compared EGFP (Fig. 3A). Application of (GU)₆ RNA had no effect on EGFP-expressing cells (Fig. 3B), while (CA)₆ RNA moderately

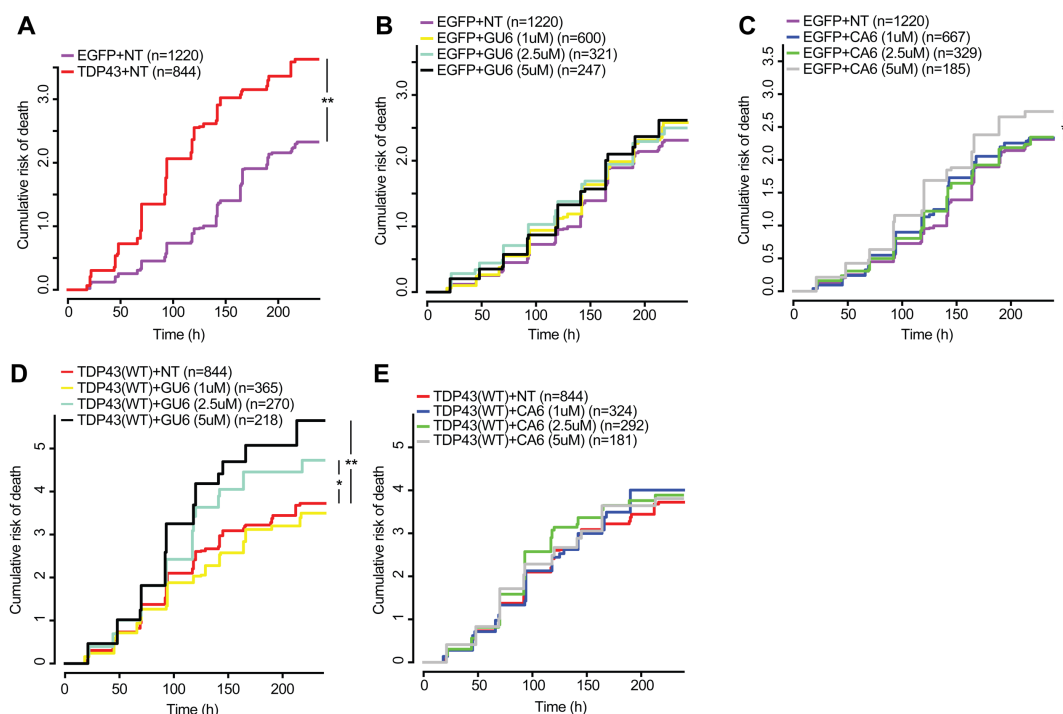


Figure 3. RNA oligonucleotide application affects the survival of primary neurons in a sequence-dependent fashion. (A) Rodent primary cortical neurons were transfected with EGFP or TDP43(WT)-EGFP, then followed for 240h by automated microscopy. Neuron death in each group was determined by unbiased image analysis, and relative survival in each condition was visualized and compared using Cox hazards analysis. In cells treated with non-targeting (NT) RNA oligonucleotides, TDP43(WT)-EGFP expression resulted in a significant increase in the risk of cell death compared EGFP alone [hazard ratio (HR)=2.3, $**p < 2 \times 10^{-16}$.] Application of (GU)₆ RNA (B) had no effect on EGFP-expressing cells, while (CA)₆ RNA (C) moderately increased the risk of death at the highest dose (HR=1.5, $*p = 1.6 \times 10^{-5}$). In contrast, (GU)₆ dose-dependent increased the risk of death in TDP43(WT)-expressing neurons (D; 2.5 μ M, HR=1.3, $*p = 7.4 \times 10^{-4}$; 5 μ M, HR=1.4, $**p = 4.9 \times 10^{-5}$) while (CA)₆ had no appreciable effect (E). All experiments performed with minimum of 3 biological replicates, 8 technical replicates each. N=number of neurons. HR and p values determined by Cox proportional hazards analysis, stratifying among biological replicates.

increased the risk of death at the highest dose (**Fig. 3C**). In contrast, (GU)₆ increased the risk of death in TDP43(WT)-expressing neurons in a dose-dependent manner, while (CA)₆ had no appreciable effect (**Fig. 3D,E**). These results suggest that RNA increases TDP-43-mediated toxicity in a sequence-dependent manner. Under these conditions, the short GU-rich sequence composed of the consensus sequence preference for TDP-43 binding enhances the toxic properties of TDP-43. Based on recent data from the Hayes lab and our own (4, 5), we speculate that this short RNA oligonucleotide induces cytoplasmic egress, which is detrimental to TDP-43 homeostasis and function.

To determine if alternative RNA sequences exert the same effect as (GU)₆ on neurotoxicity, the Barmada lab tested longer GU-rich RNA sequences, CLIP34 and (GU)₁₈. These RNA molecules were used to treat human induced pluripotent stem cells (iNeurons) expressing the ALS-associated TDP-43 mutant M337V or the isogenic WT TDP-43 control. The mutant shows a significant increased risk of death under stress, compared to WT (**Fig. 4A**). Treatment of WT cells with CLIP34, modeled after a natural TDP-43 target RNA sequence, significantly decreased cell survival of WT-expressing cells (**Fig. 4B**). We observed a similar effect of CLIP34 on the survival of M337V-expressing neurons (**Fig. 4C**). However, (GU)₁₈ showed the opposite effect as it reduced M337V cumulative risk of death, compared to control or CLIP34-treated cells. The differences observed between the two RNA sequences may be caused by differences in binding affinity, since (GU)₁₈ binds with approximately 40-fold greater affinity relative to CLIP34. In addition, we observed that while CLIP34 primarily localized to the nucleus after cell uptake, (GU)₁₈ was mostly excluded from this compartment. Therefore, it is possible that nuclear vs. cytoplasmic localization determines the ability of RNA to act as a chaperone for TDP-43. Importantly, our preliminary findings provide evidence of a sequence-

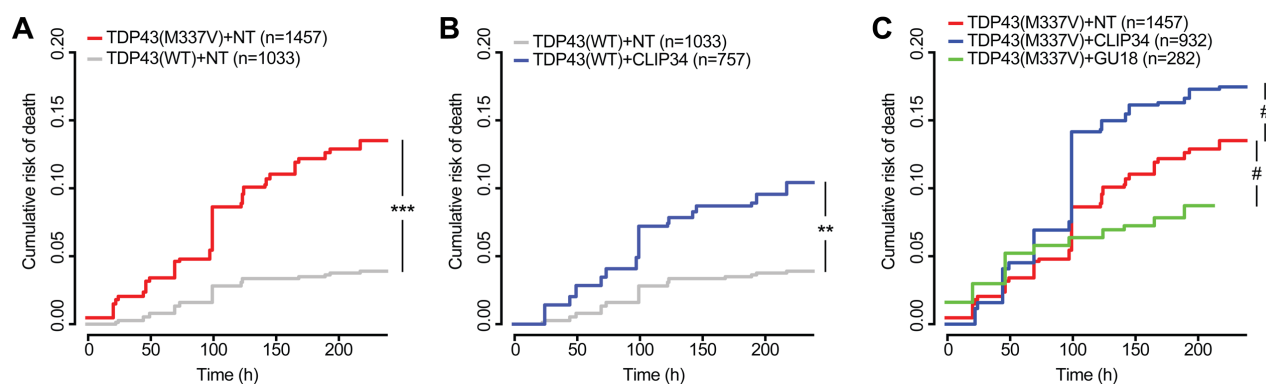


Figure 4. RNA molecules display sequence-related effects in human iNeurons. (A) Human induced pluripotent stem cell-derived neurons (iNeurons) expressing endogenous mutant TDP-43(M337V) or their wild-type (WT) isogenic controls were treated with non-targeting (NT) RNA oligonucleotides, tracked for 240h by automated microscopy, and survival analysis accomplished as before. TDP-43(M337V) iNeurons exhibited a significantly higher risk of death under conditions of nutrient deprivation in comparison to their isogenic WT controls (HR=3.4, **p=5.7x10⁻⁹.) (B, C) CLIP34 ASOs modeled after the native TDP-43 substrate significantly increased the risk of death in TDP-43(WT) iNeurons (HR=2.7, **p=2.6x10⁻⁵), and resulted in a non-significant trend towards toxicity in TDP43(M337V) iNeurons (HR=1.3, #p=0.06). Longer (GU)₁₈ RNA molecules displayed the opposite effect, with a non-significant trend towards neuroprotection in TDP43(M337V) iNeurons (HR=0.7, #p=0.09). All experiments performed with minimum of 2 biological replicates, 8 technical replicates each. N=number of neurons. HR and p values determined by Cox proportional hazards analysis, stratifying among biological replicates.

specific protection TDP-43-mediated neurotoxicity upon RNA treatment.

Aim 2. Determine whether RNA inhibits intracellular TDP-43 aggregate seeding and propagation. We focused on the prion-like behavior of TDP-43, whereby aggregate seeds are capable of nucleating de novo misfolding and aggregation in cells. This is one of the mechanisms thought to drive disease emergence and propagation of ALS and frontotemporal dementia (FTD), based on clinicopathological data (6). Therefore, by measuring the aggregate seeding activity of TDP-43 we may evaluate the toxicity of aggregate species and perhaps establish models to recapitulate features characteristic of TDP-43 proteinopathies and gain understanding of fundamental pathogenic processes. We used two alternative reporter cells based on the expression of fluorescently labelled full-length protein and C-terminal fragments of TDP-43. Nucleation of TDP-43 aggregates in these cells may be easily visualized and quantified. We assayed the effect of RNA on seeding activity with two different paradigms (**Fig. 5**). First, we generated TDP-43 aggregates in the presence of GU-rich RNA before treating cells (**Fig. 5A**) and second, we pre-treated the reporter cells with GU-repeat RNA prior to seeding with FTD-derived insoluble extracts or control tissue (**Fig. 5B**). We measured the fraction of cells positive for a pathological marker, phosphorylated TDP-43 pS409/410 (pTDP-43), and found that in either case GU-rich RNA significantly decreased seeding activity by approximately 50%, or 40% in the case of proteopathic seeds derived from tissue. Both results suggest that RNA protects cellular TDP-43 from prion-like aggregate seeding and are consistent with a chaperone-like activity of RNA to prevent TDP-43 misfolding.

