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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: *Can specific RNA molecules be used to prevent TDP-43 aggregation and decrease neurotoxicity?* 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.

15. SUBJECT TERMS

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

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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). The submitted publication was included in the previous report. We found that GU-rich RNA molecules inhibit the conversion of TDP-43 biomolecular condensates to fibrils, or maturation into irreversible aggregates. These studies are based on the paradigm that intracellular organization of RNA binding proteins into ribonucleoprotein (RNP) granules is mediated by a process of condensation or phase separation. Based on evidence emerging over the last decade, these protein and RNA-rich granules are formed via liquid-liquid phase separation (LLPS) that often show dynamic, liquid-like properties (2-4). The high concentration of the components, while retaining dynamic properties within the droplets, may be essential to determine function, cellular organization and rapid response to cellular stimuli. Numerous studies also suggest that defects in LLPS homeostasis are associated with disease, such as in the case of TDP-43, FUS (Fused in sarcoma) and other RNA binding proteins linked to neurodegeneration (5-7). Specifically, conversion of the condensates into fibrils or complexes with solid-like properties may lead to the accumulation of protein aggregates associated with pathology. Our results, discussed in the previous report, suggest that RNA binding increases the dynamic and liquid properties of TDP-43, depending on RNA sequence and specific interactions. We speculate that RNA binding prevents the conversion of TDP-43 droplets into solid-like complexes. Our work also suggests that RNA oligonucleotides may effectively block TDP-43 pathology as early as during the initial misfolding stage.

Based on this work, our recent observations summarized below:

- **TDP-43 solubility is enhanced with additional RNA oligonucleotides with sequences other than GU repeats.** We tested the ability of an RNA sequence denominated CLIP34 in promoting TDP-43 solubility. This RNA sequence is derived from a natural RNA TDP-43-binding transcript that was previously shown to decrease neurotoxicity in neurons(8). CLIP34 is encoded at the 3' UTR of the TDP-43 transcript and mediates the recruitment of TDP-43 during autoregulation (9, 10). We found that CLIP34 increases TDP-43 solubility and liquid/dynamic properties of TDP-43 complexes. To define the mechanism by which CLIP34 increases TDP-43 solubility we performed binding assays and determined a stoichiometry of 3 to 4 TDP-43 per RNA molecule. Furthermore, we investigated the role of CLIP34-mediated TDP-43 condensation in autoregulation in cells. We found that TDP-43 condensation is necessary for autoregulation. This work was published and included in the Appendix.

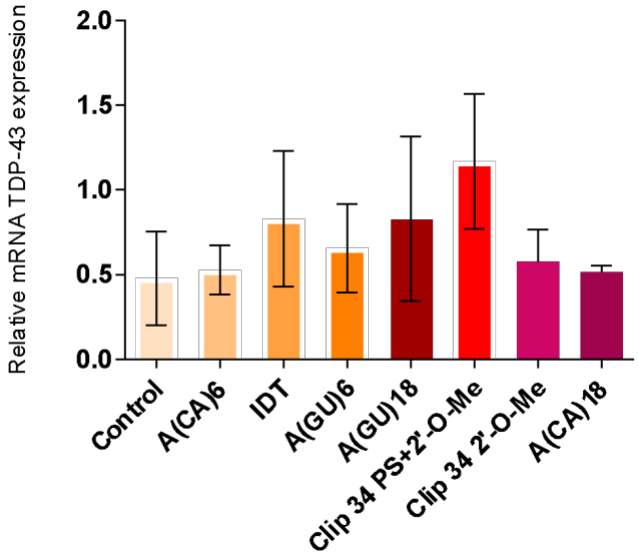

Figure 1. Fully modified CLIP34 inhibits TDP-43 autoregulatory function. HEK293 cells were treated with RNA oligonucleotides consisting of two TDP-43-specific sequences $A(GU)_6$ and CLIP34 and two non-specific controls $A(CA)_6$ and IDT. The oligonucleotide backbone was fully modified as phosphorothioate (PS), non-modified, or carried the 2' O-Me sugar modification only. Autoregulatory activity in these stable cell lines was measured by qPCR quantifying the inhibition of endogenous TDP-43 expression upon induction of HA-TDP-43 expression. Mean of four independent replicates and S.D.

- **RNA-mediated assembly of TDP-43 in cells retains the protein in the nucleus**. Our most recent findings show that interactions such as the one uncovered for TDP-43 with CLIP34 increase nuclear localization of the protein. The large size of these TDP-43-CLIP34 complexes reduces exit to the cytoplasm. Increased cytoplasmic TDP-43 localization is associated with decreased solubility and TDP-43 aggregates in this compartment are a halmark feature of ALS. Therefore, our results suggest that TDP-43-RNA intreactions that induce protein self-assembly or condensation promote TDP-43 nuclear retention and prevent aggregation.The manuscript describing these findings is in preparation.
- **Specific sequences and backbone modifications in RNA molecules inhibit normal TDP-43 cellular function in RNA processing.** During the design of this aim we considered the possibility that sequences that bind TDP-43 with tight affinity (low nanomolar range), as $(GU)_6$, may compete with TDP-43 RNA binding partners and inhibit RNA processing. To test this, we measured the effect of RNA treatment on TDP-43 autoregulatory function in cells, using well-established assays in our lab. Our preliminary data shows no significant cell toxicity under the conditions tested. Moreover, $(GU)_6$ treatment did not significantly alter TDP-43 autoregulation, suggesting that GU-rich RNA does not significantly affect physiological TDP-43 activity in cells (**Fig. 1**). However, transfection of CLIP34 with chemically modified phosphorothioate backbone plus 2' O-Methyl sugar disrupted autoregulatory activity. We also

tested the same sequence with 2'-O-Me only and found that its presence did not affect TDP-43 autoregulation. These preliminary results indicate that RNA treatment may alter normal TDP-43 function. However, this inhibition depends on RNA sequence and chemical modification. These findings will guide our design of optimal RNA oligonucleotides that decrease TDP-43 pathology without diminishing protein function, which is essential in cells.

- **RNA treatment of mouse neurons decreases TDP-43-mediated cytotoxicity depending on RNA sequence and chemical modifications.** During the course of last year we initiated a collaboration with Dr. Sami Barbada (University of Michigan) who has established assays to measure TDP-43-mediated toxicity in mouse neurons. We tested the various RNA oligonucleotides that decreased TDP-43 aggregation in our HEK293 culture model. In particular, the Barmada lab found that a specific modification of CLIP34 suppresses TDP-43-mediated toxicity (**Fig. 2**). The 2'OME sugar modification on CLIP34 alone shows no significant difference compared to control. The CLIP34*FITC oligo reduces toxicity, but not the 2'OME modified version, or the fully modified version (phosphorothioate in the backbone). These differences may be caused by alterations in the efficiency of RNA delivery inside the cells. We are currently testing this possibility.

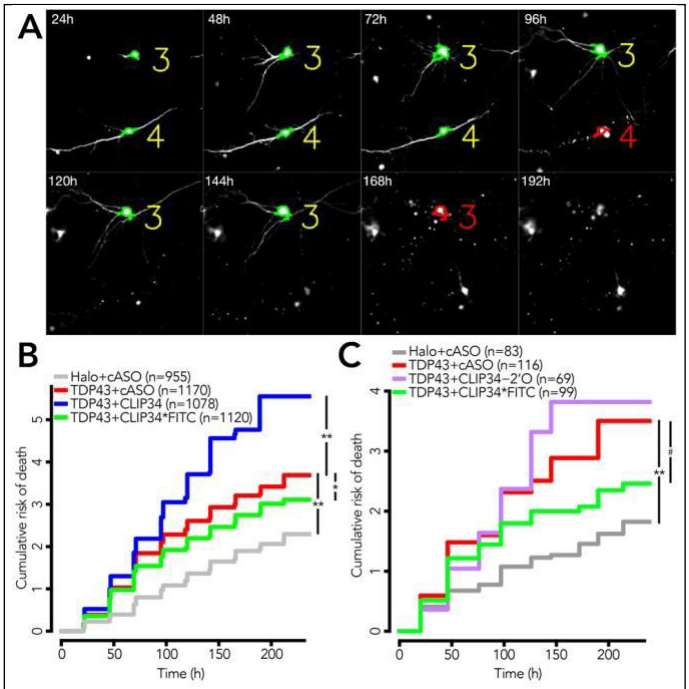

Figure 2. CLIP34 ASOs display distinct effects on the survival of TDP43-expressing primary rodent neurons. (**A**) Longitudinal imaging and automated survival analysis of primary rodent mixed cortical neurons. All cells are transfected with a marker protein (HaloTag) enabling their visualization by fluorescence microscopy. Custom-written scripts enable tracking of hundreds of individual neurons at regular 24h intervals. Image segmentation algorithms define individual neurons, draw regions of interest (green) surrounding each cell, label neurons with unique identifiers (yellow), and determine the time of death for each (red). (**B**) Cumulative risk of death plot showing the relative toxicity of TDP43 overexpression in comparison to HaloTag alone (**$p<2x10^{-16}$, hazard ratio=1.9) in cells treated with control ASO (cASO). Application of CLIP34 resulted in greater toxicity (**$p=1x10^{-10}$, hazard ratio=1.3), while FITC-labeled CLIP34 (CLIP34*FITC) extended survival (*$p=5x10^{-4}$, hazard ratio=0.86), compared to cASO. Data from 8 technical replicates per condition, in each 2 biological replicates. (**C**) Preliminary data confirming the toxicity of TDP43 overexpression (**$p=1x10^{-5}$, hazard ratio=1.9), as well as consistent protective trend for CLIP34*FITC ($^{\#}p=0.1$, hazard ratio=0.79), but little effect of 2'O-ME modified CLIP34 (CLIP-2O). Data pooled from 8 technical replicates per well.

Aim 2–Determine whether RNA inhibits intracellular TDP-43 aggregate seeding and propagation. Transfection of human cells with recombinant TDP-43 aggregates results in a 3-fold increase in cellular TDP-43 cytoplasmic aggregation relative to treatment with soluble protein (11). Indeed, additional studies show that frontotemporal dementia (FTD)-derived, TDP-43-positive extracts spread TDP-43 pathology in mouse brain (12). The ability of TDP-43 aggregates to spread intracellularly in prion-like fashion is a strong indication that TDP-43 inclusions are toxic and support a gain of function mechanism in pathogenesis. In this aim

we examine the potential of RNA-based tools to prevent disease progression by targeting a central mechanism in the spread of TDP-43 pathology. We tested whether $(GU)_6$ RNA was able to prevent propagation of TDP-43 inclusions and found that intracellular seeding triggered by pre-formed TDP-43 aggregates significantly decreased in the presence of $(GU)_6$ RNA. We have recently developed two additional models to study cellular TDP-43 aggregate seeding and spread of pathology. One approach is to analyze TDP-43 aggregate propagation in primary neurons derived from mouse brain cortex. The other technique uses biosensor cell lines from our collaborator Marc Diamond (UT Southwestern) that are used to quantify TDP-43 aggregation on the basis of Foster resonance energy transfer (FRET). We used these cell lines to detect TDP-43 aggregation and seeding using frontotemporal dementia extracts and pre-formed aggregates from purified TDP-43. Based on our promising results with the FRET sensor line, we will use these to test the effect of RNA oligonucleotide treatment on TDP-43 aggregate seeding. The advantage of this method compared to the previously developed sensor cell line is that FRET analysis may be easily adapted for medium or high throughput screening.

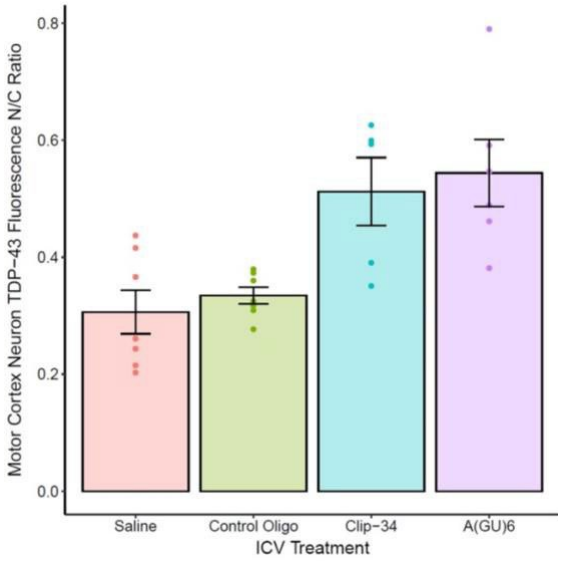

Figure 3. RNA treatment rescues TDP-43 nuclear localization *in vivo*. hTDP-43ΔNLS mice injected with saline, control oligonucleotide, or the TDP-43 specific sequences Clip-34, A(GU)6. One month following injection, TDP-43 nuclear/cytoplasmic localization was quantified in neurons. Each data point represents an individual mouse. Mean and SD are shown.

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. One of the few ALS mouse models that recapitulates TDP-43 pathology and neurotoxicity, as seen in human patients, is achieved by expressing the nuclear localization signal (NLS)-deficient TDP-43 mutant, $TDP\text{-}43^{NLS}$, in motor neurons (13). These mice, known as hTDP-43ΔNLS, express a human copy of the mutant. $TDP\text{-}43^{NLS}$ 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*.

During the previous award period we observed that all oligonucleotides were able to increase nuclear localization of TDP-43 and modestly improve behavior early in disease. Our more recent work confirmed this increase in nuclear localization and demonstrated that it was dependent on oligonucleotide sequence. However, we did not observe effects on phosphorylated TDP-43 (pTDP) accumulation, nor did we see a sustained improvement in behavior due to oligonucleotide administration.

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?

Specific Aim 1: Establish RNA-based methods to block TDP-43 aggregation. Determine the effect of selected RNA molecules in cell viability and TDP-43 RNA processing function. We will screen RNA molecules for their ability to decrease TDP-43-mediated neurotoxicity using a well-established and unique assay in collaboration with Dr. Sami Barmada at the University of Michigan (Ann Arbor, MI). Based on our results showing very promising avenues to block aggregation of the purified TDP-43 protein from our lab, we tested the top candidates in primary neurons. These experiments directly evaluate and quantify the effect of TDP-43 on the health of neurons. These assays are less costly and less time consuming compared to in vivo animal studies, which make them ideally suited to test multiple compounds. We measured the cumulative death hazard of the neurons associated with TDP-43 toxicity at a single-cell level. Our preliminary results showed that treatment with specific RNA oligonucleotides significantly decreased TDP-43-linked neurotoxicity. These are highly promising results suggesting that specific RNA molecules may be used to decrease neuronal death caused by TDP-43 dysfunction. Our plan is to continue using this assay to test modifications of the RNA oligonucleotides as well as additional sequences identified using our in vitro methods. These results will help determine the top candidates to use for future therapeutic strategies and in vivo.

Specific Aim 2: Determine whether RNA inhibits intracellular TDP-43 aggregate seeding and propagation. Treat in vitro generated TDP-43 aggregates with control and TDP-43-targeting RNA molecules prior to seeding intracellular aggregates. We will use the biosensor cell line established by our collaborator Dr. Marc Diamond (UT Southwestern Medical Center) to quantify the amount of seeding by TDP-43 aggregates (14) as a function of RNA treatment. Our findings show that disease-derived aggregates and pre-formed fibrils from purified TDP-43 significantly increase seeding compared to control. This cellular reporter is now being used to quantify differences in aggregate seeding activity upon treatment with different RNA molecules. We will test RNA oligonucleotides identified as the top candidates in Aim 1.