Aim 3. Use RNA to decrease TDP-43 pathology and neurotoxicity in an animal model of ALS. To determine the relevance and test the potential applicability of RNA molecules in decreasing TDP-43 pathology we applied this new approach in a mouse model of ALS, hTDP-43 Δ NLS (7). This is one of the few ALS mouse models that recapitulates TDP-43 pathology and neurotoxicity,

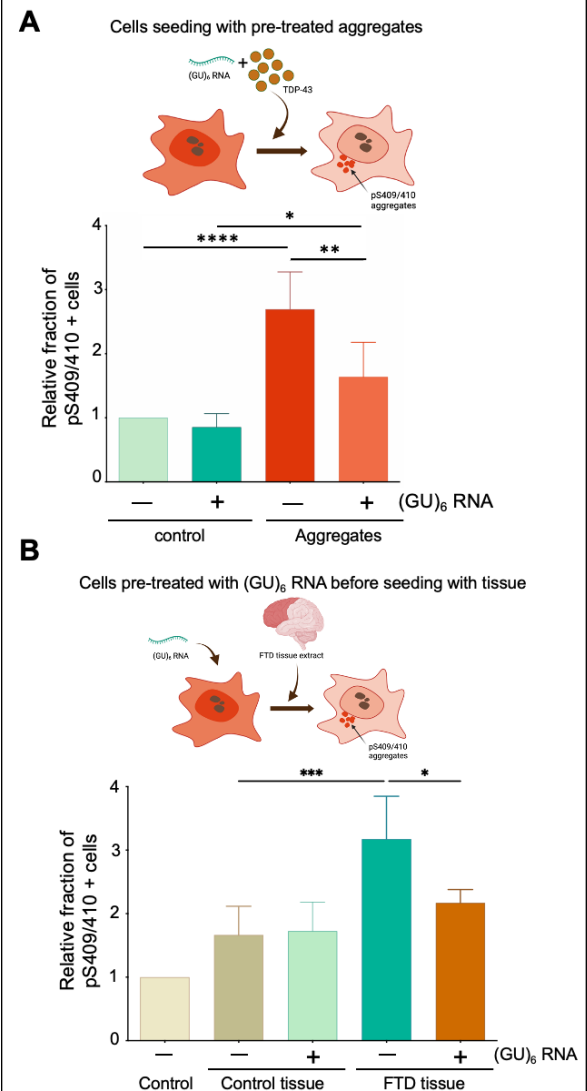
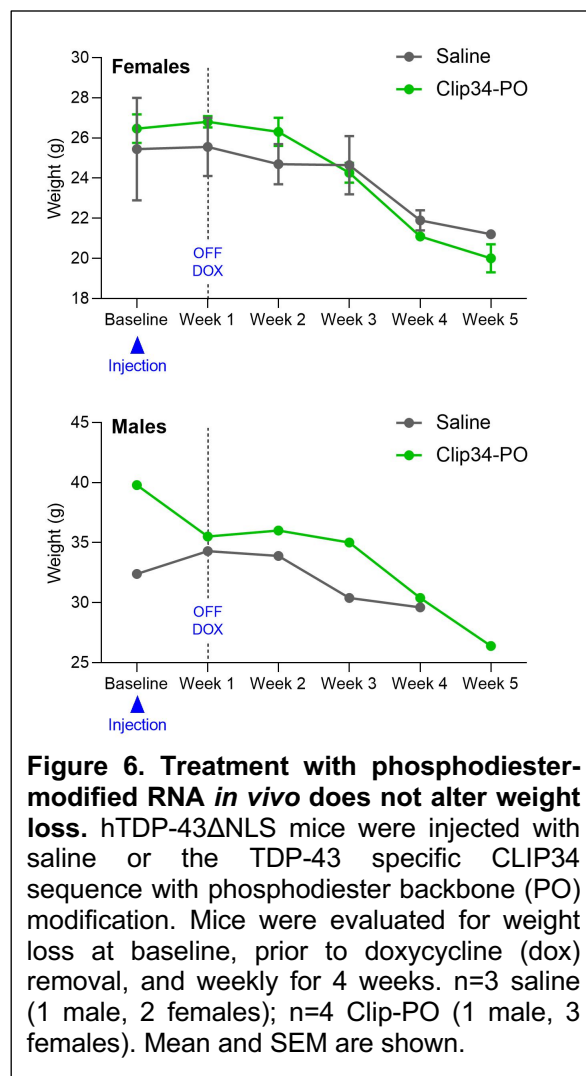


Figure 5: RNA treatment inhibits prion-like TDP-43 aggregate seeding activity. (A) TDP-43 aggregates were generated from purified protein in the presence of (GU)₆ RNA before treating reporter HEK293 cells and second. De novo aggregate formation was measured with the pathological marker for phosphorylated TDP-43 pS409/410. The fraction of cells showing pS409/410 signal in control or treated with aggregate seeds, in the presence of GU-repeat RNA or control. Plot shows relative mean $\pm$ SD, compared to control, of 3 independent replicates (>300 cells) *p<0.04, **p<0.002, ****p<0.0001 multiple comparison one-way ANOVA with Tukey's test. B) Reporter HEK293 cells were treated with 1 μ M (GU)₆ RNA prior to seeding with brain extract from frontotemporal dementia (FTD) or unaffected control patients. Aggregate seeding efficiency was quantified as in (A). ***p=0.0009.

as seen in human patients, achieved by expressing the nuclear localization signal (NLS)-deficient TDP-43 mutant, TDP-43^{NLS}, in motor neurons. These mice express a human copy of the mutant, TDP-43^{NLS}, which accumulates in cytoplasmic inclusions and the mice suffer neuronal loss and a progressive ALS-like phenotype. We generated this double transgenic mouse line under “tet off” control and used these animals to evaluate the effects of TDP-43-binding RNA molecules *in vivo*.

Previously, we observed that all oligonucleotides were able to increase nuclear localization of TDP-43 without changes in phosphorylated TDP-43 (pTDP) accumulation nor sustained improvement in behavior. The activity of oligonucleotides appeared dependent on oligonucleotide sequence. In addition, we hypothesized the backbone modification (i.e., phosphorothioate (PO)) may influence oligonucleotide affinity to proteins, including TDP-43. To test this hypothesis, we designed oligonucleotides with the CLIP34 sequence and 2'-methoxyethyl and phosphodiester (PO) backbone modifications to test within the hTDP-43 Δ NLS mouse model of TDP-43 pathology. Mice were treated with intraventricular injections of CLIP34-PO (n=4) or saline control (n=3) at ~4 months of age. Following injection, dox diet was removed to induce mutant TDP-43 expression. We evaluated weight and rotarod behavior weekly for 4 weeks, a time point at which mice are expected to exhibit motor impairments and TDP-43 aggregates. No significant differences were seen in weight or rotarod behavior between the CLIP34-PO- and saline-treated mice. Although this experiment was not fully powered to detect a survival difference, we did note that one male saline-treated mice developed seizures by week 3 post-dox removal and died prematurely.



What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We have requested a no-cost extension for the award. No additional funds are being requested for an additional period to complete the proposed work, which would have a new end date of June 30, 2024. There are no changes to the original Scope of Work. During this time, we will complete our proposed project as follows:

- Part of Major Task 1: Unbiased approach to identify RNA molecules associated with soluble TDP-43 from human cells via transcriptome wide sequencing.
- Major Task 3: Determine the effect of selected RNA molecules in cell viability and TDP-43 RNA processing function.
- Major Task 5: Treat cells with control and multiple TDP-43-targeting RNA molecules prior to seeding with in vitro generated TDP-43 aggregates.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

This proposal tests the novel idea that RNA-based tools may be used to inhibit TDP-43 aggregation and thereby decrease pathology and loss of TDP-43 function. We are currently determining whether these methods restore TDP-43 homeostasis without affecting TDP-43 function, cellular viability and animal survival. Dr. Timothy Miller, the co-Principal Investigator in this proposal, and his team are experts in using RNA oligonucleotides as a therapeutic approach to reduce ALS and other neurodegenerative disorders in various models and in first-in-kind patient clinical trials. Upon successful completion of our project we will test the efficacy of the RNA molecules in additional animal models of ALS. Our proposed studies will determine whether RNA molecules prevent the accumulation of TDP-43 pathology and toxicity upon early treatment. In addition, future studies will ask whether RNA-based therapy is able to reverse TDP-43 pathology in the mouse models. Based on the collection of our findings, the ultimate goal of our work is to generate new RNA-based treatments for human patient clinical trials. In addition, our studies will be the first to provide evidence that RNA association with target RNA binding proteins may modulate protein homeostasis and prevent decreased activity.

The long-term impact of our proposed work will be to accelerate the development of new drugs to combat ALS in the patient population. Development of therapies will help reduce disease, suffering, and premature death caused by ALS in both the civilian and military populations.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:**Changes in approach and reasons for change**

We are continuing our project as part of a one year no-cost-extension of the award.

Actual or anticipated problems or delays and actions or plans to resolve them

The award initiated shortly after the beginning of the COVID-19 pandemic, while the Ayala and Miller labs at Saint Louis University and Washington University, respectively, were locked down under institutional regulations. As a result, in person activities that required researchers to work in the lab and considered to be non-essential were halted, and mouse colonies were reduced such that the mouse line used for this work had to be completely re-established in late 2020. All the personnel involved in the award continued to work remotely and continued to hold regular monthly meetings between the two groups. Despite these unexpected circumstances, our groups made great progress during the first year of the award, especially upon returning to work in the lab. However, there was a delay relative to our original program. Therefore, we plan to continue our studies beyond the official end date of the award, if it is deemed necessary to complete our stated goals.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period.

Yes, please see other support in the Appendix section.

6. PRODUCTS:**Journal Publications.**

dos Passos, P.M., Hemamali, E.H., Mamede, L.D., Hayes, L.R. & **Ayala, Y.M.** RNA-mediated ribonucleoprotein assembly controls TDP-43 nuclear retention (submitted for publication) bioRxiv. 2023 Aug 7;2023.08.06.552215. doi: 10.1101/2023.08.06.552215. Preprint.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Dr. Sami Barmada from the University of Michigan as a collaborator.

Please see changes in other support for key personnel attached.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

-References

-Other support changes

-Journal publication

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**For New and Renewal Applications – DO NOT SUBMIT UNLESS REQUESTED
PHS 398 OTHER SUPPORT**

*Name of Individual: Ayala, Yuna
Commons ID: YAYALA

Other Support – Project/Proposal

*Title: Role of oligomeric TDP-43 aggregate intermediates in ALS and frontotemporal dementia

Major Goals: This proposal aims to detect intermediates of TDP-43 aggregates in cells and disease and to determine the mechanisms that mediate their formation. In addition, we will test their ability to seed and propagate TDP-43 pathology in mice.

*Status of Support: Active

Project Number: NS114289

Name of PD/PI: Ayala, Y

*Source of Support: NINDS

*Primary Place of Performance: Saint Louis University

Project/Proposal Start and End Date: (MM/YYYY) (if available): 02/2020 – 01/2025

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	4.6
2. 2022	4.6
3. 2023	4.6
4. 2024	4.6
5. 2025	4.6

Name of Individual: Ayala, Yuna
Commons ID: YAYALA

*Title: Functional and Pathological interactions of TDP-43

Major Goals: This is a collaboration with Nicolas Fawzi (Brown University) to uncover the structural and functional determinants that regulate TDP-43 interactions and cellular function in RNA processing. My laboratory only receives a small amount of funds through a subcontract with the goal of defining the structural requirements regulating TDP-43 RNA processing function in human cells.

*Status of Support: Active

Project Number: NS116176

Name of PD/PI: Fawzi, N

*Source of Support: NINDS

*Primary Place of Performance: Brown University

Project/Proposal Start and End Date: (MM/YYYY) (if available): 04/2020 – 03/2025

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	1.2
2. 2022	1.2
3. 2023	1.2
4. 2024	1.2
5. 2025	1.2

Name of Individual: Ayala, Yuna
Commons ID: YAYALA

*Title: RNA-Based Approach to Decrease TDP-43 Pathology and Neurotoxicity in ALS

Major Goals: The goal of this project is to test the efficacy of using RNA molecules to promote TDP-43 solubility and prevent pathology in ALS models using cell-based methods and an in vivo model of ALS.

*Status of Support: Active

Project Number: DE-W81XWH2010241

Name of PD/PI: Ayala, Y

*Source of Support: Department of Defense

*Primary Place of Performance: Saint Louis University

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2020 – 06/2024 (no-cost extension)

* Total Award Amount (including Indirect Costs):

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	2.4
2. 2022	2.4
3. 2023	2.4
4. 2024	2.4

Name of Individual: Ayala, Yuna
Commons ID: YAYALA

PENDING:

*Title: Regulation of TDP-43 aggregate seeding, propagation and neurotoxicity

Major Goals: The major goal of this project aims to identify the factors controlling TDP-43 aggregate conversion and propagation and determine whether the pathogenic mechanisms linked to TDP-43 loss of function and neurotoxicity are triggered by the seeding process.