Specific Aim 3. Use RNA to decrease TDP-43 pathology and neurotoxicity in a mouse model of ALS. We will test the *in vivo* therapeutic efficacy of an additional oligonucleotide that showed a benefit in our cellular work. When we designed the oligonucleotides to be delivered *in vivo* as a part of this project, we focused primarily on nucleotide sequence as a means of modulating TDP-43 binding affinity (leveraging its preference for GU-rich sequences) and only secondarily on oligonucleotide chemistry as a means of ensuring proper delivery and limiting toxicity. Because PO oligonucleotides have not been well described in our mouse model, we will begin by characterizing the ICV bolus distribution, longevity, and dose-limiting toxicities of the Clip-34 PO compound and a scrambled control by immunofluorescence and splint-ligation qPCR. With these characteristics in mind, we will treat our mouse model of TDP-43 pathology as previously described, evaluating TDP-43 localization and aggregation via

immunofluorescence, TDP-43 function via PCR splicing assay, and disease progression via weight, rotarod performance, and survival. Notably, our PCR splicing assays will assess TDP-43's ability to splice known targets (including SORT1, DNAJC5, ADPN2 and others) in mice treated with Clip-34 PO, directly evaluating whether oligonucleotide administration affects physiological TDP-43 function.

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 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.

1. Koehler, L.C.[§], Grese, Z.G.[§], Bastos, A.C.S., Mamede L.D., Heyduk T. & **Ayala, YM.** (2022) TDP-43 oligomerization and phase separation properties are necessary for autoregulatory function. *Frontiers in Neuroscience,* **16**, 818655. [§] equal contribution. Acknowledgement of federal support (yes)
2. Grese, Z.G., Bastos, A.C.S., Mamede, L.D., French, R.L., Miller, T.M. & **Ayala, YM.** (2021) RNA binding controls TDP-43 phase separation through multivalent interactions while maintaining liquid properties. *EMBO Reports,* **22,** e53632. Acknowledgement of federal support (yes).
3. **Ayala, Y.M.**, Nguyen, A.D. (2021) RNA-based therapies for neurodegeneration. *Missouri Medicine*, **118**, 340-345. Acknowledgement of federal support (yes)

Other publications, conference papers, and presentations.

1. Invited Seminar, “Regulation and Function of TDP-43 in Health and Disease” FAZE Medicines. Virtual/Zoom. Boston, MA. July 2022.
2. Invited Seminar, “Regulation and Function of TDP-43 Biomolecular Condensates.” 22nd Annual Robert Packard Center for ALS Research Symposium. Baltimore, MD. February 2022.

3. Invited Seminar, “Using RNA to find answers in neurodegeneration.” Hope Center for Neurological Disorders, ALS mini-seminar Series. Washington University School of Medicine, St. Louis MO. December 2021.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Dr. Sami Barmada from the University of Michigan was added 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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TDP-43 Oligomerization and Phase Separation Properties Are Necessary for Autoregulation

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Loss of TDP-43 protein homeostasis and dysfunction, in particular TDP-43 aggregation, are tied to amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). TDP-43 is an RNA binding protein tightly controlling its own expression levels through a negative feedback loop, involving TDP-43 recruitment to the 3^{0} untranslated region of its own transcript. Aberrant TDP-43 expression caused by autoregulation defects are linked to TDP-43 pathology. Therefore, interactions between TDP-43 and its own transcript are crucial to prevent TDP-43 aggregation and loss of function. However, the mechanisms that mediate this interaction remain ill-defined. We find that a central RNA sequence in the 3^{0} UTR, which mediates TDP-43 autoregulation, increases the liquid properties of TDP-43 phase separation. Furthermore, binding to this RNA sequence induces TDP-43 condensation in human cell lysates, suggesting that this interaction promotes TDP-43 self-assembly into dynamic ribonucleoprotein granules. In agreement with these findings, our experiments show that TDP-43 oligomerization and phase separation, mediated by the amino and carboxy-terminal domains, respectively, are essential for TDP-43 autoregulation. According to our additional observations, CLIP34-associated phase separation and autoregulation may be efficiently controlled by phosphorylation of the N-terminal domain. Importantly, we find that specific ALS-associated TDP-43 mutations, mainly M337V, and a shortened TDP-43 isoform recently tied to motor neuron toxicity in ALS, disrupt the liquid properties of TDP-43-RNA condensates as well as autoregulatory function. In addition, we find that M337V decreases the cellular clearance of TDP-43 and other RNA binding proteins associated with ALS/FTD. These observations suggest that loss of liquid properties in M337V condensates strongly affects protein homeostasis. Together, this work provides evidence for the central role of TDP-43 oligomerization and liquid-liquid phase separation linked to RNA binding in autoregulation. These mechanisms may be impaired by TDP-43 disease variants and controlled by specific cellular signaling.

Keywords: TDP-43 (TAR DNA-binding protein 43), RNA binding protein, ALS, frontotemporal dementia (FTD), liquid-liquid phase separation (LLPS), protein aggregation, TDP-43 autoregulation, ALS mutations

INTRODUCTION

Aggregation, cellular mislocalization and loss of nuclear TDP-43 (TAR DNA binding protein) are hallmarks of amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) (Arai et al., 2006; Neumann et al., 2006) and limbic-predominant age-related TDP-43 encephalopathy (LATE) (Nelson et al., 2019). In addition, TDP-43 inclusions are associated with multisystem proteinopathy (MSP) (Weihl et al., 2009) and other neurodegenerative disorders, including Alzheimer's disease (AD) and chronic traumatic encephalopathy (CTE) (McKee et al., 2010). TDP-43 is an RNA binding protein whose cellular levels are tightly controlled as its overexpression leads to increased cytoplasmic accumulation and aggregation. In addition, even moderate changes in TDP-43 protein expression disrupt the regulation of target genes (Arnold et al., 2013). Abnormal increase in TDP-43 levels results in neurotoxicity as observed in human neurons (Barmada et al., 2010) and in a wide range of animal models, including non-human primates (Tatom et al., 2009; Wils et al., 2010; Xu et al., 2010; Estes et al., 2011; Uchida et al., 2012; Diaper et al., 2013). So far, TDP-43 autoregulation through a negative feedback loop is the only known mechanism controlling TDP-43 expression levels. During this process, TDP-43 binds to its own mRNA 3^{0}UTR and inhibits protein synthesis (Ayala et al., 2011; Polymenidou et al., 2011; **Figure 1A**). This autoregulatory mechanism, reviewed in Tziortzouda et al. (2021), is conserved among vertebrates and its disruption in mouse models results in widespread splicing changes, neurotoxicity (Xu et al., 2010; Arnold et al., 2013; Sugai et al., 2019) and increased cytoplasmic TDP-43 aggregation (D'Alton et al., 2015; Koyama et al., 2016). The existence of mutations that are causative of ALS in noncoding regions involved in autoregulation (Gitcho et al., 2009; Pesiridis et al., 2009) and the finding that one of these mutations shows increased TDP-43 transcript expression (Gitcho et al., 2009), strongly suggest that defects in self-regulation may result in disease. This is also supported by a knock-in mouse model in which the Q331K ALS mutation exhibits cognitive impairment and disrupts autoregulation (White et al., 2018). Furthermore, accumulation of misfolded TDP-43 aggregates potentially increases protein production in affected cells by triggering a feed-forward mechanism, in which TDP-43 is sequestered and is no longer able to self-regulate, as suggested by recent studies (Sugai et al., 2019). In agreement with this model, ALS-affected motor neurons and FTD neurons that lack nuclear TDP-43 show impaired *Tardbp* autoregulation (Koyama et al., 2016; Liu et al., 2019). Collectively, these findings indicate that *Tardbp* may be one of the most critical TDP-43 targets to prevent the loss of protein homeostasis and neurotoxicity. Therefore, improved understanding of the factors controlling TDP-43 autoregulation are necessary to test this model and provide new avenues to maintain TDP-43 proteostasis. However, despite the central importance of this process in TDP-43 function and its link to disease, the mechanisms involved in TDP-43 autoregulation are ill-defined, and the factors controlling autoregulation are even less well understood.

The cellular organization of RNA binding proteins, which in turn impacts protein function, involves condensation into ribonucleoprotein (RNP) granules. This process is mediated by liquid-liquid phase separation (LLPS) into often dynamic, liquid-like complexes (Brangwynne et al., 2009; Wippich et al., 2013; Feric et al., 2016). The high concentration of the components while retaining dynamic properties within the droplets is fundamental for RNP granule function and organization in cells [reviewed in Alberti et al. (2019)]. Formation of RNP granules is mediated by multivalent interactions, including RNA binding and self-assembly through different protein domains, such as low complexity regions and oligomerization domains (Li et al., 2012; Lin et al., 2015; Zhang et al., 2015; Garcia-Jove Navarro et al., 2019). The liquid properties of these assemblies and their multivalent properties enable rapid regulation in response to cellular stimuli [reviewed in Shin and Brangwynne (2017); Wang et al. (2021)]. RNA is a principal component of RNP granules and recent studies, including our own, suggest that RNA binding modulates the LLPS properties of TDP-43 through specific interactions (Zhang et al., 2015; Garcia-Jove Navarro et al., 2019; Grese et al., 2021). However, how LLPS affects RNA processing following recruitment to the transcripts, and whether this is a gene-dependent mechanism is currently not known. Importantly, numerous studies also suggest that defects in LLPS are associated with disease, such as in the case of TDP-43, FUS (Fused in sarcoma) and other RNA binding proteins linked to neurodegeneration (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). Specifically, conversion of the condensates into fibrils or complexes with solid-like properties is thought to cause accumulation of protein aggregates associated with pathology. Therefore, further insight on the regulation of TDP-43 LLPS properties and their involvement in controlling the expression of TDP-43 targets will help elucidate important pathogenic factors. We sought to define the molecular mechanisms in TDP- 43 autoregulation and determine the effect disease-associated conditions have in this process. Our results strongly suggest that autoregulation is mediated by TDP-43 self-assembly and LLPS upon RNA binding. In addition, specific TDP-43 binding to RNA sequences that mediate autoregulation promotes liquid properties of TDP-43 condensates or liquid droplets. In addition, we find that TDP-43 posttranslational modifications and disease-associated variants may alter autoregulation by modulating TDP-43 condensation. Together, these findings highlight the potential of developing therapies based on controlling pathways linked to TDP-43 autoregulation as well as specific TDP-43-RNA binding interactions.

MATERIALS AND METHODS

Reagents and Chemicals Were Purchased From Sigma-Aldrich Unless Otherwise Specified

Plasmid Construction

The HA tagged TDP-43 sequence was cloned into pcDNA5^{TM}/FRT/TO (Thermo Fisher) between BamHI and NotI restriction sites. Quikchange- Site directed mutagenesis protocol (Agilent) was performed to create single-site and

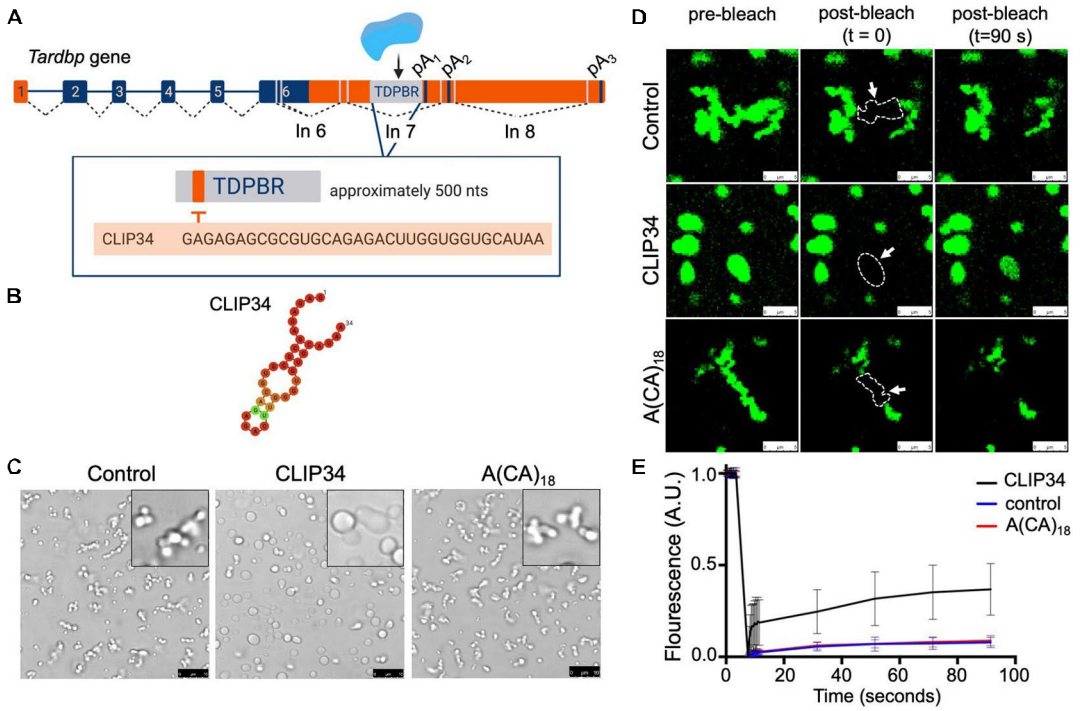

FIGURE 1 | Binding to the RNA region mediating TDP-43 autoregulation increases the liquid properties of TDP-43 condensates. (A) *Tardbp* pre-mRNA organization into introns (lines) and exons (boxes), highlighting coding regions (blue boxes). Splicing is denoted by dashed lines and the approximate positions of alternative splicing in the 3^0 UTR are shown (gray lines). TDP-43 binds to its cognate mRNA transcript in the 3^0 UTR TDP-43 binding region (TDPBR, gray box), including the 34-nt sequence CLIP34. (B) Lowest free energy secondary structure of CLIP34 predicted by RNAfold. The calculated frequency of this structure in the ensemble is 53%. (C) Condensates formed by purified TDP-43 (4 μM) as seen by brightfield microscopy without RNA (control), in the presence of CLIP34 RNA (4 μM), or the non-specific control RNA $A(CA)_{18}$ (4 μM) at 150 mM NaCl, pH 7.5. Representative images of 3 independent experiments from 2 protein preparations. Scale bars, 10 μm. (D) Fluorescence recovery after photobleaching (FRAP) of condensates (white arrow) formed by Oregon green labeled-TDP-43 (10% of total protein) mixed with CLIP34, $A(CA)_{18}$, or in the absence of RNA in the same experimental conditions as (C). Dotted line and arrow define the region of interest. Scale bars, 5 μm. (E) Quantification of fluorescence recovery in panel (D), showing mean values and SD from 3 biological replicates consisting of 16 measurements, performed from 2 different protein purifications.

multiple-site mutations. The 1CR deletion missing a.a. 316–346 was generated from gBlock sequence. pcDNA5 HA-mEGFP-TDP constructs were created by subcloning TDP-43 cDNA into the mEGFP-C1 vector (Addgene) between XhoI and HindIII. HA-mEGFP-TDP-43 was subcloned into pcDNA5^{TM}/FRT/TO using EcoRV and NotI. Oligonucleotides and gBlock sequence are listed in the **Supplementary Table 1**.

Expression and Purification of Recombinant TDP-43

Recombinant TDP-43 expression and purification was carried out as previously described (Grese et al., 2021).