*Status of Support: Pending

Project Number: GRANT13767547

Name of PD/PI: Ayala, Y

*Source of Support: NIH

*Primary Place of Performance: Saint Louis University

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2023-06/2028

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2024	3.6
2. 2025	3.6
3. 2026	3.6
4. 2027	3.6
5. 2028	3.6

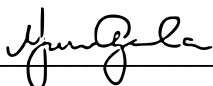
IN-KIND:

None.

***Overlap:**

None.

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature:  _____

Date: September 13, 2023

*Name of Individual: Timothy M. Miller, MD, PhD
Commons ID: TIMOTHYMILLER

**Other Support – Project/Proposal
ACTIVE**

*Title: Developing 4R Tau-Targeting Antisense Oligonucleotides in Novel Mouse Models of Tauopathy

Major Goals: Support the development of 4R tau-targeted antisense oligonucleotides as a therapeutic strategy for 4R tauopathies caused by MAPT mutations.

*Status of Support: Active

Project Number: N/A

Name of PD/PI: Miller

*Source of Support: Rainwater Charitable Foundation

*Primary Place of Performance: Washington University School of Medicine

Project/Proposal Start and End Date: 08/01/2023 - 07/31/2024

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period:

Year (YYYY)	Person Months (##.##)
1. [2024]	0.6

*Title: Development of Lovastatin or related compounds as a therapy for ALS

Major Goals: Identifying the motor neuron protective mechanisms of lovastatin and discovering the best compound to move forward to clinical trial.

*Status of Support: Active

Project Number: HT9425-23-1-0154

Name of PD/PI: Miller, Timothy M and Brad Racette

*Source of Support: Department of Defense/DOD

*Primary Place of Performance: Washington University School of Medicine

Project/Proposal Start and End Date: 07/01/2023 - 06/30/2025

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period:

Year (YYYY)	Person Months (##.##)
1. [2024]	0.6
2. [2025]	0.55

Name of Individual: Timothy M. Miller, MD, PhD
Commons ID: TIMOTHYMILLER

*Title: Development of ASOs to restore normal MYT1L levels

Major Goals: Test ability of in vivo massively parallel reporter assay technologies to predict targetable elements and identify a lead ASO compound for preclinical rescue studies.

*Status of Support: Active

Project Number: N/A

Name of PD/PI: Miller

*Source of Support: RTW Charitable Foundation

*Primary Place of Performance: Washington University School of Medicine in St. Louis

Project/Proposal Start and End Date: (MM/YYYY) (if available): 11/01/2022 – 10/31/2023

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2023]	0.6

*Title: Using the Answer ALS dataset to expand genetically defined ALS subtypes

Major Goals: Leverage the Answer ALS dataset to better understand how patients with known genetic forms of ALS compare to those without.

*Status of Support: Active

Project Number: N/A

Name of PD/PI: Miller

*Source of Support: Robert Packard Center for ALS Research

*Primary Place of Performance: Washington University School of Medicine in St. Louis

Project/Proposal Start and End Date: (MM/YYYY) (if available): 08/01/2021 – 12/31/2023

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	0.6
2. [2023]	0.6

*Title: Increasing mRNA translation to treat neurodegeneration

Major Goals: Develop universal gene-targeting strategies that stimulate mRNA translation to understand and treat neurodegenerative diseases.

Name of Individual: Timothy M. Miller, MD, PhD
Commons ID: TIMOTHYMILLER

*Status of Support: Active

Project Number: N/A

Name of PD/PI: Miller, Djuranovic

*Source of Support: Chan Zuckerberg Initiative

*Primary Place of Performance: Washington University School of Medicine in St. Louis

Project/Proposal Start and End Date: (MM/YYYY) (if available): 08/01/2022 – 07/31/2026

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2023]	1.2
2. [2024]	1.2
3. [2025]	1.2
4. [2026]	1.2

*Title: Understanding Protein Release from Cells in Neurodegenerative Disease

Major Goals: Understand the release of neurofilament proteins from the central nervous system by using ALS models and human tissue analysis.

*Status of Support: Active

Project Number: N/A

Name of PD/PI: Miller

*Source of Support: Ionis Pharmaceuticals, Inc.

*Primary Place of Performance: Washington University School of Medicine in St. Louis

Project/Proposal Start and End Date: (MM/YYYY) (if available): 03/01/2022 – 02/28/2025

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2023]	1.2
2. [2024]	1.2
3. [2025]	1.2

*Title: Molecular recording to predict cell fate decisions and animal behavior

Major Goals: Develop a robust, easy to use technology platform to record molecular events at single cell resolution in the mouse brain and connect them with behavioral outcomes, cell fate decisions, neuronal activity profiles, and anatomical location.

*Status of Support: Active

Name of Individual: Timothy M. Miller, MD, PhD
Commons ID: TIMOTHYMILLER

Project Number: RF1MH126723

Name of PD/PI: Mitra, Dougherty

*Source of Support: NIH

*Primary Place of Performance: Washington University School of Medicine in St. Louis

Project/Proposal Start and End Date: (MM/YYYY) (if available): 08/01/2021 – 7/30/2024

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2023]	0.6
2. [2024]	0.6

*Title: Longitudinal Biofluid Collection

Major Goals: Determine biomarkers changes in cerebral spinal fluid and serum from patients with ALS.

*Status of Support: Active

Project Number:

Name of PD/PI: Miller

*Source of Support: Target ALS Foundation

*Primary Place of Performance: Washington University School of Medicine in St. Louis

Project/Proposal Start and End Date: (MM/YYYY) (if available): 11/01/2020 – 10/31/2025

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	0.36
2. [2023]	0.36
3. [2024]	0.36
4. [2025]	0.36

*Title: High Resolution Analysis of Neurofilament in ALS Biofluids

Major Goals: Complete in-depth discovery mass spectrometry in CSF from ALS and controls to determine if there are unique neurofilament protein signatures in ALS.

*Status of Support: Active

Project Number: N/A

Name of Individual: Timothy M. Miller, MD, PhD
Commons ID: TIMOTHYMILLER

Name of PD/PI: Miller, Ly

*Source of Support: Target ALS Foundation

*Primary Place of Performance: Washington University School of Medicine in St. Louis

Project/Proposal Start and End Date: (MM/YYYY) (if available): 11/01/2020 – 10/31/2023

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2021]	0.6
2. [2022]	0.6

PENDING

*Title: Enhanced Protein Clearance to Improve Antisense Oligonucleotide Therapeutics for Alzheimer's Disease

Major Goals: investigate CNS-specific approaches to activate endogenous protein clearance mechanisms and evaluate the therapeutic potential of adjunctive treatments to target protein clearance in combination with ASO-mediated tau reduction

*Status of Support: Pending

Project Number: Not Assigned

Name of PD/PI: Timothy Miller

*Source of Support: National Institutes of Health

*Primary Place of Performance: Washington University School of Medicine

Project/Proposal Start and End Date: 04/01/2024 - 03/31/2029

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period:

Year (YYYY)	Person Months (##.##)
1. [2024]	3
2. [2025]	3
3. [2026]	3
4. [2027]	3
5. [2028]	3

Name of Individual: Timothy M. Miller, MD, PhD
Commons ID: TIMOTHYMILLER

*Title: Clinical Outcomes of Combined Treatment of SOD1 ALS with Tofersen and Neuromuscular Rehabilitation

Major Goals: Investigate the impact of neuromuscular rehabilitation on individuals diagnosed with SOD1 ALS who are also being treated with the newly approved drug tofersen

*Status of Support: Pending

Project Number: Not Assigned

Name of PD/PI: Timothy Miller and Sean Smith

*Source of Support: National Institutes of Health

*Primary Place of Performance: Washington University School of Medicine

Project/Proposal Start and End Date: 04/01/2024 - 03/31/2026

*Total Award Amount (including Indirect Costs):

*Person Months (Calendar/Academic/Summer) per budget period:

Year (YYYY)	Person Months (##.##)
1. [2024]	1.8
2. [2025]	1.8
3. [2026]	1.8

IN-KIND

*Summary of In-Kind Contribution:

None

***Overlap** (summarized for each individual):

None of the currently active or pending grants overlap in terms of goals, aims, or financial support.

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature: _____

Date: _____

1 **RNA-mediated ribonucleoprotein assembly controls TDP-43 nuclear retention**

2
3 Patricia M. dos Passos¹, Erandika H. Hemamali¹, Lohany D. Mamede¹, Lindsey R.
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5
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7 University School of Medicine, St. Louis MO 63104, USA

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10 *Corresponding author

11
12 **Short title:** Regulation of TDP-43 cellular distribution

1 **ABSTRACT**

2

3 TDP-43 is an essential RNA-binding protein strongly implicated in the pathogenesis
4 of neurodegenerative disorders characterized by cytoplasmic aggregates and loss of
5 nuclear TDP-43. The protein shuttles between nucleus and cytoplasm, yet
6 maintaining predominantly nuclear TDP-43 localization is important for TDP-43
7 function and for inhibiting cytoplasmic aggregation. We previously demonstrated that
8 specific RNA binding mediates TDP-43 self-assembly and biomolecular
9 condensation, requiring multivalent interactions via N- and C-terminal domains.
10 Here, we show that these complexes play a key role in TDP-43 nuclear retention.
11 TDP-43 forms macromolecular complexes with a wide range of size distribution in
12 cells and we find that defects in RNA binding or inter-domain interactions, including
13 phase separation, impair the assembly of the largest species. Our findings suggest
14 that recruitment into these macromolecular complexes prevents cytoplasmic egress
15 of TDP-43 in a size-dependent manner. Our observations uncover fundamental
16 mechanisms controlling TDP-43 cellular homeostasis, whereby regulation of RNA-
17 mediated self-assembly modulates TDP-43 nucleocytoplasmic distribution.
18 Moreover, these findings highlight pathways that may be implicated in TDP-43
19 proteinopathies and identify potential therapeutic targets.

20

1 INTRODUCTION

2
3 TDP-43 (TAR DNA binding protein), a highly conserved and essential RNA binding
4 protein in multicellular eukaryotes, is central in the pathogenesis of
5 neurodegenerative disorders. Accumulation of TDP-43 aggregates and loss of
6 nuclear protein localization are hallmark features of almost all amyotrophic lateral
7 sclerosis (ALS) and ~50% of frontotemporal dementia (FTD) patients [1-3]. In
8 addition, TDP-43 pathology is a signature feature of an Alzheimer's disease (AD)-
9 related dementia affecting the oldest population, termed limbic-predominant age-
10 related TDP-43 encephalopathy (LATE) [4]. The majority of TDP-43 inclusions are
11 observed in the cytoplasm and often correspond to loss of nuclear localization.
12 Structural defects in the nuclear pore complex linked to ALS and FTD promote TDP-
13 43 cytoplasmic mislocalization, suggesting that aberrant nucleocytoplasmic transport
14 impacts TDP-43 homeostasis in disease [5]. Aberrant accumulation of cytoplasmic
15 TDP-43 is linked to increased protein aggregation and is thought to promote
16 depletion of the nuclear pool of functional TDP-43 in affected cells [6-8]. Maharana et
17 al. proposed that the relatively lower levels of RNA in the cytoplasm is a major factor
18 contributing to aggregation of TDP-43 and other RNA binding proteins in this
19 compartment [9]. While in the nucleus, RNA binding may prevent TDP-43 misfolding
20 despite the higher concentration of TDP-43. Data from our group and others support
21 this model by showing that specific RNA binding decreases TDP-43 aggregation and
22 promotes liquid properties during phase separation [7, 10]. Therefore, defects in
23 TDP-43 nuclear retention may strongly contribute to pathogenesis by increasing the
24 cytoplasmic accumulation of the protein. At the same time, loss of nuclear
25 localization decreases TDP-43 nuclear functions, such as control of gene expression
26 [11-14], as detected in neurons extracted from ALS/FTD patient brain devoid of
27 nuclear TDP-43 [15]. Indeed, dysregulation of TDP-43 targets is observed in patients
28 [16-19], supporting the idea that loss of nuclear localization strongly impairs
29 physiological TDP-43 function contributing to disease.