Liquid-Liquid Phase Separation, Turbidity Assays, and Fluorescence Recovery After Photobleaching

The protocols developed in our lab for these assays were recently described (Grese et al., 2021). Microscopy images were taken following equilibration of condensates onto glass coverslips, approximately 60 min after the starting point of the reaction. For turbidity assays, data points were taken as the absorbance at 600 nm 1 at 25 min. Data points were then normalized using the equation $Abs_{norm} = (Abs\ Abs_{blank})/(Abs_{max}\ Abs_{blank})$, where Abs_{max} is the condition being normalized to 1. The RNA oligonucleotides used in these experiments are listed in **Supplementary Table 3**. FRAP was performed as previously described (Grese et al., 2021).

Fluorescence Anisotropy

Purified TDP-43 WT and mutants previously purified were serially diluted in a 1:2 ratio in a final range of 0–2 μM protein into a 300 mM NaCl, 10 mM Tris (pH 8.0), 5% glycerol, 5% sucrose, 0.5 mM TCEP buffer solution. The protein dilution was mixed with CLIP34 (IDT) 3^0 labeled with FITC (fluorescein isothiocyanate) in a final concentration of 100 nM and added in triplicate in a 384-well black flat bottom plate (Corning) in a total reaction volume of 30 μL protected from light. The anisotropy measurements were performed in a Spectra Max i3 plate reader (Molecular Devices) with excitation and emission wavelengths of 480 and 520 nm, respectively. To assess the binding, the anisotropy data was fitted in a nonlinear fit in 4-parameter logistic model to calculate the apparent $IC_{50,app}$. The apparent IC_{50} ($IC_{50,app}$) is the protein concentration in which 50% of the maximal anisotropy change is observed. Data analysis were performed using GraphPad Prism 9.

Cell Culture and Stable Cell Line Production

HEK293 Flp-In™ T-REX™ cells (Thermo Fisher) were maintained in DMEM (Dulbecco's Modified Eagle's Medium—high glucose, Sigma) with 10% FBS (fetal bovine serum) and incubated at 37°C and 5% CO_2. Stable cell lines were achieved through co-transfection of pcDNA5™/FRT/TO/HA or mEGFP constructs and pOG44 using Lipofectamine 3000™ (Invitrogen). Cells were selected using 75 μg/mL hygromycin (Sigma).

Cell Collection, Lysis, and Induction of LLPS Using Cell Lysates

This protocol was modified from Freibaum et al. (2021) and described in Grese et al. (2021). RNA oligonucleotides for these assays (**Supplementary Table 3**) were synthesized by Integrated DNA Technologies. In addition, oligonucleotides modified with a 2^0OMe group and phosphorothioate backbone were used in the experiments carried out at 250 mM NaCl.

Cycloheximide Post-Chase Experiment

WT and M337V HA-TDP stable cells were plated and induced with 1 μg/mL of tetracycline for 72 h to reach confluency of 85–90%. Media was then changed to DMEM, 10% FBS, 100 μg/mL cycloheximide. Samples were taken at 0, 4, 8, 16, 32, and 40 h. Each sample was washed once with PBS and collected by centrifugation. Cell pellets were immediately lysed in cell lysis buffer and sonicated before being prepared for immunoblotting.

Cell Lysate RIPA/Urea Fractionation

WT and M337V stable cell lines were plated and induced for 72 h before being harvested by centrifugation. Cell pellets were then resuspended in RIPA Buffer [50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% SOC, 0.1% SDS, 1x protease/phosphatase inhibitor (SigmaFAST EDTA-free protease inhibitor cocktail tablet)] and sonicated. Total fraction samples were taken after sonication and the samples were then centrifuged at 98,400 *g* for 30 min at 4°C. The pellet was then rinsed with RIPA buffer and centrifuged again. The resulting pellet was resuspended in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris pH 8.5). Fractions were quantified by western blot as described above.

Immunoblotting

Immunoblotting was carried out using standard western blotting techniques and the antibodies for TDP-43 (Proteintech, 10782-2-AP), FUS/TLS (Santa Cruz Biotechnologies, sc47711), matrin-3 (Abcam, ab151739), and GAPDH (Abcam, Cat. No. ab181602). Membranes were imaged using Odyssey scanning (LI-COR) and quantified using ImageStudioLite software (LI-COR).

RNA Extraction and Quantitative RT-PCR

Trizol Reagent (Thermo Fisher) was used for RNA extraction and was carried out according to manufacturer instructions. M-MLV reverse Transcriptase (Thermo Fisher) was used for cDNA production according to the manufacturer instructions. Each primer mentioned in **Supplementary Table 2** was used at a final concentration of 0.4 μM for a CFX96 Real-Time PCR detection system.

RESULTS

Specific TDP-43 Binding to an RNA Sequence Linked to Autoregulation Increases the Liquid Properties of TDP-43 Condensates

TDP-43 binds to an extended region in the 3^0 UTR of its own transcript (*Tardbp*), spanning approximately 500 nucleotides, during autoregulation (**Figure 1A**; Ayala et al., 2011; Polymenidou et al., 2011; Tollervey et al., 2011; Bhardwaj et al., 2013). This TDP-43 binding region (TDPBR) is immediately upstream of the proximal alternative polyadenylation site pA_1 and is included in an alternatively spliced intron (in7). The mechanisms proposed to follow TDP-43 recruitment to TDPBR are based on analyses of the endogenous *Tardbp* transcript and derived gene reporters. These studies support the engagement of different processes during autoregulation: alternative polyadenylation, alternative splicing producing isoforms targeted for degradation *via* nonsense mediated decay (NMD), and nuclear retention of specific transcripts. Detailed studies by Koyama et al. (2016) suggest that autoregulation results from a combination of these mechanisms, but the extent to which each of these pathways contributes to the control of TDP-43 expression remains unknown (Polymenidou et al., 2011; Bembich et al., 2014; Koyama et al., 2016). A 34-nt. region within TDPBR (CLIP34) is commonly found in cross-linking immunoprecipitation (CLIP) analyses of TDP-43-bound transcripts in human cells and mouse brain (Polymenidou et al., 2011; Tollervey et al., 2011; **Figures 1A,B**). This isolated sequence is capable of decreasing TDP-43 toxicity in neurons, suggesting that CLIP34 binding prevents TDP-43 aggregation and aberrant phase transitions (Mann et al., 2019). We recently reported that CLIP34 was among the GU-rich RNA sequences capable of modulating TDP-43 phase separation properties (Grese et al., 2021). In the present work, we sought to gain further insight of the mechanisms that mediate TDP-43 LLPS linked to CLIP34 and explore whether LLPS plays a role in TDP-43 autoregulatory function. For this, TDP-43 LLPS was analyzed using purified full-length TDP-43 and we observed a dramatic change in the behavior of TDP-43 condensates in the presence of CLIP34. At 150 mM NaCl in the absence of RNA, TDP-43 forms small condensates that fail to coalesce and cluster into chain-like structures (**Figure 1C**). As previously shown, these rapidly obtain fibril-like properties that are more similar to gel or solid complexes instead of behaving as liquid droplets (French et al., 2019). Remarkably, addition of CLIP34 promoted TDP-43 condensate fusion and coalescence increasing droplet size. This was not observed in the presence of an RNA oligonucleotide control sequence of similar length, $A(CA)_{18}$. We measured the size of the condensates in the different conditions to quantify the changes. The condensates mediated by CLIP34 are more than twofold significantly larger than

control conditions (**Supplementary Figure 1**). Addition of $A(CA)_{18}$ showed no significant difference relative to control. These observations are in agreement with recently published data from our lab, showing that CLIP34 promotes TDP-43 condensation at higher NaCl concentration (250 mM). Our findings suggest that at physiological salt conditions (150 mM NaCl) TDP-43 binding to CLIP34 specifically increases the liquid and dynamic properties of TDP-43 condensates. Fluorescence recovery after photobleaching (FRAP) of the TDP-43-CLIP34 condensates showed rapid recovery of Oregon green-labeled TDP-43 indicating a dynamic exchange between the light and dense phases. We observed approximately 40% recovery in TDP-43 condensates formed with specific-binding RNA (CLIP34), while condensates without RNA or in the presence of non-binding RNA showed virtually no recovery (**Figures 1D,E**). This suggests that while a substantial immobile fraction is present in the TDP-43-CLIP34 sample, there is a difference between the dynamics of the specific vs. non-specific RNA conditions. The immobile fraction present in the CLIP34 condition could be explained by the tight binding between TDP-43 and CLIP34 RNA, as the recovery could be partially dependent on exchange of RNA-bound and free labeled TDP-43, and thereby the off-rate of the TDP-43-RNA interaction. TDP-43-CLIP34 binding affi nity is in the nanomolar range suggesting that the off-rate may be slow and this could explain the slow fluorescence recovery. In addition, the amount of CLIP34 in our reaction may also affect the degree of recovery. High RNA concentrations in similar experiments causing FRAP values that do not recover to baseline levels have been previously observed (Zhang et al., 2015). Our results are further supported by videos of condensates that show the dynamic properties of these complexes. Here, we show the marked effect of CLIP34, which increased condensate fusion and coalescence, generating larger complexes compared to control and $A(CA)_{18}$ (**Supplementary Movies 1–3**). Collectively, our findings indicate that TDP-43 phase separation may be modulated by binding to CLIP34 specifically, preventing fibrillization of TDP-43 condensates.

To test whether condensation of CLIP34-bound TDP-43 may be observed in a cellular environment, we adapted a recently developed protocol to observe biomolecular condensates in human cellular lysate (Freibaum et al., 2021). We prepared cellular extract from human embryonic kidney cells (HEK293) cells stably expressing a single copy of monomeric mEGFP-TDP-43 wild-type (WT) (**Figure 2A**). The lysate was mixed with purified TDP-43 after addition of RNA. At 250 mM NaCl, in the absence of RNA, the mixture appeared homogeneous and lacked visible foci (**Figure 2B**). However, addition of CLIP34 readily promoted formation of mEGFP-TDP-43-positive granules. Control conditions in which no CLIP34 was added did not promote mEGFP-TDP-43 condensation. As a control, we tested the effect of $A(CA)_{18}$ RNA and observed a marked decrease in phase separation compared to CLIP34. The condensates observed with $A(CA)_{18}$, which is not expected to bind TDP-43 specifically (Buratti and Baralle, 2001), may be due to interactions of cell lysate components with the oligonucleotide and TDP-43 through non-specific binding. Additionally, the higher concentration of TDP-43 and RNA that was required

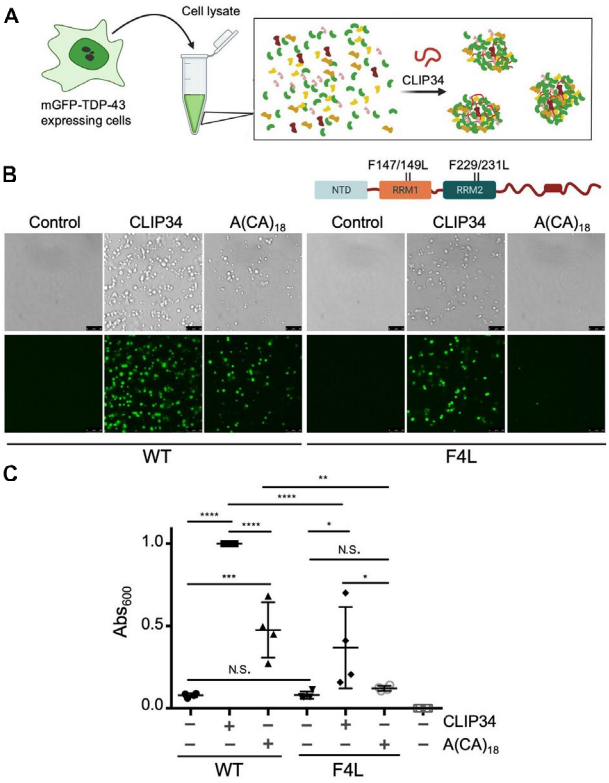

FIGURE 2 | CLIP34 RNA promotes TDP-43 condensation in a cellular lysate. (A) Cell lysates prepared from HEK293 cells stably expressing a single mGFP-tagged copy of either wild-type (WT) or F147/149/229/231L (F4L) TDP-43. Recombinant TDP-43 (WT or F4L, 5.3 μM) was added to the cell lysate at 250 mM NaCl, pH 7.5. Samples were mixed with CLIP34 (7.6 μM), $A(CA)_{18}$ (7.6 μM), or no RNA control. (B) Condensates were imaged by brightfield and fluorescence microscopy. Representative images of 3 biological replicates from 2 protein preparations. Scale bars, 10 μm. (C) Phase separation of TDP-43 in cell lysates measured by turbidity in the same experimental conditions as panel B. Mean and SD of 4 biological replicates from 2 recombinant protein preparations. Analyzed by one-way ANOVA [$F(6,21) = 38.51$, $P < 0.0001$]. Sidak's multiple comparisons test was used to compare selected groups. *$P \leq 0.05$ **$P \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.0001$, N.S., no significance. The last column corresponds to blank, buffer only sample.

to generate condensate formation under these conditions could promote liquid-liquid phase separation through less specific interactions. We also found that, at lower salt concentrations (150 mM NaCl), purified TDP-43 and cell lysate resulted in the formation of the small condensates clustering together into fibril-like structures (**Supplementary Figure 2**). However, addition of CLIP34 generated larger round-shaped condensates, suggesting increased liquid properties consistent with our experiments using purified TDP-43 alone. This was not observed upon addition of $A(CA)_{18}$ RNA (**Supplementary Figure 2B**). To further test the requirement of specific TDP-43 binding to CLIP34, we analyzed RNP granule formation of cellular lysate from HEK293 cells expressing an RNA binding-deficient mutant mEGFP-TDP-43 F4L (Phe147/149/229/231Leu) (Buratti and Baralle, 2001). Addition of CLIP34 to the F4L cellular lysate showed a

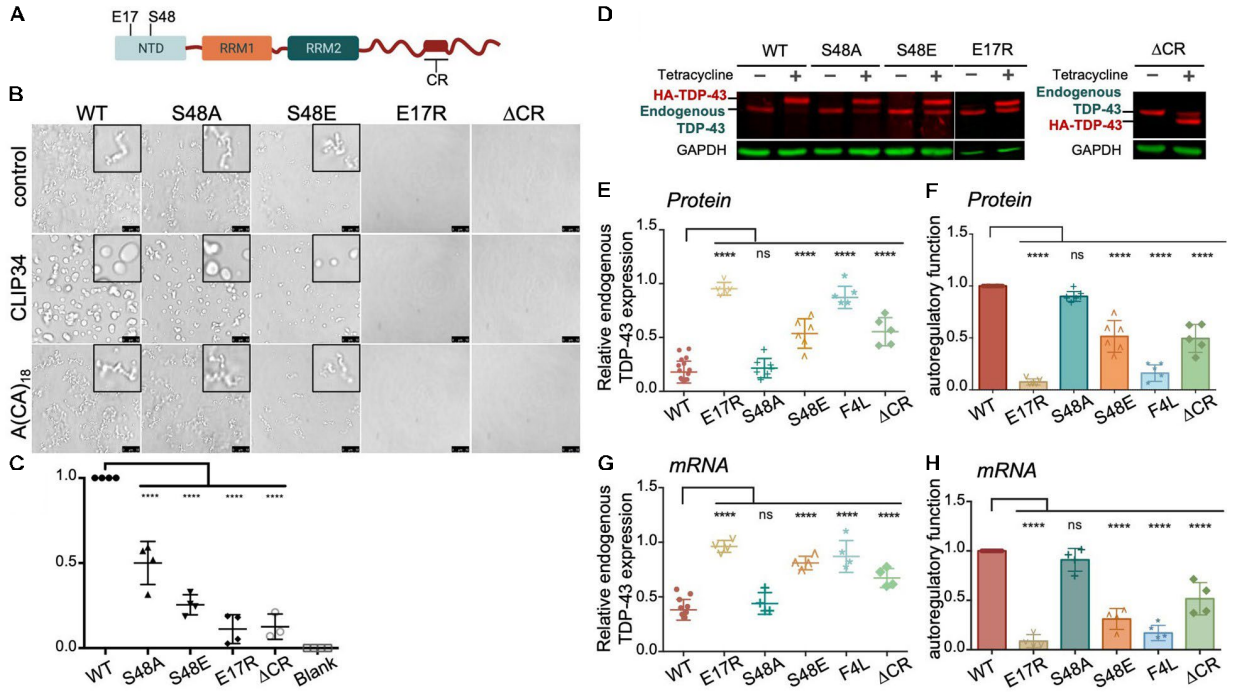

FIGURE 3 | TDP-43 autoregulation in human cells depends on protein self-assembly and phase separation. (A) Single site amino acid substitutions at E17 and S48 in the N-terminal domain of TDP-43 and deletion of the highly conserved region (CR) in the C-terminal domain are indicated. (B) Phase separation of purified WT and mutant TDP-43 (4.8 μM) as seen by brightfield microscopy without RNA (control), in the presence of CLIP34 RNA (4.8 μM), or the non-specific control RNA $A(CA)_{18}$ (4.8 μM) at 150 mM NaCl, pH 7.5. Representative images of three biological replicates from 2 protein preparations. Scale bars, 10 μm. (C) Phase separation of WT and mutant TDP-43 measured by turbidity in the same experimental conditions as panel (B). Mean and SD of at least three biological replicates from 2 recombinant protein preparations. Analyzed by one-way ANOVA [$F(4,14) = 81.45$, $P < 0.0001$]. Dunnett's multiple comparisons test was used to compare groups to WT. ****$P \leq 0.0001$. The last column corresponds to blank, buffer only sample. (D) Representative immunoblots of HEK293 cell lines stably expressing HA-tagged TDP-43 upon tetracycline induction for 72 h. Transgene and endogenous TDP-43 signals are indicated. Immunoblots were probed with TDP-43 antibody (red) and GAPDH as loading control (green). (E) Reduction of endogenous TDP-43 protein levels upon expression of the transgene quantified from panel (D). Cells expressing the RNA binding-deficient mutant F4L were used for comparison. (F) Autoregulatory activity of each mutant, relative to WT, calculated from values in panel (E) as endogenous $TDP\text{-}43^{WT}$/endogenous $TDP\text{-}43^{mutant}$. (G,H) Autoregulation in terms of *Tardbp* transcript levels measured by qPCR and quantified as in panels (E,F). Mean and SD of 5 and four biological replicates for protein and RNA, respectively. Analyzed by one-way ANOVA, (E) [$F(10,69) = 111.6$, $P < 0.0001$], (F) [$F(11,58) = 75.02$, $P < 0.0001$], (G) [$F(10,43) = 54.64$, $P < 0.0001$] and (H) [$F(9,37) = 97.40$, $P < 0.0001$]. Dunnett's multiple comparisons test was used to compare selected groups. ****$P \leq 0.0001$.

decrease in condensate formation relative to WT (**Figure 2B**). We also observed a dramatic decrease in the in the number and size of granules formed in F4L cell lysates mixed with $A(CA)_{18}$, relative to WT. To further compare and quantify TDP-43 condensation in the various conditions, we measured lysate turbidity, as this often correlates with phase separation (Babinchak and Surewicz, 2020; Grese et al., 2021). Addition of CLIP34 increased the turbidity more than threefold compared to lysate or buffer only controls (**Figure 2C**). Lysate turbidity upon mixing with CLIP34 was also significantly higher than $A(CA)_{18}$-mixed lysate, consistent with our imaging data. Addition of CLIP34 to F4L cellular lysate showed 50% reduction in turbidity compared to WT. As the imaging studies showed, addition of $A(CA)_{18}$ to F4L lysate did not significantly increase turbidity compared to control. This data suggests that in the context of cellular lysate, the remaining TDP-43-RNA contacts of F4L are sufficient for condensate formation, albeit with lower efficiency relative to WT. Collectively, these results indicate that CLIP34 binding specifically increases the liquid properties of TDP-43 condensates and promotes phase separation of TDP-43 in cell-like conditions.

TDP-43 Autoregulation Is Mediated by Oligomerization and Phase Separation

Based on our results showing modulation of TDP-43 phase separation by CLIP34, we next asked whether LLPS plays a role during TDP-43 autoregulation in human cells. For this, we introduced site directed mutations in different TDP-43 domains that disrupt self-assembly and phase separation (**Figure 3A**). We targeted the N-terminal domain (NTD) with specific substitutions that inhibit oligomerization. Residue E17 of each NTD monomer makes multiple contacts upon dimerization, whereby the N-terminal domains are arranged in head-to-tail configuration (Afroz et al., 2017; Wang et al., 2018). Consequently, the E17R mutation greatly impairs NTD oligomerization. We also evaluated the role of S48 in the NTD, which undergoes phosphorylation in human cells

(Rigbolt et al., 2011; Hornbeck et al., 2015; Wang et al., 2018). The phosphomimetic substitution S48E disrupts oligomerization and LLPS of full-length TDP-43 (Wang et al., 2018), suggesting that S48 phosphorylation modulates TDP-43 self-assembly. In agreement with these previous results, we observed fewer S48E condensates in the presence or absence of CLIP34 compared to WT or to the phospho-null substitution S48A (**Figure 3A**). At the same time, S48A showed moderately decreased condensation compared to WT under control and CLIP34 conditions. We found that in stark contrast with WT TDP-43, E17R failed to phase separate and addition of CLIP34 was unable to promote condensation of this mutant (**Figure 3B**). As further validation of the role of LLPS in CLIP34-mediated condensates, we deleted an evolutionarily conserved region in the C-terminal domain (a.a. 316-343) (*1*CR) that forms a partially a-helical structure, which is important for self-assembly and phase separation (Conicella et al., 2016, 2020; Schmidt and Rohatgi, 2016). As seen with the NTD oligomerization mutants, *1*CR inhibited LLPS in the presence or absence of RNA (**Figure 3B**). To better compare the differences in LLPS among the variants, we quantified phase separation in the presence of CLIP34 by turbidity (**Figure 3C**). Compared to WT, disruption of NTD-mediated oligomerization by E17R and S48E showed greater than 80 and 70% significant reduction in turbidity, respectively. Consistent with the microscopy data, S48A reduced turbidity compared to WT, but not as much as S48E. The *1*CR mutant showed greater than 80% significant reduction in turbidity. We examined whether the defects in CLIP34-driven LLPS were caused by decreased mutant binding to the RNA, quantifying the TDP-43-CLIP34 interactions by fluorescence anisotropy. We recently estimated that more than one TDP-43 molecule may associate with the CLIP34 sequence (Grese et al., 2021). However, further studies are necessary to determine the actual number of TDP-43 molecules binding CLIP34. Due to this limitation, we analyzed the apparent IC_{50} ($IC_{50,app}$) for the interaction between WT and mutant TDP-43 and CLIP34, as an estimate of the binding affinity. $IC_{50,app}$ is the protein concentration in which 50% of the maximal anisotropy change is observed. Our results indicate that NTD mutations and *1*CR do not significantly affect CLIP34 binding (**Table 1** and **Supplementary Figure 3**). As expected, we could not determine the binding of F4L for CLIP34 as these mutations greatly disrupt sequence-specific contacts (Buratti and Baralle, 2001; Lukavsky et al., 2013). Based on our results, we propose that the mutants bind CLIP34 as WT TDP-43 but fail to form condensates due to impaired self-assembly.

To test the role of TDP-43 oligomerization and LLPS on autoregulation, we compared autoregulatory function in human cells expressing each of the LLPS-defective mutants. Stable HEK293 cells were generated expressing a single copy of the tagged TDP-43 transgene upon tetracycline induction, as previously described (Ayala et al., 2011). To determine autoregulatory activity of the mutants we measured protein and mRNA levels of endogenous TDP-43 in these cells at 72 h post transgene induction, which typically results in 70% reduction of endogenous TDP-43 levels in the case of WT (**Figures 3D–H**). In **Figures 3F,H** we report autoregulatory activity of the different mutants compared to WT at the protein and RNA levels calculated from the values of endogenous TDP-43 measured in **Figures 3E,G**. The *1*CR deletion showed a 50% decrease in autoregulatory activity at the protein and transcript level (**Figures 3F,H**), suggesting that LLPS is required for this function. We found a dramatic disruption of autoregulation in E17R cells at the protein and mRNA levels, resulting in >90% loss in activity compared to WT (**Figures 3F,H**). Similarly, S48E showed reduced autoregulatory function of approximately 50 and 70% compared to WT at protein and RNA levels, respectively. S48A function was not significantly different from WT, indicating that introduction of the negative charge at this site specifically disrupts autoregulation. In addition, these results suggest that the decrease in phase separation observed with S48A (**Figures 3B,C**) is not sufficient to significantly disrupt autoregulation in the cellular assay. Instead, our findings suggest that TDP-43 phosphorylation at Ser48 may be able to modulate autoregulatory activity and control TDP-43 levels in cells. These results indicate a crucial role of NTD-directed self-assembly in autoregulation, which is particularly highlighted by the degree of functional loss in E17R. This mutation was even more disruptive than the RNA-binding deficient mutant F4L (Ayala et al., 2011), here used as control (**Figures 3E–H**). Based on these observations, we propose that TDP-43 autoregulation requires TDP-43 self-assembly/oligomerization and condensate formation mediated by both N- and C-terminal domains. It is possible that these domains may also mediate heterotypic protein interactions that are important during autoregulation and this should be further investigated.

TABLE 1 | Binding affinity of wild-type and mutant TDP-43 to CLIP34.

Protein	$IC_{50,app}$ (μM)	$IC^{Mut}{}_{50,app}$ / $IC^{WT}{}_{50,app}$*
Wild-type (WT)	0.52 ± 0.08	1
S48A	0.66 ± 0.05	1.3
S48E	0.55 ± 0.04	1.1
E17R	0.43 ± 0.04	0.8
*1*CR (del. 316–346)	0.52 ± 0.08	1
*1*C (a.a. 1–269)	0.55 ± 0.08	1.1
S1 (a.a. 1–278)	1.1 ± 0.2	2
F4L	*Not determined*	

Values were obtained from binding experiments in Supplementary Figure 3.
**Estimated loss (> 1) or gain (< 1) in binding affinity relative to WT.*

TDP-43 Disease-Associated Variants Show Defects in CLIP34 LLPS and Autoregulatory Function

We and others have shown that specific ALS-associated TDP-43 mutations alter the dynamic properties of TDP-43 condensates (Grese et al., 2021). Here, we investigated whether these TDP-43 mutations or variants linked to disease alter CLIP34-driven LLPS and consequently diminish autoregulatory function. We selected mutations positioned in the conserved region of the CTD (**Figure 4A**; Conicella et al., 2020). Phase separation of purified TDP-43 mutants A321G, Q331K and M337V was analyzed upon mixing with CLIP34 and compared to $A(CA)_{18}$ or no RNA control (**Figure 4B**). We found that as in the case of WT

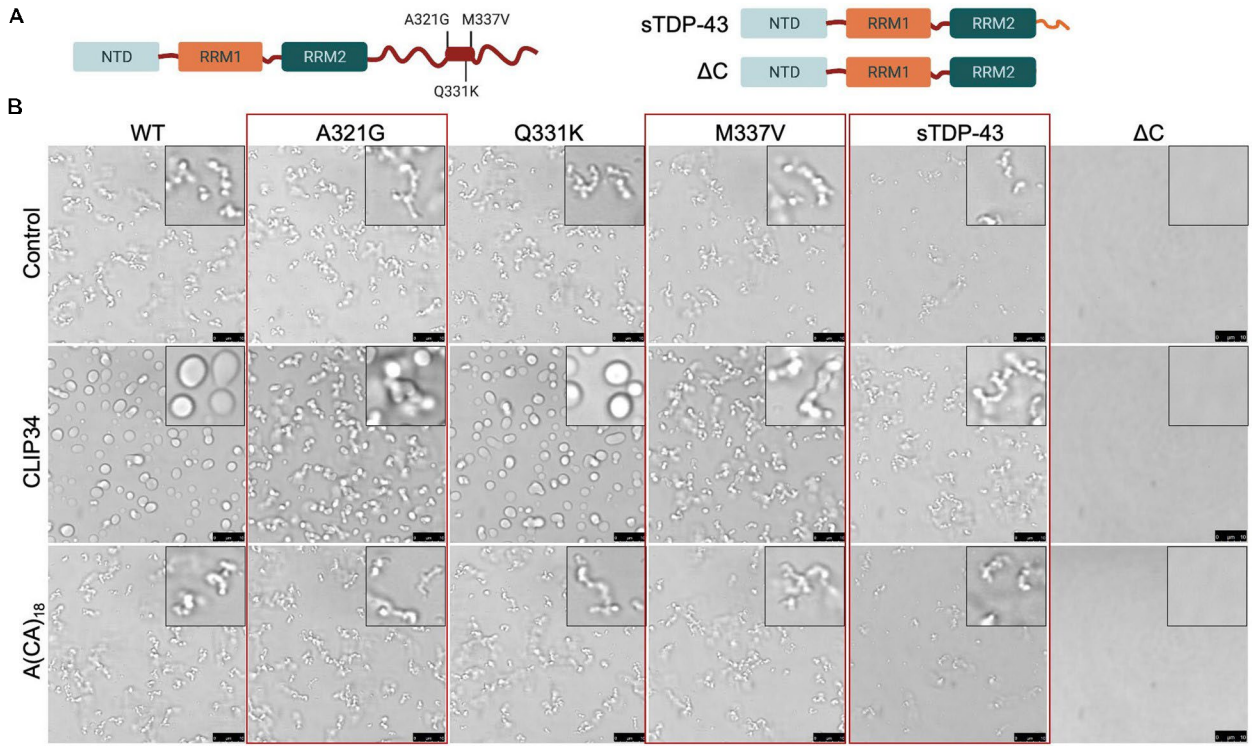

FIGURE 4 | Specific ALS-associated TDP-43 mutations disrupt the liquid properties of Clip34-induced LLPS and alter autoregulation. (A) Single site amino acid substitutions associated with ALS A321G, Q331K and M337V, part of a highly conserved region and partially α-helical structure in the C-terminal domain (boxed region), are indicated. In addition, sTDP-43 incorporating a unique 18 a.a. C-terminal sequence is compared to the truncation mutant lacking the entire CTD, *1*C. (B) Phase separation of TDP-43 WT and mutants (4 μM) observed by brightfield microscopy in the presence of no RNA control, CLIP34 or $A(CA)_{18}$ RNA (4 μM) at 150 mM NaCl, pH 7.5. Red boxes highlight variants that show differences from WT. Representative images of 3 biological replicates from 2 protein preparations. Scale bars, 10 μm.