30
31 TDP-43 shuttles continuously between the nuclear and cytoplasmic compartments,
32 but is normally predominantly nuclear as shown by fractionation experiments and cell
33 imaging [20]. TDP-43 undergoes active nuclear import mediated by a basic bipartite
34 nuclear localization signal (NLS) (**Fig.1A**) and binding to importins α/β .

(karyopherins α/β)[6, 21]. In addition, alternative mechanisms may also actively or passively promote nuclear entry [22-24]. A nuclear export signal was previously proposed to interact with exportin 1 (XPO/CRM1)[6]; however, multiple lines of evidence indicate that TDP-43 passively exits the nucleus in a size-dependent manner [23-26] through nuclear pore channels, which increasingly exclude cargoes above 30-60 kDa in size [22, 27]. Recent findings suggest that binding to nuclear RNAs restricts TDP-43 availability for passive exit through nuclear pore channels (Duan et al., 2022). Thus, TDP-43 incorporation into high molecular weight nuclear ribonucleoproteins (RNPs) may be a key mechanism promoting TDP-43 nuclear accumulation.

TDP-43 is a heterogeneous ribonucleoprotein (hnRNP) composed of multiple independently folded domains and a mostly disordered C-terminal region (**Fig 1A**). The N-terminal domain (NTD) resembles ubiquitin-like Dishevelled/Axin (DIX) domains and forms dimers/oligomers through head-to-tail interactions [28, 29]. Two tandem RNA recognition motifs (RRMs) bind RNA with strong preference for GU-rich sequences, which are recognized with affinity in the low nanomolar range [30, 31]. The C-terminal domain (CTD) is a low complexity sequence region also referred to as prion-like domain. The isolated CTD undergoes liquid-liquid phase separation (LLPS) in vitro [32] and is believed to largely contribute to TDP-43 condensation in cells [33]. In particular, an evolutionarily conserved region (CR, a.a. 320-343) in the CTD that forms a partial α -helical structure [34] mediates homotypic assembly and LLPS in vitro and in cells [32, 33]. This process of biomolecular condensation is increasingly believed to mediate the formation of protein and RNA-rich assemblies or RNP complexes [35, 36]. In cells, TDP-43 forms and is recruited to RNPs, such as Cajal bodies, and stress granules [37, 38]. We recently showed that RNA binding to extended GU-rich sequences promotes TDP-43 biomolecular condensation in vitro and in mammalian cell lysate reconstitution assays [10]. Consistent with the strong contribution of biopolymers and multivalent interactions to the formation of biomolecular condensates [39], we found that RNA-mediated TDP-43 condensates require NTD and CTD-mediated interactions, as shown in our model (**Fig. 1A**). These TDP-43 assemblies are necessary for the regulation of RNA processing, as shown in the case of TDP-43 autoregulation [40]. This notion is supported by

Hallegger et al., who also showed that TDP-43 phase separation determines the recruitment to RNA transcripts with specific sequence composition [41].

Here, we investigated the role of RNA-mediated TDP-43 self-assembly through oligomerization and phase separation in regulating cellular distribution, and examined the relative contribution of each of these interactions to the native molecular weight of these RNP complexes. We demonstrate that RNA binding and multi-domain interactions form large macromolecular assemblies that act in combination to maintain TDP-43 nuclear localization by preventing nuclear exit. Our work highlights important mechanisms regulating TDP-43 cellular homeostasis by showing that these macromolecular complexes increase nuclear retention in a size-dependent manner and are tightly regulated by RNA-mediated phase separation.

RESULTS

TDP-43 multivalent interactions are necessary for TDP-43 nuclear localization.

Mounting evidence, including our previous studies, indicate that RNA binding increases nuclear TDP-43 localization [20, 26]. In addition, our recent work showed that RNA binding, in the presence of multivalent interactions, induces biomolecular condensation or LLPS [10]. We therefore asked whether these RNA-bound complexes play a role in TDP-43 nuclear retention by investigating how disruptions in their assembly alter cellular distribution. We analyzed site-specific mutations in different TDP-43 domains previously established to impair self-assembly (**Fig. 1A**), using an engineered human cell line stably expressing a single copy of hemagglutinin (HA)-tagged wild-type (WT) or TDP-43 variants upon induction with tetracycline (Tet) [40]. This collection of human embryonic kidney isogenic cell lines (HEK293^{HA-TDP-43}) expressed WT and mutant TDP-43 at close to endogenous levels. The small HA tag was specifically used to minimize size-dependent disruption of cellular trafficking. To measure nuclear-cytoplasmic distribution, nucleus and cytoplasm were biochemically separated after 72 h of transgene induction. TDP-43 levels in nuclear and cytoplasmic fractions were measured by immunoblotting (**Fig. 1B, Fig. S1**) and expressed as the nuclear-cytoplasmic ratio (N/C) (**Fig. 1C**). Lamin A/C and tubulin were used to normalize samples and to determine the integrity of nuclear and cytoplasmic fractions for each experiment. The same assays were

previously utilized to show that TDP-43 predominantly localizes in the nucleus [20]. The anti-TDP-43 antibody detected low levels of endogenous TDP-43 upon HA-WT TDP-43 expression (**Fig. 1B**), which is consistent with TDP-43 autoregulatory function [12, 42]. A mutant disrupting the nuclear localization signal (NLS^{mut}, K⁸²RK⁸⁴/K⁹⁵VKR⁹⁸ to A⁸²AA⁸⁴/A⁹⁵VAA⁹⁸) [6] was used as a control (**Fig. 1A**). Each independent replicate was performed in parallel with WT-expressing cells, and the N/C was calculated relative to WT (**Fig. 1C**). As expected, NLS^{mut} levels increased in the cytoplasm, resulting in a 50% reduction in N/C, relative to WT. The immunoblot also showed loss of autoregulation as seen by the higher levels of endogenous TDP-43 in these cells compared to WT (**Fig. 1B**). Endogenous TDP-43 nuclear-cytoplasmic distribution in NLS^{mut}-expressing cells was not affected by the presence of the mutant copy. In agreement with our previous results [20, 26], nuclear retention was reduced by mutations in the RNA recognition motifs (RRMs) that greatly impair specific RNA binding affinity F147/149/229/231L (F4L) [10, 43]. Next, we probed the role of NTD-driven oligomerization by disrupting interactions previously shown to be important for self-assembly [29]. The E17R substitution is sufficient to inhibit oligomerization [44] and impair RNA-driven LLPS [40]. We found that E17R significantly reduced N/C by approximately 70%, relative to WT. Notably, this effect was even more pronounced than loss of the nuclear localization signal (NLS^{mut}). The interaction between Glu17 and Ser48 from two independent NTDs is required for oligomerization [29]. Ser48 is subject to phosphorylation [44-46] and this is predicted to inhibit oligomerization as observed by the decrease in TDP-43 dimerization and LLPS in the presence of the phosphomimetic S48E substitution [44]. Consistent with the effect of E17R, we observed that S48E significantly reduced TDP-43 N/C by approximately 50% compared to WT. In contrast, the phospho-null mutant S48A, used as control, showed a moderate yet not significant increase in N/C relative to WT. Importantly, these results suggest that phosphorylation at Ser48 may regulate TDP-43 nuclear localization. We also found that deletion of the conserved region (Δ CR) in the C-terminal domain decreased the relative N/C by approximately 50%. Altogether, our observations suggest that RNA-dependent multivalent interactions through the NTD and conserved α -helix at the C-terminus strongly promote TDP-43 nuclear localization.

To distinguish whether enhanced cytoplasmic localization of TDP-43 mutants (**Fig. 1C**) was caused by defects in nuclear import, or increased nuclear efflux, we performed heterokaryon assays using WT and mutant HEK293^{HA-TDP-43} cells. HEK293^{HA-TDP-43} cells were fused with NIH3T3 mouse cells resulting in multinucleated cells (**Fig. 2A**). Within one hour of outer membrane fusion, WT HA-TDP-43 was observed in HEK293 nuclei (donor) as well as in mouse nuclei (acceptor) of heterokaryon cells, consistent with our previous findings [20] (**Fig. 2B**). Mutants E17R, F4L and Δ CR were also present in acceptor nuclei, indicating that the mutations did not impair nuclear import. Interestingly, we observed that at this time point WT TDP-43 levels in the donor nuclei remained greater compared to the acceptor, based on the fluorescence signal. In contrast, E17R, F4L and Δ CR showed similar levels of HA-TDP-43 fluorescence in donor and acceptor nuclei. To further investigate these differences, we quantified the fluorescence ratio of donor vs. acceptor nuclei (**Fig. 2C**). WT TDP-43 donor to acceptor ratio was approximately 1.5, suggesting that at 1 h post heterokaryon formation, TDP-43 exits donor nuclei and is imported into the acceptor, but that a significant amount of protein, 50% more, is retained in donor nuclei. The mutants, in contrast, showed significant reductions of donor to acceptor ratio (approximately 50-25%), relative to WT. These results indicate that the increased cytoplasmic levels of E17R, F4L and Δ CR, shown in figure 1, are not the result of impaired nuclear import. Rather, disruption of TDP-43 self-assembly through the NTD and CR as well as RNA binding deficiency increase the rate of TDP-43 egress from the nucleus.

RNA binding and multi-domain TDP-43 interactions prevent nuclear exit synergistically.

To define the relative contribution of NTD, CR-driven assembly and RNA binding to TDP-43 nuclear retention, we investigated the role of RNA on the localization of NTD and Δ CR mutants. For these experiments we measured nuclear and cytoplasmic TDP-43 levels in HEK293^{HA-TDP-43} cells using confocal high-content imaging [26]. In agreement with the biochemical fractionation assays, E17R, F4L and Δ CR showed significant reductions of TDP-43 N/C, compared to WT (**Fig. 3A**). F4L caused a significantly greater loss of nuclear localization relative to E17R and Δ CR, highlighting a central role of RNA binding in maintaining high nuclear TDP-43 concentration. Treatment with the pan-transcriptional inhibitor Actinomycin D (Act D)

reduced WT nuclear localization (**Fig. 3B**), as previously observed [20, 26]. Similarly, the specific RNA polymerase II inhibitor NVP2 [47] decreased WT TDP-43 N/C by approximately 40%, indicating that the observed changes are specific to decreased transcription. We found that Act D and NVP2 treatment significantly decreased E17R and Δ CR N/C by approximately 45 and 20%, respectively, compared to untreated control cells. In contrast, localization of F4L was unaffected, consistent with the idea that transcriptional inhibition alters TDP-43 localization by decreasing the abundance of nuclear pre-mRNAs available for TDP-43 to bind. We then transfected cells with increasing amounts of (GU)₆ RNA oligonucleotides carrying a phosphorothioate backbone and 2'-O-methyl modifications to prevent degradation by RNases (reviewed in [48]). The (CA)₆ RNA sequence which shows no significant TDP-43 binding [43] was used as non-specific control. Efficient cellular uptake and nuclear import of these RNA molecules was previously quantified [26]. In agreement with Duan et al., (GU)₆ RNA induced a dose-dependent efflux of WT TDP-43 to the cytoplasm (**Fig. 3C**), while the non-specific RNA did not have an effect. E17R and Δ CR mutant N/C was decreased by (GU)₆ in the same manner as WT, suggesting that increasing levels of GU-rich RNA further impair nuclear retention of TDP-43 mutants with defects in self-assembly. On the other hand, F4L nuclear-cytoplasmic distribution was insensitive to the presence of (GU)₆ RNA, as expected. Our results indicate that RNA binding, NTD oligomerization and CR-mediated self-assembly together contribute to TDP-43 nuclear retention and that the sum of these interactions is required to prevent protein efflux to the cytoplasm.