TDP-43, CLIP34 promoted Q331K condensate liquidity, shown by increased fusion and coalescence into larger droplets. In contrast, A321G and M337V remained in clusters or chain-like structures in the presence of CLIP34, suggesting that these mutations retain their gel/solid properties and do not acquire liquid properties upon binding to this RNA molecule. Addition of $A(CA)_{18}$ RNA did not significantly change the LLPS behavior of the mutants compared to control, as seen with WT TDP-43. Previous studies of the isolated CTD showed that these mutations disrupt LLPS through alterations in a-helix structure and helix-helix interactions (Conicella et al., 2016). Q331K and M337V similarly disrupt helix-helix contacts, while A321G shows helix-breaking behavior. However, we find that in the context of the full-length protein and RNA-driven condensation Q331K is distinct from M337V and A321G. The differences in the behavior of the mutants between these studies and the previous report (Conicella et al., 2016) may due to the use of the isolated CTD in previous work compared to the full-length protein in our studies, which specifically analyzed RNA binding-driven LLPS. The specific properties acquired by M337V and A321G, but not Q331K, should be further investigated as these may highlight important disease mechanisms. Next, we sought to determine whether a disease-associated short isoform of TDP-43 (sTDP-43), recently characterized by the Barmada group (Weskamp et al., 2020), alters protein condensation associated to autoregulation. Alternative splicing of *Tardbp* produces sTDP-43, which lacks the CTD and is replaced by a unique 18 amino acid long sequence (**Figure 4A**; Wang et al., 2004; D'Alton et al., 2015; Weskamp et al., 2020). This variant is toxic upon overexpression in neurons and is more highly expressed in ALS neurons and glia (Weskamp et al., 2020). We analyzed LLPS properties of sTDP-43 in the presence of CLIP34 and anticipated that loss of the entire CTD would strongly impair LLPS of sTDP-43, as in the case of *1*C (a.a.1-269) which lacks the entire CTD (**Figure 4A**) and prevented LLPS regardless of RNA presence, under the conditions tested (**Figure 4B**). In contrast, we found that the sTDP-43 variant spontaneously formed fibril-like structures (**Figure 4B**). Addition of CLIP34 did not promote liquid behavior, but increased abundance of the fibril complexes. The presence of $A(CA)_{18}$ RNA did not significantly alter properties compared to control. The stark difference between sTDP-43, WT and *1*C suggests that the short peptide at the C terminus dramatically alters the conformation of the rest of the protein by conferring aggregation-prone properties. Alternatively, the short tail, unique to sTDP-43, may drive intermolecular interactions that promote fibrilization.

We then investigated whether changes in condensate behavior seen with the ALS mutations affect autoregulation. We created

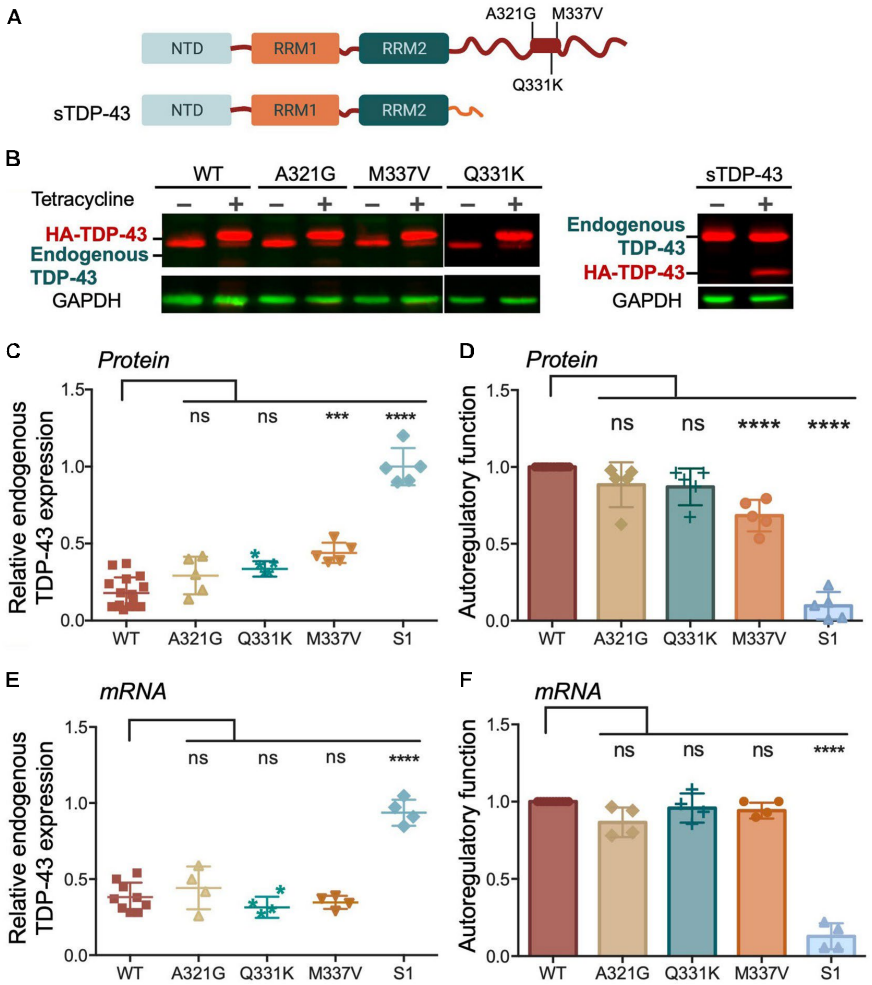

FIGURE 5 | Disease associated TDP-43 variants suppress autoregulation. (A) Single site amino acid substitutions associated with ALS A321G, Q331K, M337V and sTDP-43 are depicted. (B) Representative immunoblots of HEK293 cell lines stably expressing HA-tagged TDP-43 upon tetracycline induction for 72 h. The transgene and endogenous TDP-43 signals appeared as indicated. Immunoblots were probed with TDP-43 antibody (red) and GAPDH as loading control (green). (C) Reduction of endogenous TDP-43 protein levels upon expression of the transgene quantified from panel (B). Values calculated by the ratio of endogenous TDP-43 levels upon tetracycline induction, relative to non-induced conditions, both normalized to GAPDH. (D) Autoregulatory activity of the ALS mutants and sTDP-43, relative to WT, calculated from the values in panel (C) as endogenous TDP-43^{WT}/endogenous TDP-43^{mutant}. (E,F) Autoregulation in terms of *Tardbp* transcript levels measured by qPCR and quantified as in panels (C,D). Mean and SD of 5 and 4 biological replicates for protein and RNA, respectively. Analyzed by one-way ANOVA, (B) [$F(9,35) = 43.11$, $P < 0.0001$], and (C) [$F(9,34) = 82.45$, $P < 0.0001$]. Dunnett's multiple comparisons test was used to compare selected groups. ***$P \leq 0.001$, ****$P \leq 0001$. (D) Protein turnover estimated by measuring TDP-43 protein normalized to GAPDH at different time points post cycloheximide (CHX) treatment, as indicated. Cells expressing WT and M337V, as well as endogenous TDP-43, were treated with CHX (100 μg/mL) after 72 h of induced transgene expression. Mean and SD of 3 biological replicates.

stable HEK293 cell lines expressing a single copy of each mutant transgene upon tetracycline induction as for **Figures 3D–H**. To compare autoregulatory function between WT and mutants we quantified endogenous TDP-43 protein and mRNA levels in uninduced cells and after 72 h of induction (**Figures 5A–F**). We observed no significant differences in autoregulation in A321G and Q331K expressing cells relative to WT at protein and transcript levels (**Figures 5B-F**), suggesting that the mutants were similarly capable of autoregulation as WT TDP-43. Our results regarding the Q331K mutation are in contrast with observations using a knock-in mouse model expressing this mutant which results in altered *Tardbp* mRNA processing and increased TDP-43 protein and transcript levels (White et al., 2018). However, our observations are supported by similar behavior of Q331K dynamics in cells and autoregulatory activity compared to wild-type seen by others (Hallegger et al., 2021). The observed discrepancies between our cell-based model and the animal model may be caused by species or cell type-specific factors that influence TDP-43 condensation and/or autoregulation. As previously reported by Weskamp et al. (2020), we observed strong defects in sTDP-43 autoregulation using our stable cell line upon quantifying protein and mRNA levels of endogenous TDP-43 (**Figures 5B–F**). These results are consistent with the important role of the CTD in this function (Ayala et al., 2011).

Because of the dramatic decrease in autoregulation associated with sTDP-43, we measured its binding affinity for CLIP34 RNA, comparing it to WT and *1*C (**Table 1** and **Supplementary Figure 3**). While *1*C did not significantly change binding relative to WT, sTDP-43 caused a significant twofold increase in IC_{50}, suggesting a moderate decrease in binding affinity of sTDP-43. The spontaneous fibrilization of sTDP-43 may account for this reduction in binding and should be further investigated. Nevertheless, based on previous studies, we predict that a decrease of binding affinity in this range is unlikely to alter cellular RNA processing function (Lukavsky et al., 2013). We also found that sTDP-43 was consistently expressed at lower protein levels compared to WT and the other mutants in the stable cell lines (**Figure 5B**). This may be caused by increased clearance of this isoform because of its propensity to aggregate/misfold, as seen in our assays (**Figure 4B**). Importantly, the decreased sTDP-43 protein levels may partly account for the reduction in its autoregulatory activity. Therefore, we propose that the extreme defects in autoregulation in the case of sTDP-43 arise from impaired LLPS, decreased protein expression, and impaired protein-protein interactions in the absence of the CTD. The behavior observed for sTDP-43 may partly explain the association of this isoform with neurotoxicity and TDP-43 pathology in neurons and in patients (Weskamp et al., 2020).

We found that M337V did not affect autoregulatory function when analyzing mRNA transcript levels (**Figures 5E,F**). These findings suggest that although M337V decreased the liquid properties of the condensates, these defects are not sufficient to significantly alter RNA processing in the case of autoregulatory function. Intriguingly, we found a 40% reduction in autoregulatory function when quantifying endogenous TDP-43 protein levels in M337V cells (**Figures 5B–D**). This difference derives from significantly greater accumulation of endogenous TDP-43 protein in M337V expressing cells, relative to WT (**Figure 5C**). To investigate the mechanism associated with this phenotype, we asked whether M337V increases protein aggregation in cells, as we previously observed using the purified protein (French et al., 2019). The M337V protein was found to be less soluble than the WT counterpart upon cell lysate fractionation into soluble and insoluble fractions (**Supplementary Figures 4A–C**). These results are in agreement with increased levels of soluble and insoluble TDP-43 in human induced pluripotent stem cells (iPSCs)-derived neurons expressing M337V (Bilican et al., 2012). Next, investigated whether M337V prevents the normal turnover and clearance of endogenous TDP-43 (**Figure 6A**). M337V-expressing cells were treated with cycloheximide (CHX), a translation inhibitor, and we measured the levels of induced, exogenous M337V and endogenous TDP-43 protein at different time points post-treatment. Cells expressing WT TDP-43 were similarly treated as control. Quantification of the protein levels for up to 40 h post-CHX treatment showed a stark increase in the half-life of induced M337V protein relative to induced WT protein (**Figure 6B**). Furthermore, the half-life of endogenous TDP-43 in M337V-expressing cells was also drastically increased relative to that measured in WT-expressing cells (**Figure 6B**). These results strongly suggest that M337V protein homeostasis is impaired and that these defects lead to aberrant accumulation of M337V that affects non-mutated TDP-43 in cells as well. We explored whether these defects may be reflected as differences in TDP-43 cellular localization. However, we did not observe significant changes in the nuclear-cytoplasmic localization of M337V compared to WT, or in the diffuse vs. focal organization of the transgene and endogenous proteins in the nucleus, based on immunofluorescence (**Supplementary Figure 4D**). Interestingly, we found that the levels of two other RNA binding proteins associated with ALS, FUS and matrin-3, also showed abnormal accumulation post-CHX treatment in M337V, compared to WT-expressing cells (**Figure 6B**). The stark differences in the proteostasis of M337V may be caused by defects in the loss of liquid properties unique to this mutation as seen by our *in vitro* condensation assays. These intriguing findings also suggest that the defects caused by M337V alter general pathways of cellular proteostasis, affecting aggregation-prone proteins that are relevant to disease.

DISCUSSION

The autoregulatory activity of TDP-43 is central to its function in RNA processing as even moderate changes in TDP-43 levels may significantly alter gene expression. The importance of autoregulation for TDP-43 metabolism is further highlighted by two lines of evidence. First, *in vivo* models of TDP-43 pathology and patient-derived tissue show aberrantly increased levels of TDP-43 expression (Gitcho et al., 2009; White et al., 2018; Liu et al., 2019). Second, defects in autoregulation cause TDP-43-associated neurotoxicity (Tatom et al., 2009; Barmada et al., 2010; Wils et al., 2010; Xu et al., 2010; Estes et al., 2011; Uchida et al., 2012; Diaper et al., 2013; White et al., 2018). These observations strongly indicate that impaired autoregulation may result in elevated protein levels, increasing the aggregation propensity of TDP-43. Here, we show that this essential TDP-43 function is mediated by TDP-43 condensation into ribonucleoprotein complexes through a mechanism implicating specific RNA binding and protein domain interactions. We show that TDP-43 binding to CLIP34 RNA specifically, whose sequence forms part of the TDP-43 binding region mediating autoregulation, increases the liquid properties of TDP-43 condensates. Importantly, we find that TDP-43 ALS-associated mutations and the short variant isoform sTDP-43 affect different interactions involved in this process. In particular, our studies provide insight into possible pathogenic mechanisms associated with the M337V mutant and the short TDP-43 isoform associated with neurotoxicity in ALS. These findings provide mechanistic insight of an important process regulating TDP-43 homeostasis and may help in the design of new therapeutic strategies to control TDP-43 function and solubility.

Based on our results, TDP-43 autoregulation requires TDP-43-self-assembly and condensate formation. We find that disruption of TDP-43 LLPS activity through targeted mutations strongly impairs autoregulatory activity (**Figure 3**). These results are supported by studies from the Ule lab published while this manuscript was in preparation (Hallegger et al., 2021).