Nuclear TDP-43 condensate formation promotes nuclear retention.

RNA binding proteins, including TDP-43 and related ALS/FTD-associated proteins, undergo phase separation and condensate formation as purified components and in cells [49-52]. The conserved α -helical region within the C-terminal domain of TDP-43 is essential for promoting phase separation in the context of the isolated domain and full-length protein [32, 41]. We showed that this region is also necessary to form RNA-mediated TDP-43 condensates [40]. To determine whether the decrease in nuclear localization upon deletion of the CR (**Fig. 2C**) is caused by defects in phase separation, we measured TDP-43 nuclear-cytoplasmic distribution under conditions that inhibit condensate formation. 1,6-hexanediol (1,6-HD) is widely used to inhibit phase separation [53] and impairs droplet formation of purified TDP-43 [7, 54]. 1,6-

HD also decreases the formation of nuclear TDP-43 foci in cells [41], suggesting that it reduces TDP-43 RNP condensates. We treated cells with 5% 1,6-HD for 5 minutes and observed a 5-fold decrease in N/C of endogenous TDP-43, compared to non-treated control cells (**Fig. 4A**). We considered that 1,6-HD may also disrupt the phenylalanine-glycine (FG) permeability barrier in the central channel of the nuclear pore complex [55]. This effect could also contribute to the increased nuclear efflux of TDP-43 upon 1,6-HD treatment. To test disruption of TDP-43 condensate formation more specifically, we introduced mutations in the CR to modulate phase separation by altering α -helix structural properties (**Fig. 4B**) [32, 41]. A326P, M337P and the ALS-linked mutation M337V are helix breaking mutants that result in aberrant CTD self-assembly and impaired LLPS [10, 32, 40]. Moreover, these single-site substitutions closely resemble the inhibition properties of Δ CR on the formation of TDP-43-rich nuclear foci and phase separation-dependent RNA processing of TDP-43 targets in cells [41]. We found significant decrease in nuclear TDP-43 localization with each of these mutations (**Fig. 4B**), consistent with the effect of 1,6 HD treatment and deletion of the entire CR in figure 1C. Altogether, these data strongly suggest that nuclear condensate formation plays a key role in regulating nuclear-cytoplasmic TDP-43 trafficking by promoting nuclear localization.

The size of RNA-bound TDP-43 macromolecular complexes controls nuclear retention.

Previous evidence indicates that TDP-43 nuclear efflux is largely a passive, size dependent process, whereby larger fusion constructs reduce TDP-43 export [23, 24]. TDP-43 forms complexes of a wide range of sizes in cells, from monomers to large higher-order complexes greater than 1.5 MDa, as measured by size exclusion chromatography (SEC) [56, 57]. Based on these findings, we tested whether the contribution of TDP-43 RNA-mediated complexes to nuclear retention is size-dependent, requiring the formation of large macromolecular complexes. We analyzed the size distribution of TDP-43 in HEK293^{HA-TDP-43} cell lysates expressing WT and mutant TDP-43 using gel filtration chromatography. Of note, the use of these isogenic cell lines expressing TDP-43 variants at endogenous-like levels was designed to avoid unintended effects of TDP-43 overexpression. The relative HA-TDP-43 levels in each eluted fraction was analyzed by immunoblotting to compare the approximate size distribution of WT vs mutant TDP-43 assemblies (**Fig. 5A, Fig**

S3). We observed a broad profile of WT TDP-43 distribution starting at the estimated 45 kDa fraction to 700 kDa, which is the upper detection limit of the SEC column. The highest level of detected WT protein eluted at approximately 115 kDa and this was set as 100% (**Fig. 5B**). Disruption of NTD-mediated oligomerization, E17R, showed a shift to higher elution volume compared to WT, whereby the peak of highest concentration moved to smaller estimated molecular weight, between 45 and 72 kDa (**Fig. 5C**). In addition, TDP-43 levels in the fractions corresponding to higher molecular weight complexes were markedly decreased compared to WT. These results are consistent with previous cross-linking assays indicating a loss of multimeric TDP-43 species in cells upon disruption of NTD-mediated oligomerization [29]. Similar behavior was observed with the RNA-binding-deficient F4L mutant. The most abundant F4L fractions shifted to smaller estimated sizes compared to WT together with a dramatic sharpening of the elution profile. An even greater shift in size distribution was observed with Δ CR, showing an elution peak of estimated 45 kDa. In stark contrast with WT, little or no detection of Δ CR was observed above the estimated 115 kDa fraction. Based on the estimated size separation in our experiments, the altered behavior of Δ CR is unlikely to be caused by the 20 amino acid difference between this construct, WT and the other mutants analyzed here. To further test the role of CR-mediated assembly and LLPS on the size of TDP-43 complexes, we studied the effect of A326P. In agreement with Δ CR, A326P also reduced the size of the most abundant TDP-43 complexes, eluting at the estimated 45 kDa fraction. These results are consistent with an important role of CR-mediated homotypic assembly and LLPS in forming higher-order RNP bodies. Collectively, our SEC experiments provide biochemical evidence that disruption of NTD oligomerization, RNA binding and phase separation decrease the size of TDP-43-rich complexes by impairing formation of the largest macromolecular species in cells. In addition, our data strongly suggest that the decreased ability to form these higher order macromolecular assemblies reduces TDP-43 nuclear retention by increasing the population of protein freely diffusing to the cytoplasm.

DISCUSSION

The normal distribution of TDP-43 between nuclear and cytoplasmic compartments, in which the protein is predominantly found in the nucleus, plays a central role in

1 physiological function. Disruption of homeostasis is associated with pathogenesis in
2 neurodegenerative disorders, wherein pathological decrease in nuclear TDP-43 and
3 elevated protein levels in the cytoplasm are linked to TDP-43 loss of function and
4 aggregation, respectively [1, 2, 15]. TDP-43 is recruited to RNA and protein-rich
5 ribonucleoprotein (RNP) complexes through RNA binding, TDP-43 self-assembly
6 and condensate formation/phase separation [58]. The mechanisms by which these
7 complexes regulate TDP-43 function and homeostasis are only beginning to be
8 explored. Recently, we showed that RNA-mediated TDP-43 phase separation
9 depends on multivalent interactions involving N-terminal domain (NTD)
10 oligomerization and assembly through an α -helical conserved region at the C-
11 terminus (CR) (**Fig 1A**) [10, 40], which is essential for TDP-43 condensation in vitro
12 and in cells [32, 41, 59]. Here, we find that disruption in any of the assembly points
13 necessary to form RNA-mediated TDP-43 complexes is sufficient to markedly
14 increase cytoplasmic localization. Thus, TDP-43 recruitment into RNP bodies plays a
15 critical function in maintaining nuclear retention.

16
17 Based on emerging evidence, RNA binding plays a key role in TDP-43 molecular
18 dynamics and proteostasis. We recently showed that specific RNA binding
19 modulates TDP-43 phase separation properties [10] and that formation of these
20 complexes is essential in regulating TDP-43 target expression, such as its own
21 transcript during autoregulation [40, 41]. The importance of RNA in TDP-43 nuclear
22 localization was previously demonstrated with RNA binding-deficient mutants and by
23 showing that inhibition of RNA transcription promotes cytoplasmic localization [20,
24 26]. In addition, Duan et al. recently quantified the effect of TDP-43-RNA interactions
25 in cells and showed that inhibition of splicing increases TDP-43 nuclear levels
26 probably by increasing the retention of introns with TDP-43 recruitment sites [26]. In
27 addition, small GU-rich RNA oligonucleotides, which bind TDP-43 with nanomolar
28 affinity [30], promote the cytoplasmic egress of TDP-43 [26]. These data suggest that
29 these RNA molecules promote the release of nuclear TDP-43 by competing with
30 transcripts for TDP-43 binding. Here, we find that mutants deficient in NTD and CR-
31 driven interactions are similarly affected by (GU)₆ RNA compared to WT (**Fig. 3C**).
32 Thus, our observations indicate that RNA binding, NTD and CR interactions
33 contribute synergistically in maintaining nuclear localization. Importantly, we and
34 others showed that specific RNA interactions prevent TDP-43 aggregation [7, 60]

1 and promote liquid-like behavior of TDP-43 condensates [10]. These results
2 collectively suggest that RNA binding plays multiple essential roles in maintaining
3 TDP-43 function. Specific RNA binding increases nuclear TDP-43 retention by
4 mediating the formation of RNP assemblies. In addition, RNA interactions increase
5 solubility in the nucleus despite the higher protein concentration in this compartment.
6 This idea is consistent with previous observations that solubility and liquid properties
7 of the related protein FUS are maintained by the abundance of RNA in the nucleus
8 [9].

9
10 Our data agree with recent work from the Polymenidou lab published while our
11 manuscript was in preparation [61]. Using cell-based models, including human
12 neurons, Pérez-Berlanga et al. show that NTD-mediated oligomerization and RNA
13 binding act cooperatively to increase nuclear localization and that both interactions
14 are important to prevent TDP-43 aggregation. Here, we extend beyond these
15 findings by showing that condensate formation is required for the assembly of
16 complexes that increase TDP-43 nuclear retention (**Fig 4**), highlighting the
17 importance of multivalent interactions in TDP-43 function. In addition, our
18 heterokaryon assays demonstrate that the defects in nuclear localization observed
19 upon disruption of TDP-43 RNA binding, oligomerization or phase separation are not
20 caused by diminished nuclear import (**Fig 2**). Instead, our results suggest that
21 disruption of these interactions accelerate protein efflux to the cytoplasm. Moreover,
22 our size exclusion chromatography studies are the first to biochemically correlate the
23 ability of TDP-43 to form RNA-bound condensates with the formation of large higher-
24 order macromolecular complexes in cells (**Fig 5**). Based on our observations, we
25 propose that TDP-43 nuclear retention is largely dependent on the formation of large
26 RNP granules, reducing the population of smaller species available for passive
27 export to the cytoplasm (**Fig 6**). These results are important because they support a
28 size-dependent nuclear retention mechanism of RNA-bound TDP-43 condensates
29 and illustrate multiple possible mechanisms to modulate TDP-43 nucleocytoplasmic
30 distribution. Furthermore, our observations raise the intriguing possibility that the
31 cellular dynamics of other related RNA-associated proteins may be controlled in
32 similar fashion.

1 Condensate formation plays a central role in RNP granule assembly and function
2 [39] and our work suggests that it promotes TDP-43 nuclear localization, as seen by
3 deleting and/or disrupting the CR in the C-terminus. This region also interacts with
4 different RNA binding proteins including hnRNPs A1, A2, A3, B1 and C [62].
5 Therefore, association with these hnRNPs may also contribute to RNP granule
6 formation and nuclear retention, and this possibility requires further investigation. We
7 also observe a significant decrease in nuclear localization in the presence of A326P,
8 M337P and the ALS-linked mutation M337V and a reduction in the size of TDP-43
9 complexes with the A326P mutation, similar to the effect of Δ CR. We predict that
10 these single-site substitutions do not abolish TDP-43 association with the partner
11 hnRNPs. On the other hand, the substitutions significantly disrupt TDP-43 phase
12 separation, as seen in vitro and in cells [32, 41]. Therefore, we posit that CR
13 contributes to TDP-43 nuclear localization primarily through homotypic assembly and
14 phase separation.

15
16 Collectively, our findings suggest the intriguing possibility that functional defects
17 previously observed with oligomerization-deficient and other mutants/conditions
18 affecting TDP-43 self-assembly may be at least partially caused by decreased
19 nuclear localization. Since the structural characterization of TDP-43 NTD-mediated
20 oligomerization [29, 44], this interaction has been increasingly shown to play a
21 central role in cellular functions. NTD-driven oligomerization is required for TDP-43
22 function in alternative splicing regulation [29, 44], phase separation [10, 44],
23 autoregulation [40] and in preventing R-loop accumulation [14]. Here, we find that
24 impaired oligomerization greatly reduces nuclear TDP-43 localization (**Fig 1C**),
25 indicating that loss of function due to inhibition of NTD-mediated oligomerization may
26 be caused by multiple mechanisms. These defects may include decreased self-
27 assembly upon RNA target engagement, reduced RNP body formation, and
28 decreased nuclear concentration. Importantly, our work suggests that TDP-43
29 nuclear efflux to the cytoplasm may be regulated by posttranslational modifications,
30 mainly through phosphorylation at Ser48 in the NTD. We show that the
31 phosphomimetic mutation S48E, which inhibits NTD-driven oligomerization [44],
32 decreases nuclear TDP-43 localization by almost 50%. Thus, phosphorylation at
33 Ser48 may normally tune TDP-43 nuclear-cytoplasmic distribution and aberrant

1 activation of the phosphorylation pathways, which remain unknown, may contribute
2 to increased cytoplasmic TDP-43 accumulation.

3
4 Together, our studies highlight multiple mechanisms that control TDP-43 RNP
5 assembly and nuclear-cytoplasmic distribution through different types of interactions
6 mediated by distinct TDP-43 domains, including binding to various RNA sequences
7 with different affinities. This idea also implies that dysfunction of these regulatory
8 factors may result in abnormal or pathological cytoplasmic TDP-43 accumulation.
9 Therefore, these mechanisms may be targeted for therapeutic intervention to restore
10 TDP-43 homeostasis and function.

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21 **Disclosure and competing interests statement**

22 The authors declare that they have no conflict of interest.

23 24 **MATERIALS AND METHODS**

25
26 Chemicals were purchased from Sigma Aldrich unless otherwise specified.

27
28 **Cloning, cell culture and stable cell production-** Generation of HEK293^{HA-TDP-43}
29 stable cell lines, expressing HA-tagged WT, E17R, S48A, S48E, M337V and Δ CR
30 constructs and stable cell lines was previously described [40]. Mutants A326P and
31 M337P were generated by site-directed mutagenesis as previously described[10]
32 using the primers listed in Supplementary Table 1. Briefly, the HEK293-derived cells
33 Flp-InTM T-RexTM 293 Cell Line (ThermoFisher Scientific) were stably transfected to

express HA-TDP-43 upon induction with tetracycline (1 µg/mL). Cells were grown and maintained in DMEM (Dulbecco's Modified Eagle's Medium – High Glucose, Corning) supplemented with 10% FBS (Fetal Bovine Serum) and incubated in a humid atmosphere at 37 °C and 5% CO₂. Expression of HA-tagged TDP-43 construct was induced at 30% confluence.

Cell fractionation- Stable cell lines cultured in 10 mm dishes (TPP) were induced with 1 µg/mL tetracycline for 72 h prior to harvesting and were pelleted through centrifugation at 300g for 5 minutes. Cell pellets were washed twice with PBS (phosphate buffered saline) and gently resuspended in 2 mL of Lysis Buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM TCEP, protease inhibitor cocktail, 10 mM NaF, 1.5 mM orthovanadate, 10 mM β-glycerol phosphate). Resuspended samples were incubated for 5 min on ice and Dounce homogenized with 10 strokes (Pyrex, No 7727). Samples were pelleted at 200g for 5 min and supernatants were collected as cytoplasmatic fractions. The pellets were rinsed in 2 mL of PBS and pelleted at 200g for 5 min. The pellets were resuspended in 500 µL of PBS to obtain the nuclear fraction. All fractionation steps were carried out at 4°C using cold buffers. Fractions were mixed with Laemmli Buffer (0.2 M Tris pH 6.8, 20% Glycerol, 10% SDS, 1.43 M β-mercaptoethanol, 0.05% bromophenol blue) and heated for 5 min at 95°C. Equal volumes were resolved in standard 12% SDS-PAGE and immunoblotted against specified antibodies.

Heterokaryon assay- HEK293^{HA-TDP-43} cells were grown on glass coverslips and heterokaryon assays were performed as previously described [20].

1,6-Hexanediol Treatment- 5% 1,6-hexanediol was added to HEK293^{HA-TDP-43} cells cultured at 80 to 90% confluency for 5 minutes prior to harvesting.

Size exclusion chromatography- HEK293^{HA-TDP-43} cells were harvested at 90% - 100 % confluence following 72 h of tetracycline induction. Cells were washed with PBS and resuspended in lysis buffer (25 mM Tris HCl pH 7.5, 0.1 mM Na₃VO₄, 10 mM MgCl₂, protease inhibitor cocktail, 150 mM NaCl, 0.5% NP-40, 2 mM DTT, 5 mM β glycerophosphate) and processed using a Dounce homogenizer with 20 strokes (Pyrex, No 7727). Following centrifugation, soluble proteins were applied to the size

exclusion gel filtration column (Superdex 200 Increase 10/300 GL, GE Healthcare), using an Akta P-920 Pump FPLC system (Amersham) and Running Buffer: 25 mM Tris-HCl (pH 7.5), 150 mM NaCl. Fractions were collected at 0.5 ml min⁻¹. The resulting dilution from input lysate was approximately 60-fold. Fractions were analyzed by standard SDS-PAGE followed by immunoblotting. The approximate molecular weight of each fraction was determined based on the elution volumes of thyroglobulin (670 KDa), γ globulin (158 KDa), ovalbumin (44 KDa), myoglobin (17 KDa), vitamin B12 (13.5 KDa), according to manufacturer instructions.

High content analysis of RNA-dependent TDP-43 localization- HEK293^{HA-TDP-43} cell lines (WT, F4L, E17R, and delta-CR) were plated on Ultimatrix-coated (1:100, R&D), 96-well optical plastic plates (Ibidi) to achieve ~75% confluence. Doxycycline (1 μ g/mL) was added to culture media at the time of plating, targeting 24 h of induction at the time of fixation. Transcriptional blockade was by NVP2 (250 nM, Tocris) or actinomycin D (1 μ M) for 2h. RNA oligonucleotides (IDT, see table) were transfected using Lipofectamine RNAiMax (Thermo Fisher) according to the manufacturer's instructions except that the lipofectamine amount was doubled based on initial optimization studies (not shown). Cells were fixed for analysis 5 h post-transfection. To determine the localization of HA-tagged TDP-43 and mutants, cells were fixed with 4% paraformaldehyde in PBS, permeabilized and blocked in 0.1% Tx-100 and 10% normal goat serum (Vector Labs), and immunostained with rabbit anti-HA (1:1500, Cell Signaling Technologies) overnight at 4°C in 10% NGS/PBS. After two PBS rinses, Alexa Fluor-labeled secondary antibodies were added for 1 h at room temperature (1:1000, Thermo). Cells were rinsed with PBS containing Hoechst 33342 and transferred to 50% glycerol/PBS for automated imaging and analysis using an ImageXpress Micro Confocal high content microscope with MetaXpress software (Molecular Devices) as described (Duan et al., 2022). A minimum of four independent biological replicates was analyzed per experiment.

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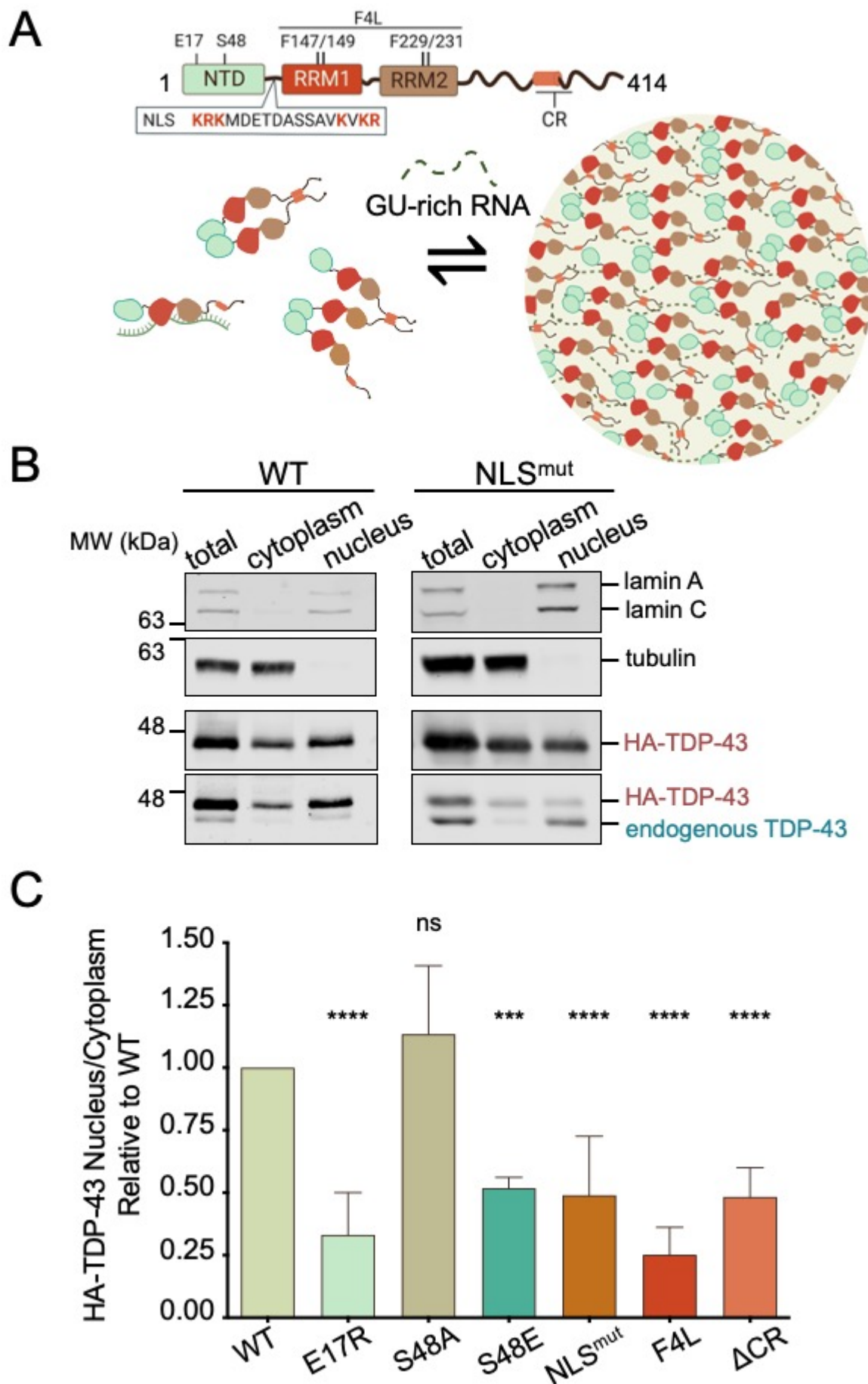


Figure 1. Defects in RNA-mediated TDP-43 self-assembly decrease nuclear localization. A) TDP-43 domain organization and model of RNA-mediated TDP-43 self-assembly involving multivalent interactions through the N-terminal domain (NTD) oligomerization and C-terminal assembly. Mutations in the NTD, RNA recognition motifs (RRMs), and deletion of the conserved α -helical region (Δ CR) in the C-tail (a.a. 316-343) are highlighted. The basic residues (red) in the nuclear localization signal (NLS) were substituted to generate NLS^{mut}, used as control. B) Immunoblot of total, cytoplasmic and nuclear fractions of HEK293^{HA-TDP-43} cells. Lamin A/C and tubulin were used as nuclear and cytoplasmic controls, respectively. C) HA-TDP-43 nuclear to cytoplasmic signal ratio (N/C) quantified from immunoblotting, normalized to WT in each independent experiment. Mean $\pm$ SD is shown for 6 independent replicates. ns, not significant, *** p <0.001, **** p <0.0001 multiple comparison one-way ANOVA with Tukey's test.