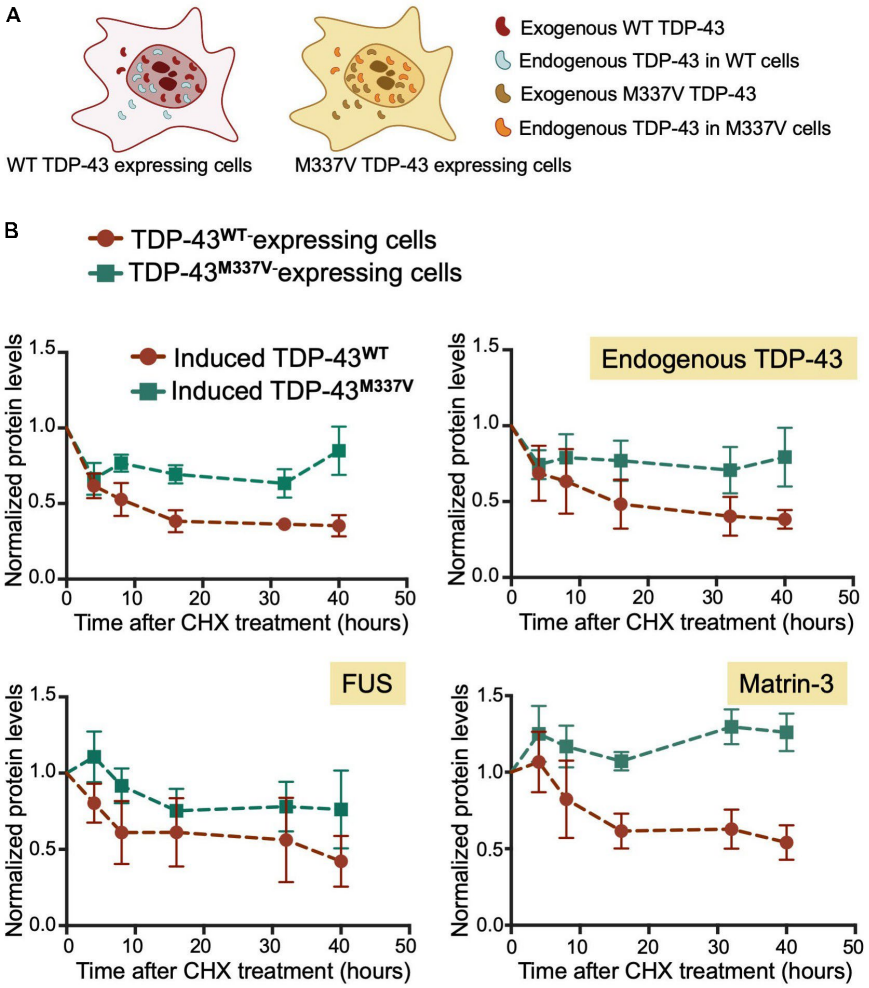

FIGURE 6 | M337V suppresses protein clearance of TDP-43 and other RNA binding proteins linked to ALS. (A) Cells stably expressing WT (red) or M337V (cyan) TDP-43 upon tetracycline induction in the presence of endogenous TDP-43. (B) Turnover of induced and endogenous TDP-43, FUS and matrin-3 monitored by measuring protein levels normalized to GAPDH at different time points post-cycloheximide (CHX) treatment (100 μg/mL). Cells were treated with CHX after 72 h of induced transgene expression. Mean and SD of 3 biological replicates.

Hallegger et al. (2021) showed that condensation activity is correlated with autoregulatory function by disrupting the conserved a-helical region in the LCD. Here, we show that in addition to interactions mediated by the LCD, self-assembly through the N-terminal domain of TDP-43 is essential for autoregulation (**Figure 3**). This mechanism of oligomerization is necessary for TDP-43-mediated splicing regulation of numerous targets and regulation of R-loop metabolism, according to our previous data and others (Afroz et al., 2017; Wang et al., 2018; Wood et al., 2020), suggesting that these processes and autoregulation share common mechanisms. Our results showing that autoregulation-linked condensation requires RNA binding as well as NTD and interactions is consistent with the pivotal role of multivalent interactions in mediating condensate networks. We also note that, at this time, we may not rule out that the N and C-terminal regions are required for oligomerization or interactions with protein partners, in the absence of phase separation. While this possibility needs to be investigated, we provide strong evidence for an important role of TDP-43 condensation during autoregulation. We showed that CLIP34 specifically and potently induces liquid-liquid phase separation of purified TDP-43 as well as in the presence of cellular lysate and promotes liquid properties of TDP-43 droplets. In addition, our work shows inhibition of autoregulation following the introduction of mutations that inhibit LLPS. Interestingly, our findings suggest that TDP-43 autoregulation may be controlled by phosphorylation at residue S48 in the N-terminal domain, which strongly inhibits self-assembly and phase separation (**Figure 3**; Wang et al., 2018). The cellular pathway controlling S48 phosphorylation is unknown, yet our data suggests that it may be a powerful modulator of TDP-43 function and proteostasis.

To explore possible links between the condensation-autoregulation process and TDP-43-associated disease, we

investigated the function of TDP-43 mutations causative of ALS. Among the disease mutations within the CTD conserved region, we found that A321G and M337V were able to form condensates on their own, similar to WT. However, CLIP34 RNA did not confer liquid properties to the condensates formed by these mutants, in contrast to WT and the other mutations tested (**Figure 4**). These defects were consistent with our recent observations that GU-repeat RNA binding is not capable of increasing the liquid properties to A321G and M337V condensates, in contrast to its effect on WT TDP-43 (Grese et al., 2021). The altered behavior of A321G and M337V suggests less dynamic assemblies compared to WT, which may result in accelerated conversion into fibrils or aggregates. This model is consistent with our previous findings that M337V accelerates aggregate formation and increases aggregate seeding capacity in cells (French et al., 2019). In addition, aberrant M337V condensate behavior is supported by defects in stress granule function observed in a previous M337V mouse model (Gordon et al., 2019). However, despite the observed loss in liquid properties, A321G and M337V mutants did not significantly decrease autoregulatory function at the transcript level, compared to WT (**Figure 5**). This is in contrast with mutations that greatly decreased or even blocked LLPS entirely, such as *1*CR and NTD mutations (**Figure 3**). We propose that the ability of A321G and M337V to form condensates, albeit of decreased liquidity, is suffi cient for autoregulation in cells where additional protein interactions (e.g., chaperones) may counteract defects in A321G and M337V complexes. It is also possible that additional regulatory factors in cells, such as 3^{0}UTR RNA sequences flanking CLIP34 in the entire TDPBR are able to rescue the defects observed with the purified A321G and M337V proteins.

Our present studies led to intriguing observations of disease-associated factors disrupting normal TDP-43 homeostasis. We found significantly increased accumulation of endogenous TDP-43 protein in M337V expressing cells, compared to wild-type (**Figures 5B–D**). These results are consistent with previous reports of neurons derived from M337V carrier patients, which show decreased survival and higher levels of total TDP-43 compared to control (Bilican et al., 2012). We also find that elevated endogenous TDP-43 protein levels were caused by a dramatic decrease in its clearance upon co- expression of M337V (**Figure 6**). The M337V protein also shows greatly reduced turnover compared to WT, as was previously reported in a different human cell line (Yin et al., 2021). Of note, the WT and M337V proteins were expressed in isogenic cell lines in our studies, suggesting that M337V homeostasis is intrinsically different from WT. Based on these results, we speculate that structural and functional defects in M337V disrupt the clearance of non-mutated TDP-43 in the affected cells, having the overall effect of increasing TDP- 43 levels. Moreover, our observations that FUS and matrin-3 proteins also showed abnormal accumulation in M337V cells suggest strong defects in cellular protein homeostasis. Based on the accelerated maturation of M337V condensates, we speculate that M337V sequesters TDP-43, and perhaps other related proteins (e.g., FUS, matrin-3), into insoluble aggregates. It is also possible that the decreased solubility of M337V (**Supplementary Figure 4**) reduces the capacity of cellular protein clearance through a yet uninvestigated mechanism. These findings are relevant to patients carrying the M337V mutation as heterozygous, whereby expression of the M337V protein also affects the homeostasis of the WT allele as well as other proteins.

Our findings show that TDP-43 binding to CLIP34, specifically modulates liquid-liquid phase separation properties of TDP-43 (**Figure 1**). We previously showed evidence that TDP-43 LLPS is upregulated with increasing number of available RNA binding sites. According to band shift analyses, we estimated that 3-4 TDP-43 molecules bind to CLIP34 (Grese et al., 2021). The increased valency of the RNA-bound complex is likely to increase TDP-43 phase separation. Importantly, this sequence-specific RNA binding activity is also demonstrated in a cellular environment using human cell lysate (**Figure 2**). These results are consistent with our recent findings that interactions with specific RNA sequences promote TDP-43 condensation, and at the same time, increase the liquidity of phase separation (Grese et al., 2021). Additionally, Mann et al. (2019) previously showed that CLIP34 RNA decreases TDP-43-mediated neurotoxicity. Collectively, this evidence indicates that specific TDP-43-RNA interactions are important to prevent TDP-43 aggregation in the condensed state, during the processing of specific targets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LK, ZG, AB, and YA designed the study. ZG and LM generated the recombinant protein and performed the phase separation analysis. LK carried out the cell biology experiments and analyses. AB measured the RNA binding affi nity. TH participated in the design, analysis and interpretation of the RNA binding assays. LM generated the DNA constructs and mutagenesis. YA wrote the manuscript. LK, ZG, AB, LM, and YA reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.818655/full#supplementary-material

Supplementary Figure 1 | CLIP34 RNA specifically increases the liquid properties of TDP-43 condensates. The size of TDP-43 condensates in the presence and absence of RNA was quantified as an estimation of differences in their liquid properties. The area of condensates composed of purified TDP-43 control, in the presence of CLIP34 or $A(CA)_{18}$ RNA shown in Figure 1C was calculated by ImageJ. Mean and SD of >600 condensates from three biological replicates using two different protein preparations. Analyzed by one-way ANOVA [$F(2,1948) = 353.5$, $P < 0.0001$]. Dunnett's multiple comparisons test was used to compare groups to WT. ****$P \leq 0.0001$. ns, no significance.

Supplementary Figure 2 | CLIP34 RNA promotes TDP-43 condensation in a cellular lysate at 150 mM NaCl. (A) Cell lysates were prepared from HEK 293 cells stably expressing a single mGFP-tagged copy of wild-type (WT) TDP-43. 4% of this lysate was added to recombinant TDP-43 (5.3 μM) mixed with CLIP34 (7.6 μM), $A(CA)_{18}$ (7.6 μM), or no RNA control at 150 mM NaCl, pH 7.5. Representative images of 3 biological replicates from 2 protein preparations. Scale bars, 10 μm.

Supplementary Figure 3 | TDP-43-CLIP43 binding assays. (A–G) Representative binding analysis for WT and mutants measured by fluorescence anisotropy of FITC-labeled CLIP34 RNA (100 nM) titrated with increasing TDP-43 WT and mutants (0–2 μM). Each point is the average of > 3 biological replicates (SEM). The calculated apparent IC_{50} ($IC_{50,app}$) is the protein concentration in which 50% of the maximal anisotropy change is observed. The values obtained are summarized in Table 1. (H) ANOVA analysis of the IC_{50} values comparing WT and mutant TDP-43. ***$P \leq 0.001$; ns, no significance.

Supplementary Figure 4 | The ALS-associated M337V mutation decreases solubility and affects the solubility of co-expressed endogenous TDP-43. (A) TDP-43 solubility measured in HEK293 cells stably expressing a single copy of WT or M337V upon tetracycline induction. (B) Lysates of cells induced for 72 h were fractionated into RIPA and Urea-soluble fractions. The levels of transgene and endogenous TDP-43 were measured by immunoblotting (representative blots shown). Blots were probed with TDP-43 and GAPDH as loading control. High exposure of the immunoblot is included to compare levels in the Urea fraction. (C) The proportion of soluble WT and M337V transgenes is plotted in addition to that of endogenous TDP-43 from WT and M337V-expressing cell lines. Mean and SD of 3 biological replicates. (D) Representative immunofluorescence of HA-TDP-43 WT and M337V-expressing cells showing the predominantly nuclear detection of endogenous and HA-tagged TDP-43, $n = 3$ biological replicates. Scale bars, 10 μm.

Supplementary Figure 5 | M337V expression decreases the clearance of RNA binding proteins linked to ALS. (A,B) Representative immunoblots of HEK293 cells stably expressing HA-tagged WT or M337V TDP-43 for 72 h upon tetracycline induction. Cells were then treated with cycloheximide (CHX, 100 μg/mL) and samples were taken at different time points post-CHX treatment, as indicated. Membranes were probed with TDP-43, Matrin-3, and FUS antibodies. GAPDH was used as a loading control.

Supplementary Table 1 | List and sequence of DNA oligonucleotides used for cloning and mutagenesis.

Supplementary Movies 1–3 | Specific RNA promotes liquid properties of TDP-43 condensates. TDP-43 (4 μM) in no-RNA conditions (1) or mixed with CLIP34 (4 μM) (2) or $A(CA)_{18}$ (4 μM) (3) at 150 mM NaCl. Related to Figure 1. Scale bar, 25 μm.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Previous, Current and Pending Support

Principal Investigator, Ayala, Yuna
Institution: Saint Louis University
CDMRP Log Number: AL190060, RNA-Based Approach to Decrease TDP-43 Pathology and Neurotoxicity in ALS

CURRENT SUPPORT:

1. **Department of Defense, Congressionally Directed Medical Research Programs**
   Amyotrophic Lateral Sclerosis Research Program Therapeutic Idea Award W81XWH2010241

Start-End dates: 07/01/2020-06/30/2023
Title: RNA-Based Approach to Decrease TDP-43 Pathology and Neurotoxicity in ALS
Goals: 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.
Specific Aims/Task:
Aim 1: Establish RNA-based methods to block TDP-43 aggregation
Aim 2: Determine whether RNA inhibits intracellular TDP-43 aggregate seeding and propagation
Aim 3: Use of RNA to decrease TDP-43 pathology and neurotoxicity in a mouse model of ALS
Role: PI (2.4 calendar)
Total cost:
Point of contact: Sarah Dougherty, Ph.D., Science Officer, sarah.e.dougherty5.civ@health.mil.

2. **National Institutes of Health/NIA NINDS R01NS114289**

Start-End dates: 02/01/2020-01/31/2025
Title: Role of oligomeric TDP-43 aggregate intermediates in ALS and frontotemporal dementia
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.
Specific Aims/Task:
Aim 1: Determine the presence of early-stage oligomers during TDP-43 aggregation in cells, in ALS and FTD
Aim 2: Determine the molecular mechanism for oligomer assembly during TDP-43 aggregation
Aim 3: Determine aggregate seeding and propagation of TDP-43 oligomers in vivo
Role: PI (4.56 calendar)
Total cost:
Point of contact: Amelie Gubitz, Program Officer, gubitza@mail.nih.gov

3. **National Institutes of Health/NIA NINDS R01NS116176**

Start-End dates: 04/01/2020-03/31/2025
Title: Functional and Pathological interactions of TDP-43
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.
Specific Aims/Task:
Aim 1: Structural basis for TDP-43 helical subdomain assembly
Aim 2: Modulation of TDP-43 CTD interactions by post-translational modifications and disease mutations
Aim 3: Structural basis for TDP-43 N-terminal region contributions to function and aggregation in disease
Role: co-PI (1.2 calendar)
Total cost:
Point of contact: Amelie Gubitz, Program Officer, gubitza@mail.nih.gov

PAST SUPPORT:

1. ALS Finding a Cure and ALS Association

Start-End dates: 11/15/2016-12/31/2021

Title: Generation of a PET Tracer for TDP-43 Aggregates for ALS and FTD

Goals: This is a multiple-PI project to develop a PET Tracer for TDP-43 aggregates in ALS/FTD that could be used to study the protein's role in disease, enable more accurate diagnoses, and assess therapeutic efficacy of novel drugs.

Specific Aims/Task:

Aim 1: Develop bioassays to assess binding affinity to aggregated TDP-43

Aim 2: Identify lead compounds with high binding affinity to aggregated TDP-43 using two-pronged approach

Aim 3: Synthesis of TDP-43 targeted PET radiopharmaceuticals and assessment of radiochemistry, blood brain barrier permeability, and binding affinity to aggregated TDP-43

Aim 4: Optimization of lead TDP-43 PET radiopharmaceuticals via pharmacokinetic studies in rodent models

Aim 5: Evaluation of translational potential of TDP-43 targeted PET radiopharmaceuticals via preclinical MicroPET validation studies and analyses

Role: PI (0.01 calendar)

Total cost:

Point of contact: Christine Collins, Grants Officer. 23 Barry Place, Stamford, CT 06902

2. National Institutes of Health/NIA R56NS105806

Start-End dates: 07/15/2019-06/30/2021

Title: Regulation of TDP-43 function in ALS and frontotemporal dementia via posttranslational modification

Goals: Test the role of TDP-43 phosphorylation in ALS/FTD pathogenesis. We propose to determine how TDP-43 homeostasis is regulated by phosphorylation using a combination of cell-based, animal and patient-derived models.

Specific Aims/Task:

Aim 1: Identify pT153/Y155-regulated mechanisms that promote TDP-43-stress granule assembly

Aim 2: Determine the effect of pT153/Y155 on TDP-43 self-assembly and protein aggregation

Aim 3: Confirm the link between pT153/Y155 and TDP-43 pathology in ALS/FTD disease models

Role: PI (0.96 calendar)

Total cost:

Point of contact: Allison Bailey, Grants Management Specialist, baileyall@mail.nih.gov

3. Spinal Cord Injury/Disease Research Program, The Curators of the University of Missouri

Start-End dates: 06/01/2018-05/31/2020

Title: Aggregate Formation and Propagation of ALS-Associated TDP-43

Goals: The aim of this work is to identify the regulatory pathways of TDP-43 propagation through small compound and gene knock-down screens in a cellular reporter developed in the lab.

Specific Aims/Task:

Aim 1: ALS-linked TDP-43 mutations accelerate aggregation and increase cellular propagation

Aim 2: Detection of TDP-43 aggregate transmission from ALS-derived samples in our cellular reporter assays

Aim 3: Establish a cellular sensor of TDP-43 propagation for high-throughput screening assays

Role: PI (4.8 calendar)

Total cost:

Point of contact: Mark McIntosh, Vice President for Research and Economic Development, UM System, University of Missouri

4. National Institutes of Health, NINDS K01 NS082391

Start-End dates: 04/01/2013 – 01/31/2017

Title: The Role of TDP-43 Phosphorylation in Protein Function and Neurodegeneration

Goals: The aim of this project was to identify novel phosphorylation sites of TDP-43 that control function using in vitro assays and in cellular models. We developed phospho-specific antibodies for these sites to detect the control of phosphorylation under normal disease conditions. This work provided the basis for current studies to understand the control of TDP-43 activity in cells in previously unexplored cellular mechanisms in gene expression, and in the maintenance of protein homeostasis.
Specific Aims/Task:
Aim 1: Study the role of phosphorylation on TDP-43 function
Aim 2: Investigate changes in TDP-43 phosphorylation associated with patient-derived mutations
Aim 3: Determine the role of TDP-43 phosphorylation on stress granule formation
Role: PI (11.4 calendar)
Total cost:

5. Presidential Research Fund, Saint Louis University
Start-End dates: 05/01/2017 – 04/30/2019
Title: Cellular Model to Detect Propagation of TDP-43 Aggregates in Neurodegeneration
Goals: The aim of this exploratory project was to develop a cellular reporter to detect TDP-43 aggregate propagation in cells. In addition, this project tested the propagation efficiency of aggregates derived from recombinant TDP-43 as well as patient derived tissue.
Specific Aims/Task:
Aim 1: Determine the seeding capacity of specific species of wild-type TDP-43 and disease-linked aggregates
Aim 2: Determine the propagation of TDP-43 aggregates from patient-derived samples
Role: PI (0 calendar)
Total cost:

6. Presidential Research Fund, Saint Louis University
Start-End dates: 10/01/2014 – 09/30/2015
Title: Characterizing the Role of TDP-43-RNA Interactions in Preventing Protein Aggregation
Goals: The aim of this pilot award was to develop methods to express recombinant TDP-43 and to study the role of RNA binding in preventing TDP-43 aggregation.
Specific Aims/Task:
Aim 1: Determine the specific RNA sequences required to maintain TDP-43 solubility
Aim 2: Study the effect of TDP-43-RNA interactions on protein solubility and nuclear localization in human cells and in *Drosophila melanogaster*
Role: PI (0 calendar)
Total cost:

Other Financial Support – Timothy Miller

There is no overlap between the project and any of the following Active, Pending, or Prior financially-supported projects.

ACTIVE

Research Grant (Miller, PI) 08/01/2022 – 07/30/2023 .60 Calendar Rainwater Charitable Foundation
Patrick Brannelly, Grants Officer
777 Main Street, Suite 2250
Fort Worth, TX 76102
"Elucidating Mechanisms and Therapeutic Targeting of 4R Tau-induced Toxicity in Astrocytes"
Major Goals: Discover novel therapeutics for 4R tauopathies by developing new 4R tau-targeted antisense oligonucleotides and by targeting the complement pathway.
Aim 1: Develop an ASO that decreases 4R tau in the setting of human tau mutations.
Aim 2: Define pathways of complement activation mediating astrocytic 4R tau-induced neuronal hyperexcitability.
Aim 3: Delineate other complement proteins and alternative mediators of 4R tau-induced astrocyte and neuronal toxicity using unbiased methods.

Research Grant (Miller, Co-PI) 08/01/2022 – 07/31/2026 1.2 Calendar per year (Miller portion)
Chan Zuckerberg Initiative
Margaret Sutherland, Grants Officer
394 Pacific Avenue, 2nd Floor
San Francisco, CA 94111
"Increasing mRNA translation to treat neurodegeneration"
Major Goals: Develop universal gene-targeting strategies that stimulate mRNA translation to understand and treat neurodegenerative diseases.
Aim 1: Test the therapeutic potential of TBK1-upregulating ASOs for TBK1 haploinsufficiency.
Aim 2: Test the ability for 3'UTR-masking ASOs to boost neurotrophic factors and mitigate neurodegeneration.
Aim 3: Develop a cell type-specific catalog of 3'UTR elements responsive to ASO masking.

Sponsored Research Agreement (Miller, PI) 03/01/2022 – 02/28/2025 1.2 Calendar
Ionis Pharmaceuticals, Inc.
Jamie Morgan, Senior Contract Manager
2855 Gazelle Court
Carlsbad, CA 92010
"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.
Aim 1: Define correlations between serum, CSF, and tissue neurofilament mRNA and protein levels during the course of motor neuron disease.
Aim 2: Delineate the mechanisms of neurofilament release using an *in vitro* model.

Research Grant (Miller, PI) 08/01/2021 – 07/31/2022 .60 Calendar
Robert Packard Center for ALS Research
Kwang Sauer, Grants Officer
855 N. Wolfe Street
Baltimore, MD 21205

"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.
Aim 1: Investigation of subgroups of ALS.
Aim 2: Identification of SOD1-related participants.

Research Grant (Miller, Co-PI) 08/01/2021 – 7/30/2024 .60 Calendar National Institutes of Health per year (Miller portion)
Betty Nkansah, Grants Officer
6001 Executive Blvd, Room 6200 MSC 9663
Bethesda, MD 20892
"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.
Aim 1: Develop the ability to identify critical genomic events that predict behavioral outcomes.
Aim 2: Develop the ability to identify critical genomic events that predict cell fate decisions and outcomes.
Aim 3: Develop spatial and activity-dependent Calling Cards for circuit mapping applications.

Research Grant (Miller, PI) 11/01/2020 – 10/31/2025 .36 Calendar
Target ALS per year
Ken Devaney, Grants Officer
P.O. Box 1598
New York, NY 10101
"Longitudinal Biofluids Core"
Major Goals: Determine biomarkers changes in cerebral spinal fluid and serum from patients with ALS.
Aim 1: Generate a biorepository of longitudinal blood (plasma, serum), CSF and urine linked to genetics and longitudinal clinical information that are made available to the research community.

Research Grant (Miller, Co-PI) 11/02/2020 – 11/01/2022 .60 Calendar
Target ALS (Miller portion)
Ken Devaney, Grants Officer
P.O. Box 1598
New York, NY 10101
"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.
Aim 1: Identify and characterize unique neurofilament isoforms and post-translational modifications in ALS biofluids by immunoprecipitation-mass spectrometry.
Aim 2: Determine effect of therapy on neurofilament isoforms in biofluids from SOD1-ALS participants administered SOD1-lowering antisense oligonucleotides.

Research Grant (Miller, Site PI) 03/01/2020 – 02/28/2022 .24 Calendar
Department of Defense
Brett Chaney, Science Officer
Congressionally Directed Medical Research Programs (CDMRP)
1077 Patchel St.
Fort Detrick, MD 21702
"RNA-Based Approach to Decrease TDP-43 Pathology and Neurotoxicity in ALS"
Major Goals: Accelerate the development of new therapeutic molecules to treat ALS by inhibiting TDP-43

pathology and decrease the associated toxicity.
Aim 1: Establish RNA-based methods to block TDP-43 aggregation.
Aim 2: Determine whether RNA inhibits intracellular TDP-43 aggregate seeding and propagation.
Aim 3: Use of RNA to decrease TDP-43 pathology and neurotoxicity in a mouse model of ALS.

Research Grant (Miller, Site PI) 04/01/2020 – 03/31/2022 .60 Calendar
Department of Defense
Jamie Shortall, Grants Officer
Assistance Agreements Group
Fort Detrick, MD 21702
"Use of High-Quality Chemical Tools to Rescue TBK1 Function and Identify Novel ATP-Competitive Targets in ALS"
Major Goals: Yield several key compounds and biological assays that target essential proteins in ALS-propagating pathways.
Aim 1: Screen kinase chemogenomic set (KCGS) to identify new ATP-binding protein targets that reduce seeding of protein aggregates.
Aim 2: Develop a TBK1-activating activation-targeting chimera (ATTAC).
Aim 3: Develop CK2 inhibitors that inhibit p62 (nucleoporin 62) phosphorylation and indirectly activate TBK1.

Research Grant (Miller, Site PI) 09/01/2016 – 08/31/2022 0.42 Calendar
ALS Finding a Cure / ALS Association
Christine Collins, Grants Officer
23 Barry Place
Stamford, CT 06902
"Dominantly Inherited ALS Network (DIALS)"
Major Goals: To establish a multicenter research platform to evaluate people at high risk for ALS to discover genes that rescue or prevent ALS, identify early diagnostic signs, and ultimately prevent or delay onset of ALS.
Aim 1: Provide a platform for the testing of known ALS causative genes in people at risk for familial ALS.
Aim 2: Perform longitudinal evaluation of people at risk for inherited forms of ALS, in order to define biomarkers of disease initiation (i.e. molecular, electrophysiologic, imaging) and novel measures of clinical disease onset.

PENDING

Research Grant (Miller, Co-I) 4 years 1.2 Calendar
Chan Zuckerberg Initiative
Margaret Sutherland, Grants Officer
394 Pacific Avenue, 2nd Floor
San Francisco, CA 94111
"Understanding glia-centric pathogenesis of Mitchell Syndrome"
Major Goals: Characterize Mitchell Syndrome, a recently described, devastating, early-onset neurodegenerative disease via deep clinical phenotyping, animal models, and patient-derived iPSCs.
Aim 1: Phenotype Mitchell Syndrome patients via retrospective and prospective clinical characterization paired with community building and advocacy.
Aim 2: Investigate glia-centric mechanisms underlying Mitchell Syndrome using knock-in fly and mouse models.
Aim 3: Evaluate cellular mechanisms of ACOX1 in human iPSCs and iPSC-derived glial cells.

PRIOR SUPPORT

Research Grant (Miller, PI) 12/01/2020 – 11/30/2021
Rainwater Tau Consortium
Patrick Brannelly, Grants Officer
777 Main Street, Suite 2250
Fort Worth, TX 76102
"*Characterizing the impact of increased 4R tau in human astrocytes and their potential as a therapeutic target"*
Major Goals: Provide an in-depth analysis of tau protein in astrocytes and establish whether astrocytic tau may be an important target for therapeutics.
Aim 1: Evaluate efficacy of tau lowering and tau splicing in commercially available ASOs.
Aim 2: Identify if altering tau isoforms changes tau half-life in neurons.
Aim 3: Measure protein levels of tau in astrocytes.

R01NS078398 (Miller, PI) 04/01/2017 – 03/31/2022
National Institutes of Health, NINDS per year
James Washington, Grants Officer
6001 Executive Blvd., Suite 3254, MSC 9537
Bethesda, MD 20892
"Using motor neuron specific miRNA to understand and develop novel therapeutics for motor neuron disease."
Major Goals: To employ a motor neuron specific microRNA approach to define important microRNA contributions to ALS pathogenesis, provide insight into the mechanisms underlying motor neuron degeneration, and potentially develop novel therapeutic targets for ALS and other motor neuron diseases.
Aim 1: Elucidate the impact of miR-218 release on ALS disease progression through non-cell autonomous mechanisms.
Aim 2: Assess miR-218 release from MNs and presence in CSF during disease in ALS models and patients.
Aim 3: Determine whether miRNAs define susceptibility of specific motor neuron pools.

Research Grant (Miller, co-PI) 12/01/20 – 05/31/22
Chan Zuckerberg Initiative (Miller portion)
Katja Brose, PhD, Program Officer
394 Pacific Ave., 2nd Floor
San Francisco, CA 94111
"*Using antisense oligonucleotides to increase mRNA translation for neurodegeneration"*
Major Goals: Determine if antisense oligonucleotides targeting the 3" UTR of mRNA transcripts can be used as a viable strategy to substantially increase protein expression.
Aim 1: Develop a screen to identify ASOs that increase expression of PGRN and TBK1.
Aim 2: Determine efficacy of candidate ASOs in PGRN and TBK1-deficient human iPSC-derived neuronal cells.

Research Grant (Miller, Co-I) 07/01/2019 – 06/30/2022
Hope Center for Neurological Disorders (Miller Lab portion)
Anneliese Schaefer, Grants Officer
660 S. Euclid Ave.
St. Louis, MO 63110
"Identification of Repurposed Prescription Medications as Neuroprotective Therapy for Amyotrophic Lateral Sclerosis"
Major Goals: Identify prescription medications that can be repurposed to modify the disease course for ALS.
Aim 1: Perform a population-based case-control study using 2009 Medicare data to identify medications associated with a higher or lower risk of ALS.

Aim 2: Test the two top inversely associated medications in an SOD1 G93A rodent model of ALS.

Research Grant (Miller, Co-PI) 11/15/2016 – 12/31/2021
ALS Finding a Cure / ALS Association
Christine Collins, Grants Officer
23 Barry Place
Stamford, CT 06902
"Generation of a PET Tracer for TDP-43 Aggregates for ALS and FTD"
Major Goals: To develop a PET Tracer for TDP-43 aggregates in ALS/FTD that could be used to study the protein's role in disease, enable more accurate diagnoses, and assess therapeutic efficacy of novel drugs.
Aim 1: Develop bioassays to assess binding affinity to aggregated TDP-43
Aim 2: Identify lead compounds with high binding affinity to aggregated TDP-43 using two-pronged approach.
Aim 3: Synthesis of TDP-43 targeted PET radiopharmaceuticals and assessment of radiochemistry, blood brain barrier permeability, and binding affinity to aggregated TDP-43.
Aim 4: Optimization of lead TDP-43 PET radiopharmaceuticals via audioradiography and immunohistochemical correlations and pharmacokinetic studies in rodent models.
Aim 5: Evaluation of translational potential of TDP-43 targeted PET radiopharmaceuticals via preclinical MicroPET validation studies and analyses.