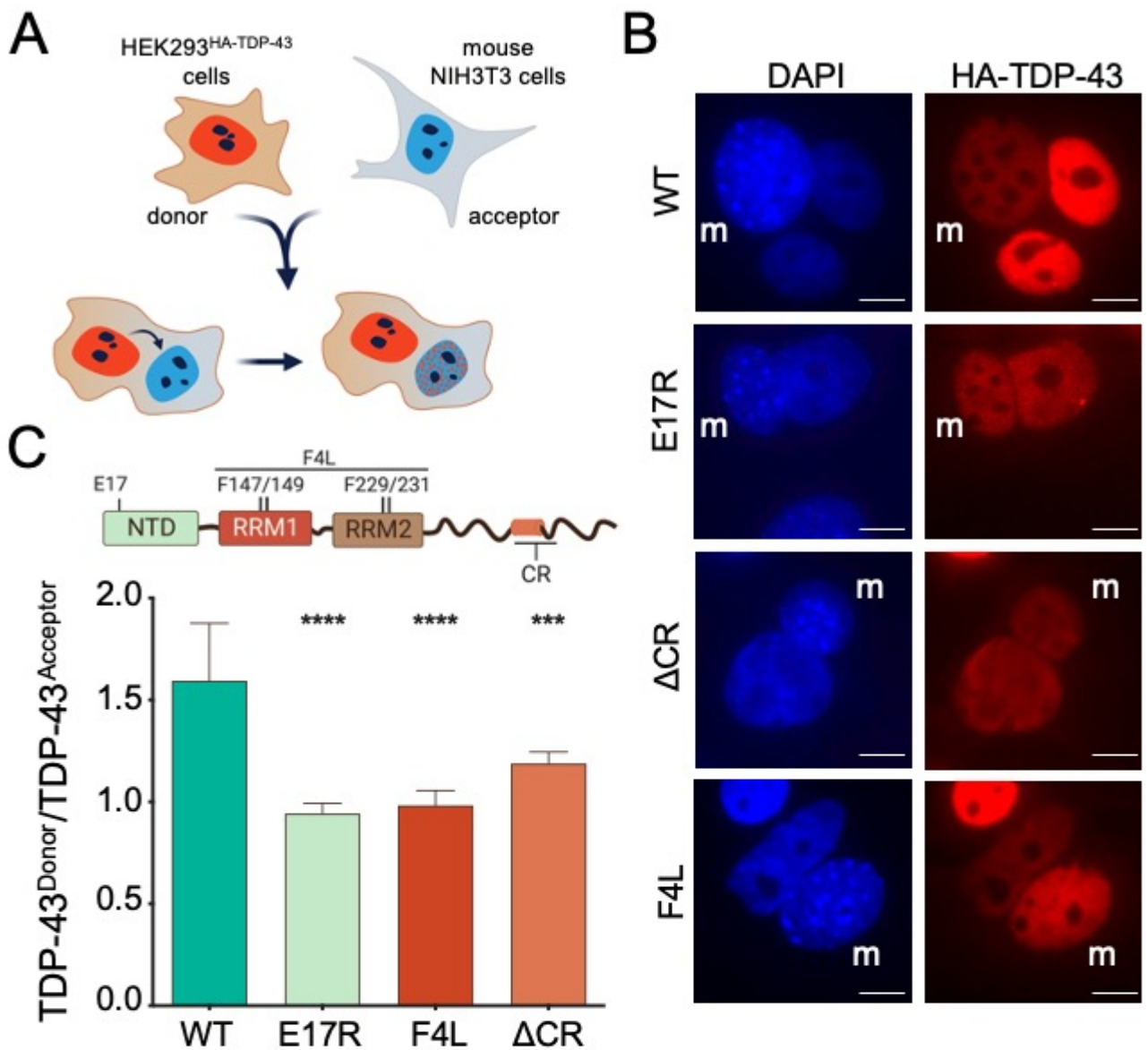


Figure 2. Disruption of RNA binding or TDP-43 self-assembly accelerates TDP-43 nuclear efflux. **A)** Heterokaryon assays in which HEK293^{HA-TDP-43} cells are fused to mouse NIH3T3 cells. HEK293^{HA-TDP-43} nuclei (donor) and HA-TDP-43 signal are depicted in red, mouse NIH3T3 nuclei (acceptor) in blue. **B)** Immunofluorescence of heterokaryons highlighting mouse nuclei (m). Scale, 10 μ m. **C)** Quantified HA-TDP-43 signal ratio of HEK293^{HA-TDP-43} (donor) to mouse (acceptor) nuclei of heterokaryons. Mean $\pm$ SD shown for 7 independent replicates, 50 or more heterokaryons were quantified, *** p <0.001, **** p <0.0001, one-way ANOVA with Tukey's test compared to WT.

Figure 3

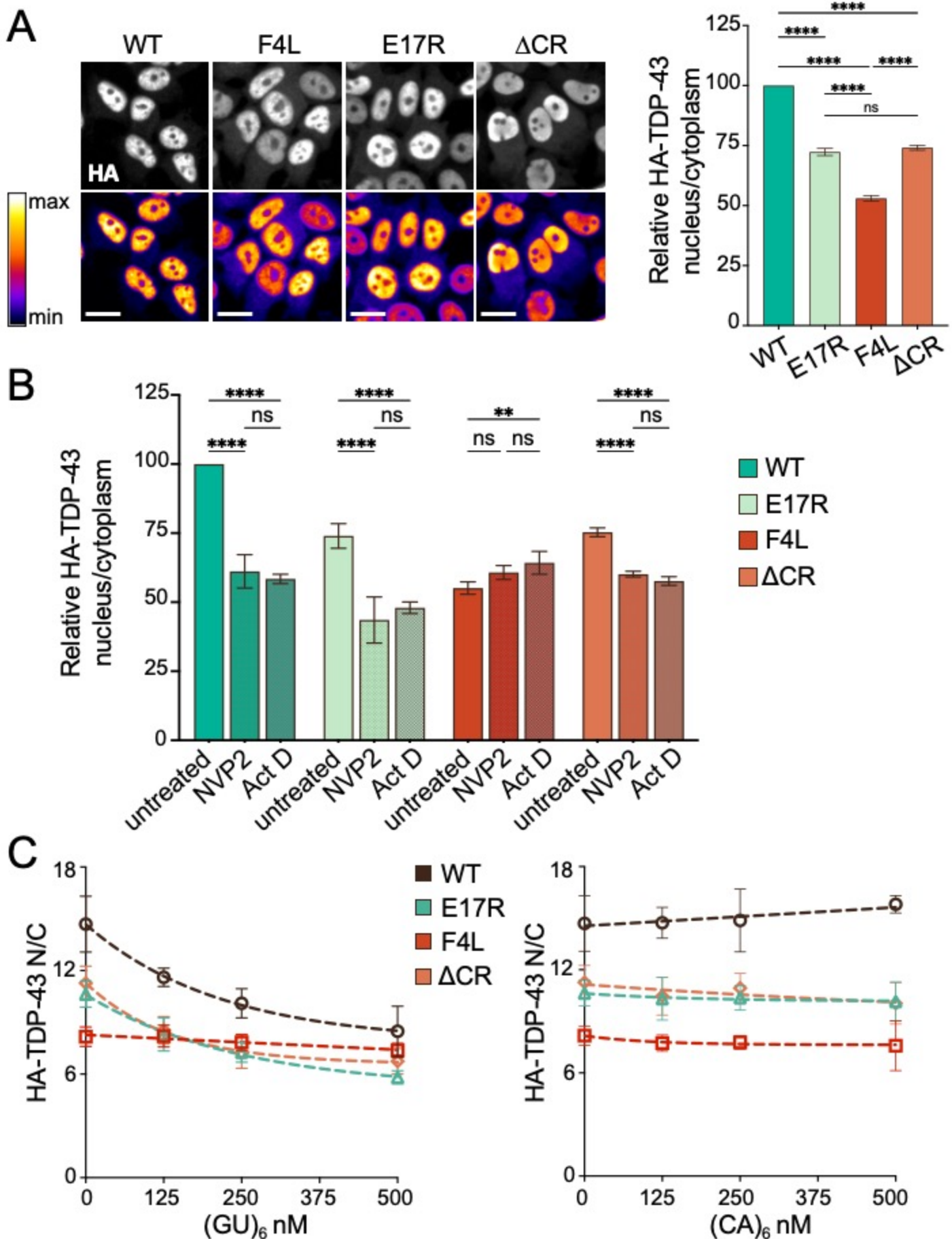
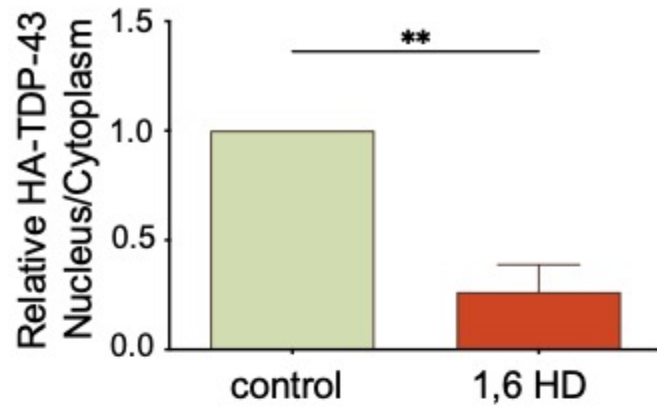


Figure 3. RNA binding and TDP-43 self-assembly exert independent and additive effects on TDP-43 nuclear localization. A) HEK293^{HA-TDP-43} cells expressing HA-tagged WT and mutant TDP-43 probed with HA antibody (top: immunofluorescence, bottom: pseudo-color look-up table). The intensity histogram for each image was independently maximized across the full range. Scale bar, 20 μ m. Graph shows automated quantification of the TDP-43 nuclear/cytoplasmic ratio (N/C) at steady state, normalized to WT. Mean $\pm$ SD is shown for $\sim$ 1600 cells/well in 7 independent replicates. ns, not significant, **** p < 0.0001, one-way ANOVA with Tukey's test. B) HA-TDP-43 N/C quantified 2 h-post transcriptional blockade with actinomycin D (Act, 1 μ M) or NVP2 (250 nM) versus DMSO control, normalized to WT control cells. Mean $\pm$ SD is shown for $\sim$ 1700 cells in 4 independent replicates. ns, not significant, ** p < 0.01, **** p < 0.0001, two-way ANOVA with Tukey's test. C) HA-TDP-43 N/C quantified 5 h post-transfection with increasing concentrations of (GU)₆ or (CA)₆ oligonucleotides. Mean $\pm$ SD is shown for $\sim$ 1700 cells/well in 5 independent replicates.