Research Grant (Miller, Co-Investigator) 09/01/2018 - 08/31/2021
National Institutes of Health (Miller portion)
Betty Nkansah, Grants Officer
6001 Executive Blvd, Room 6200 MSC 9663
Bethesda, MD 20892
"Parallel Analysis of Transcription and Protein-DNA Interactions in Single CNS Cells"
Major Goals: To enable the parallel analysis of transcription factor binding and mRNA expression levels from tens of thousands of single cells in the brain.
Aim 1: To develop electroporation-based and viral toolkits for the delivery of self-reporting transposons to CNS cells in vivo.
Aim 2: To develop a mouse genetic toolkit for delivery of self-reporting transposons.
Aim 3: To develop robust data visualization and analysis software to jointly analyze single-cell DIPbinding and mRNA expression data in the brain.

Research Grant (Miller, PI) 07/01/2018 – 06/30/2021
BrightFocus Foundation
Diane Bovenkamp, Grants Officer
22512 Gateway Center Dr.
Clarksburg, MD 20871
"Antisense-mediated TREM2 Knockdown to Lessen Amyloid and Tau Pathology"
Major Goals: Determine whether lowering TREM2 mRNA affects amyloid and tau pathology in mouse models.
Aim 1: Assess plaque deposition and cognitive behavior in APP/PS1 mice treated with *TREM2* lowering ASO
Aim 2: Quantify microglia and immune response following ASO-mediated *TREM2* reduction.
Aim 3: Investigate tau pathology and tau spread following TREM2 knockdown in mutant TauP301S mice.

R01NS097816 (Miller, PI) 06/01/2016 – 05/31/2021 per year
National Institutes of Health, NINDS
Allison Bailey, Grants Officer
6001 Executive Blvd., Suite 3254, MSC 9537

Bethesda, MD 20892
"Understanding SOD1 Kinetics in Amyotrophic Lateral Sclerosis"
Major Goals: To study the half-life of SOD1 in the cerebrospinal fluid of healthy participants and those with symptomatic ALS, in order to increase the likelihood of success of SOD1-targeted therapies.
Aim 1. Determine SOD1 protein half-life in CSF of 12 participants with SOD1 mutations.
Aim 2. Determine SOD1 protein half-life in CSF of 19 controls and 19 sporadic ALS participants without SOD1 mutations

Research Grant (Miller, Site PI) 07/01/2015 – 03/31/2020
Packard Center / ALS Finding a Cure per year
Christine Collins, Grants Officer
23 Barry Place
Stamford, CT 06902
"Answer ALS"
Major Goals: To collect iPSC stem cells and pair them with carefully collected observational clinical data to determine progression and mechanisms of disease.
Aim 1: To collect clinical information, serum, and blood for IPSCs from 1000 participants with ALS
Aim 2: To generate IPSCs and IPSC-derived neurons from 1000 participants with ALS
Aim 3: To complete extensive testing of IPSC-derived neurons including genomics, transcriptomics, RNA-seq, lipidomics, proteomics, to understand biochemically and genetically defined subsets of ALS and match these data with clinical information.

Research Grant (Miller, Site PI) 09/01/2015 – 03/31/2020
ALS Association per year
Neil Thakur, Grants Officer
1275 K Street, NW, Suite 250
Washington D.C. 20005
"Genomics Translation for ALS Clinical Care (GTAC)"
Major Goals: To perform next generation genetic sequencing and detailed clinical phenotyping of ALS patients in order to provide a basis for the development of precision medicine or individually tailored therapies for ALS.
Aim 1: Collect clinical data and environmental exposure data for 1500 people with ALS
Aim 2: Perform whole genome sequencing for 1500 people with ALS
Aim 3: Store whole blood to develop induced pluripotent stem cells

Research Grant (Miller, PI) 05/31/2018 – 05/31/2020
Spinal Cord Injury/Disease Research Program
(SCIDRP)
Ashley Berg, Grants Officer University
of Missouri
321 University Hall
Columbia, MO 65211
"Understanding Normal and Aberrant miRNA-mediated Motor Neuron Astrocyte Communication"
Major Goals: To understand if deletion of miRNA binding sites abrogates miRNA related toxicity in a mouse model.
Aim 1. Define miR-218 role in astrocytic EAAT2 cellular
Aim 2. Determine how removing miR-218-related astrogliosis affects ALS disease model
Aim 3. Test whether glial restricted precursors from EAAT2 mice show increased therapeutic benefit in rat model of ALS.

Research Grant (Miller, PI) 08/01/2019 – 07/31/2020
Rainwater Tau Consortium
Patrick Brannelly, Grants Officer
777 Main Street, Suite 2250
Fort Worth, TX 76102
"Identifying the Impact of Increased 4R Tau in Human Astrocytes and Developing Astrocyte Targeted Therapeutics"
Role: Principal Investigator
Major Goals: Study the mechanism by which 4R tau deposition impacts astrocytic function and develop methods to target ASOs to astrocytes.
Aim 1: Understand the effect of altering tau splicing in human astrocytes.
Aim 2: Identify receptor-ligand pairs that will yield preferential targeting of ASO to astrocytes.
Aim 3: Verify efficacy of widely available ASOs in reducing total tau levels.
Aim 4: Determine the effect of Tsc1 haploinsufficiency to drive tau pathology and behavioural changes.

Research Grant (Miller, PI) 08/01/2018 - 12/31/2020
ALS Biomarker Consortium
ALS Association/ALS Finding a Cure
Christine Collins, Grants Officer
23 Barry Place
Stamford, CT 06902
"Determining If Motor Neuron-specific miRNA Are Biomarkers for Motor Neuron Disease"
Major Goals: To determine if miRNAs in CSF can be used as biomarkers.
Aim 1: Continue measurement of miR-218 in CSF from SMA Type 1, 2, 3 pre and post nusinersen
Aim 2 Establish miR-218 assay characteristics including how CSF processing affects the miR-218 signal
Aim 3: Test new reagents and methods to further optimize miRNA signal in CSF
Aim 4: Measure and compare miR-218 levels in CSF from ALS and controls.

Research Grant (Miller, PI) 01/08/2018 – 01/07/2021
ALS Association
Neil Thakur, Grants Officer
1275 K Street, NW, Suite 250
Washington D.C. 20005
"Pathological Analysis of Selective Autophagy in Sporadic and Familial ALS"
Major Goals: To determine whether autophagic/mitophagic dysregulation in a prominent feature of sporadic ALS and determine the feasibility of targeting mitophagy-related genes as a therapeutic strategy in the broader sporadic ALS population.
Aim 1: Determine whether selective autophagy is disrupted in sporadic spinal cord and motor cortex.
Aim 2: Determine whether selective autophagy markers are misregulated in sporadic ALS and MRG mutant spinal cord and motor cortex.

Research Grant (Miller, PI) 08/01/2017 – 07/31/2020
Muscular Dystrophy Association per year
Karen Smith, Grants Officer
161 N. Clark, Suite 3550
Chicago, IL 60601
"Understanding RAN Dipeptide Size and Kinetics in C9orf72 ALS"
Major Goals: To test hypotheses concerning the correlation between DPR size and toxicity and to develop novel methods to understanding DPR kinetics. Applying this information to human cerebral spinal fluid may provide important prognostic and diagnostic value, and will aid in the planning of C9orf72 directed therapeutics.

Aim 1: Correlate size of DPRs with degeneration and clinical characteristics
Aim 2: Develop a method to determine the turnover rates of DPRs.

Research Grant 05/31/2018 – 11/30/2019
Rainwater Tau Consortium
Patrick Brannelly, Grants Officer
777 Main Street, Suite 2250
Fort Worth, TX 76102
"Targeting Tau in Astrocytes to Modulate Pathology and Neurodegeneration"
Role: Principal Investigator
Major Goals: Determine how increased 4R Tau may cause increased pathology in astrocytes.
Aim 1: Delineate astrocyte-specific 4R tau toxicity through cell-targeted ASOs.
Aim 2: Establish a genetic model for cell type-specific human tau manipulation.

R21NS099766 09/01/2016 - 08/31/2018
National Institutes of Health, NINDS
James Washington, Grants Officer
6001 Executive Blvd., Suite 3254, MSC 9537
Bethesda, MD 20892
"Endogenous Peptides as Markers of Abnormal SOD-1 Protein in Familial and Sporadic ALS"
Role: Collaborator
Aim 1: Determine whether the seven-amino acid deamidated SOD1 peptide is a biomarker of SOD1 ALS by assessing whether its levels in the CSF of mutant SOD1 patients
Aim 2: Test whether SOD1 contributes to sporadic ALS by determining the prevalence of the newly identified SOD1 peptide in the CSF of sporadic ALS patients
Aim 3: Exploratory study: Apply similar untargeted approach as used in initial discovery in SOD-1 samples to the sporadic samples used for quantification of the putative peptide marker

U01MH10913301 09/18/2015 – 06/30/2019
National Institutes of Health, NIMH per year
Betty Nkansah, Grants Officer
6001 Executive Blvd, Room 6200 MSC 9663
Bethesda, MD 20892
"An Inducible Molecular Memory System to Record Transient States of CNS Cells"
Role: Co-PI
Major Goals: To adapt the novel technology called transposon calling cards for use in vivo enabling a retrospective genomic analysis of molecular events that occur in neuronal progenitor cells.
Aim 1: Adapt the Calling Card technology to record general transcriptional states in the mouse brain
Aim 2: Adapt the Calling Card technology to record activity-dependent transcription in the brain
Aim 3: Adapt the Calling Card technology to stereotactic applications in multiple species

Research Grant 11/01/2014 – 07/31/2018
ALS Association per year
Neil Thakur, Grants Officer
1275 K Street, NW, Suite 250
Washington D.C. 20005
"Understanding Clinical Phenotype and Collecting Biomarker Samples in C9ORF72 ALS"
Role: Principal Investigator
Aim 1: Recruit 15 C9ORF72ALS subjects per site

Aim 2: Determine C9ORF72 hexanucleotide repeat expansion size in all subjects
Aim 3: Define ALS disease course in C9ORF72 subjects
Aim 4: Determine to what degree the disease course correlates with expansion size
Aim 5: Collect biomarker samples including blood, CSF

Sponsored Research Agreement — 09/15/2013 – 12/31/2017
Biogen Idec, Inc. — per year
Toby Ferguson, Officer
225 Binney Street
Cambridge, MA 02142
"Natural History and Biomarkers for C9ORF72 ALS"
Role: Principal Investigator
Aim 1: Identify 60 C9ORF72 ALS subjects
a. Identify 30 already known C9ORF72 subjects in ALS clinics
b. Identify 30 new C9ORF72 subjects by completing genetic testing on 200 at risk ALS subjects
Aim 2: Determine C9ORF72 hexanucleotide repeat expansion size in all subjects
Aim 3: Define ALS disease course in C9ORF72 subjects
Aim 4: Determine to what degree the disease course correlates with expansion size
Aim 5: Collect biomarker samples including blood and CSF

Research Grant — 05/01/2016 – 04/30/2017
Packard Center at Johns Hopkins
1800 Orleans Street
Baltimore, MD 21287
"miRNAs enriched in ALS-vulnerable motor neurons as therapeutic targets"
Role: Principal Investigator
Aim 1: Determine actively translated mRNAs in motor neurons from susceptible vs. resistant motor neurons in brainstem and spinal cord
Aim 2: Determine the effect of ablating miR-182 on ALS disease progression

U01NS08497002 — 04/01/2014 – 08/31/2016
National Institutes of Health, NINDS — per year
James Washington, Grants Officer
6001 Executive Blvd., Suite 3254, MSC 9537
Bethesda, MD 20892
"Development of an Antisense Oligonucleotide Therapy for SOD1 Familial ALS"
Role: Principal Investigator
Aim 1: Confirm viability of lead antisense oligonucleotides by performing tolerability screen in rodents and non-human primates, and in vitro screen for off target or inflammatory effects.
Aim 2: Test whether lead SOD1 antisense oligonucleotides lower SOD1G93A and slow down disease progression in SOD1G93A rats
Aim 3: Perform GLP toxicology studies on lead antisense drug needed to support IND application

Research Grant — 07/01/2014 – 06/30/2016
University of Missouri SCIDRP — per year
Ashley Berg, Grants Officer
University of Missouri
321 University Hall
Columbia, MO 65211
"Comprehensive miRNA Analysis of Specific Cell Types in Spinal Cord"

Role: Principal Investigator
Aim 1: Determine cell type specific miRNA expression within the mouse spinal cord by performing miRNA microarrays following GFP affinity capture from mice expressing GFP-myc-Ago2 fusion protein from a conditional (lox-stop-lox) locus.
Aim 2: Determine cell type specific miRNA changes in ALS model mice by crossing $SOD1^{G93A}$ mice into mice double transgenic for cell type specific Cre-recombinase/lox-stop-lox-GFP-myc-Ago2.

R21AG0447190 07/01/2013 – 06/30/2016
National Institutes of Health, NIA per year
6001 Executive Blvd., Suite 3254
Bethesda, MD 20892
"Does a Shift from 4R TO 3R Tau Project Against Amyloid Beta-Induced Cognitive deficits?"
Role: Principal Investigator
Aim 1: Determine how shifting 4R to 3R tau affects tau localization and components of synpatosomes
Aim 2: Correlate tau knockdown or change in 4R:3R ratio with decrease in neuronal excitability
Aim 3: Determine whether shifting 4R to 3R tau protects against Aβ induced cognitive deficits in PSAPP mice

P50AG005681 05/01/2010 – 04/30/2015
National Institutes of Health, NIA per year
6001 Executive Blvd., Suite 3254
Bethesda, MD 20892
"Alzheimer's Disease Research Center: Project 2"
Role: Principal Investigator
Aim 1: Develop Tau antisense oligonucleotides
Aim 2: Determine whether decreasing tau protein levels rescues behavioral deficits in Alzheimer's disease mice (J20 APP transgenic amyloid-β depositing mice)
Aim 3. Determine whether decreasing the ratio of 4R:3R tau (by decreasing inclusion of exon 10) rescues behavioral deficits and tau protein accumulation in tau N279K mice

Research Grant 10/15/2013 – 10/14/2015
Target ALS / Columbia University per year
Ken Devaney, Grants Officer
1740 Broadway, 15th Floor New
York, NY 10019
"Targeting miR-155 with antisense oligonucleotides as a therapy for ALS"
Role: Principal Investigator
Aim: Determine whether peripheral vs. central vs. peripheral + central delivery of anti-miR-155 provides maximum extension of lifespan in $SOD1^{G93A}$ mice

Research Grant 08/01/2013 – 12/31/2014
ALS Association
Neil Thakur, Grants Officer 1275 K Street, NW, Suite 250
Washington D.C. 20005
"*Determining the Half-Life of SOD1 in Human CSF*"
Role: Principal Investigator
Aim: Determine SOD1 protein half-life in CSF of 12 participants with SOD1 mutations.

R21NS078242/sub #C00038097-1 12/01/2012 – 11/30/2014
National Institutes of Health, University of Missouri per year
6001 Executive Blvd., Suite 3254, MSC 9537

Bethesda, MD 20892
"Therapeutic Development for Amyotrophic Lateral Sclerosis in a Canine Model"
Role: Collaborator (Joan Coates, PI)
Aim 1: Evaluate the safety and effectiveness of intrathecal delivery of an antisense SOD1 oligonucleotide in normal dogs. Refinement of the canine antisense SOD1 oligonucleotide protocol is required before instituting a clinical trial in privately owned DM-affected dogs.
Aim 2: Develop measures of disease progression in dogs with DM. If DM is to serve as an effective disease model for ALS, we must have measures of disease progression.