Figure 4

A



B

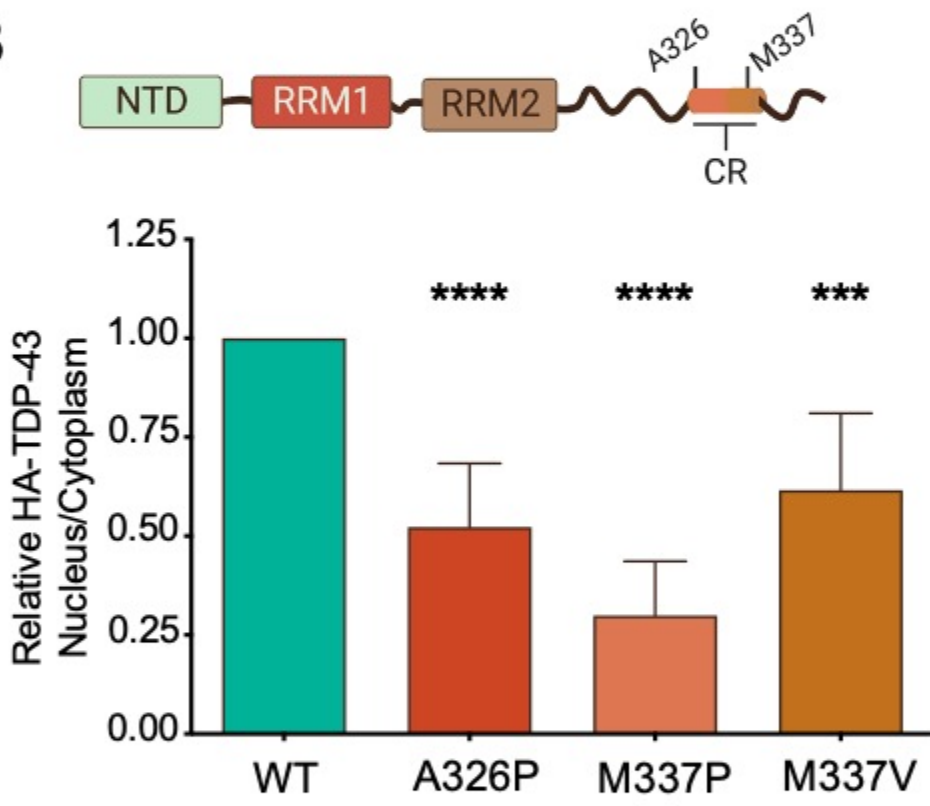


Figure 4. Inhibition of phase separation disrupts TDP-43 nuclear retention. A) Relative nuclear to cytoplasmic signal ratio (N/C) of endogenous TDP-43 in HEK293 cells treated with 1,6 hexanediol (HD, 5% for 5 min) quantified from immunoblotting (Fig. S2A), expressed relative to non-treated control. Mean $\pm$ SD is shown for 6 independent replicates. B) HEK293^{HA-TDP-43} cells expressing HA-tagged WT and A326P, M337P and M337V mutants located in the conserved α -helical region (CR). Quantification of N/C of HA-TDP-43 relative to WT in each replicate from immunoblotting (Fig. S2B). Mean $\pm$ SD is shown for 6 independent replicates. ***p<0.001, ****p < 0.0001, one-way ANOVA with Tukey's test.

Figure 5

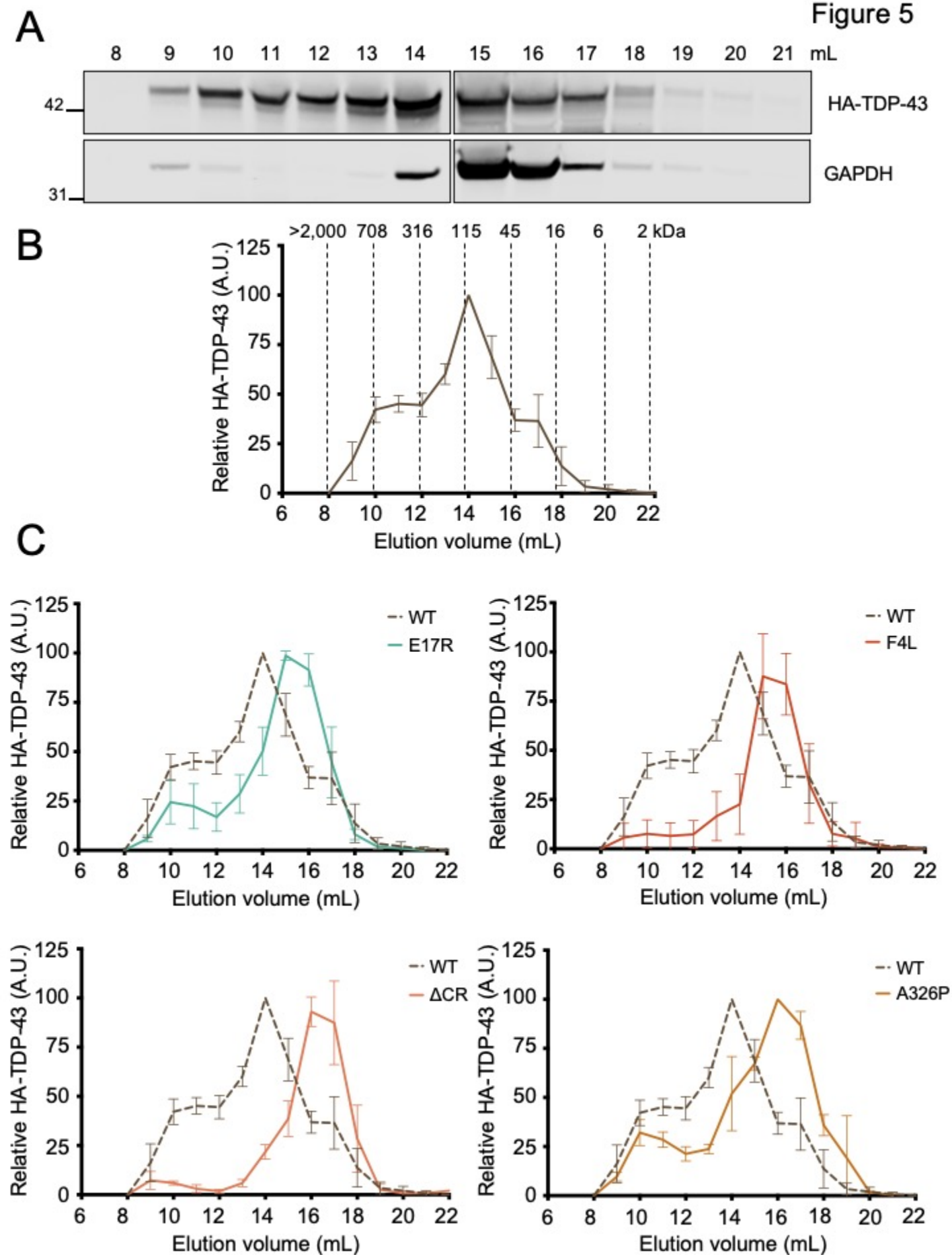


Figure 5. Disruption of RNA binding, NTD oligomerization and phase separation inhibits the assembly of large macromolecular TDP-43 complexes in cells. A) Immunoblot detecting HA-TDP-43 levels in fractions eluted from gel filtration size exclusion chromatography of HEK293^{HA-TDP-43} cell lysate. Membranes were probed with HA antibody and GAPDH as control. B) WT HA-TDP-43 levels quantified in each elution fraction expressed as percent relative to the most abundant TDP-43 fraction. Mean $\pm$ SD is shown for 4 independent replicates. The estimated molecular weight is shown for each fraction based on calibration experiments. C) Comparison of elution profiles for WT (dashed) vs. mutant (solid) TDP-43. Mean $\pm$ SD is shown for ≥ 3 independent replicates.

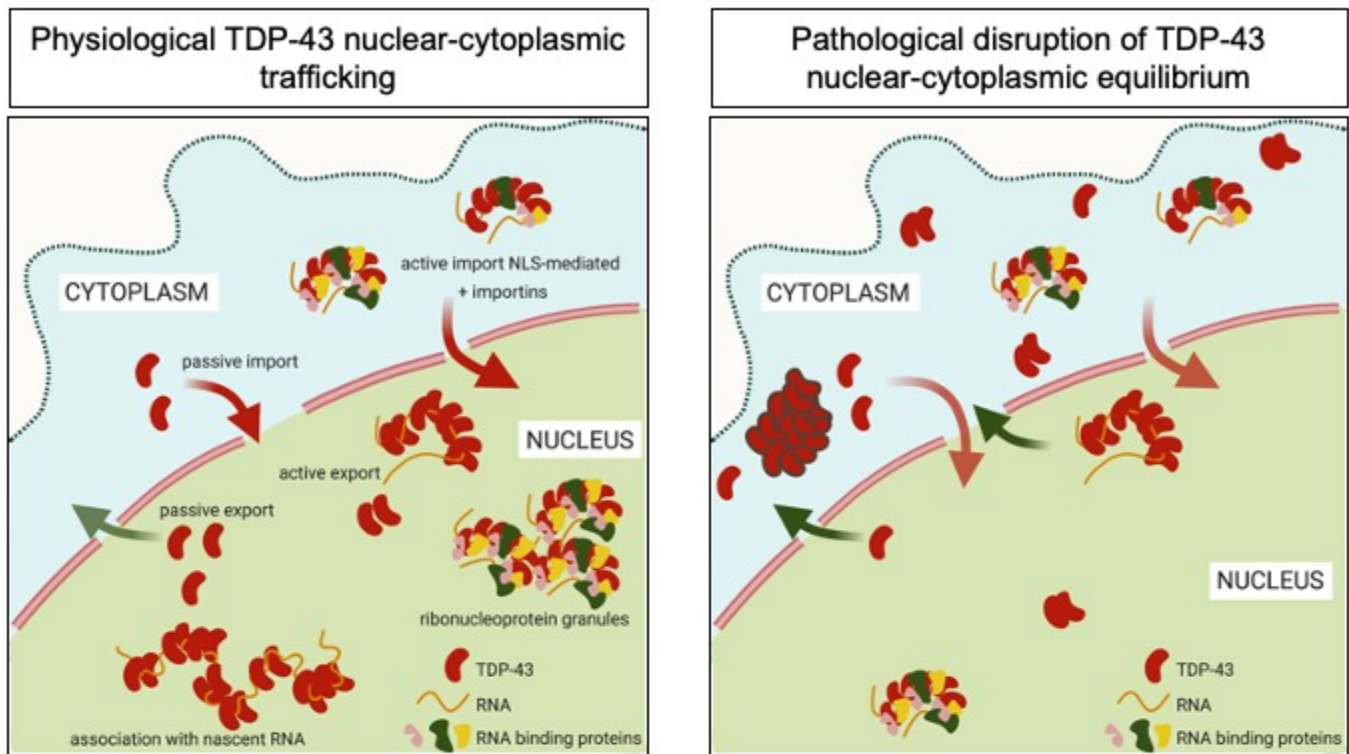


Figure 6. TDP-43 nuclear localization depends on the assembly of RNP complexes mediated via multivalent interactions. Ribonucleoprotein TDP-43 complexes in the nucleus contribute to TDP-43 nuclear localization through the formation of higher-order macromolecular assemblies unable to passively diffuse to the cytoplasm. Under aberrant conditions, disruption of RNA binding or self-assembly increases levels of smaller TDP-43 species inducing greater cytoplasmic localization. Chronic defects in RNP assembly may increase TDP-43 misfolding and aggregation in the cytoplasm.