RNA editing of *TARDBP* reduces TDP-43 aggregation while restoring its nuclear localization and splicing function in ALS disease models

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Introduction: RNA editing is a therapeutic strategy to enable site-specific base changes at the transcript level, resulting in correction of disease-causing mutations or introducing beneficial changes without permanently altering the genome. Delivery of a complementary oligonucleotide to the target RNA site recruits endogenous Adenosine Deaminase Acting on RNA (ADAR) enzymes to catalyze the deamination of adenosine to inosine. This A-to-I conversion is interpreted as guanosine during translation, enabling precise amino acid substitutions. The advantages of RNA editing include transient and reversible effects, dose-dependent control, and minimal risk of permanent genomic alterations or off-target changes compared to DNA editing technologies. In Amyotrophic Lateral Sclerosis (ALS), which has high unmet need and lack of effective therapies, cytoplasmic mis-localization and aggregation of TDP-43 protein (encoded by the *TARDBP* gene) represent pathological features that correlate with disease progression and loss of TDP-43's essential nuclear splicing functions. A novel RNA editing strategy targeting *TARDBP* preserves normal nuclear splicing function and demonstrates aggregation resistance. **Methods**: We evaluated candidate edit sites within the *TARDBP* transcript predicted to encode de novo TDP-43 proteins with enhanced resistance to aggregation. De novo TDP-43 proteins were expressed in lentivirally-transduced neuronal cell lines and evaluated for expression of the TDP-43 splicing targets *POLDIP3* and *STMN2* and impact on TDP-43 aggregation. De novo TDP-43 proteins that did not cause loss of splicing function were then prioritized based on reduction of insoluble TDP-43 fraction in cell stress induced and genetic overexpression models of TDP-43 aggregation. RNA editing oligonucleotides were designed to target the edit site corresponding to the lead validated de novo TDP-43 protein and evaluated in neuronal cell lines and iPSC-derived motor neurons. Editing efficiency, aggregate formation, TDP-43 nuclear localization, and splicing function were assessed using digital PCR, biochemical fractionation, confocal immunofluorescence microscopy, and quantitative PCR for splicing-sensitive reporter transcripts, respectively. **Results**: Several de novo TDP-43 proteins maintained normal nuclear function as shown by no change in *POLDIP3* and *STMN2* splicing and demonstrated significant reduction in two models of TDP-43 aggregate formation. Lead oligonucleotide candidates achieved sustained RNA editing in neuronal cells, with >70% editing efficiency. Under cell stress challenge that typically causes TDP-43 dysfunction, oligo treatment reduced the extent of insoluble TDP-43 aggregate formation as well as cytoplasmic TDP-43 localization in neuronal cells. Furthermore, RNA editing of the selected TDP-43 candidate site reduced mis-splicing of TDP-43 targets *POLDIP3, STMN2,* and *UNC13A. In vivo* biodistribution studies and editing efficiency assessments in relevant tissues are currently underway to support the therapeutic potential of this mechanism. **Conclusions**: This novel RNA editing strategy represents a unique therapeutic approach that addresses a critical gap in the ALS treatment landscape by specifically targeting TDP-43 pathological aggregation and mis-localization without compromising its essential nuclear functions.

TDP-43 Dysfunction in Sporadic ALS

1) **Cytoplasmic aggregation:** TDP-43 mislocalizes from the nucleus and forms insoluble cytoplasmic aggregates, which is a hallmark in >97% of sporadic ALS
2) **Nuclear mislocalization**: loss of nuclear TDP-43 results in failure of its essential roles in RNA binding and processing
3) **Splicing dysfunction:** absence of nuclear TDP-43 disrupts splicing of downstream neuronal targets such as *POLDIP3*, *STMN2*, and *UNC13A*

Methods

TDP-43 Domain Structure

Edit Site Evaluation Workflow

Multiple *TARDBP* sites are addressable by A>I editing and predicted to impact post-translational modifications associated with mislocalization and aggregation. Workflow identified and validated a de novo TDP-43 protein with no negative impact on normal TDP-43 splicing functions and decreased aggregation in cell stress-induced aggregation models.

Results

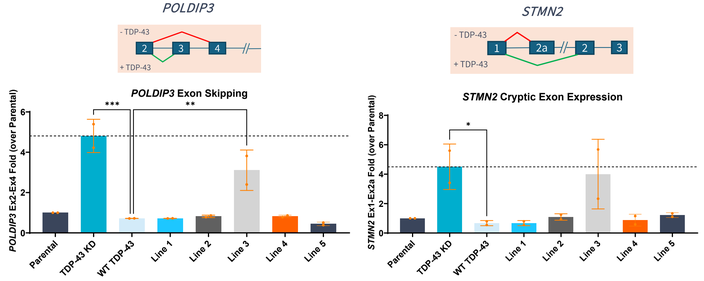

Figure 1. Lentiviral lines expressing de novo TDP-43 proteins #1-5 show either normal or disrupted function as assessed by *POLDIP3* and *STMN2* splicing. TDP-43 shRNA knockdown (KD) represents a positive control for mis-splicing. De novo TDP-43 protein 3 caused mis-splicing and did not advance to aggregation assay evaluation.

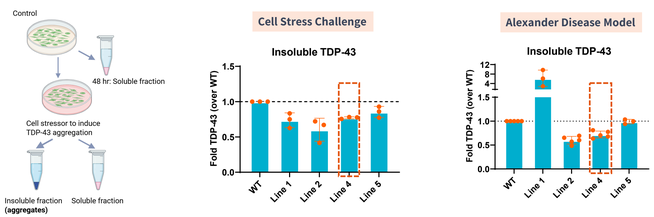

Figure 2. De novo TDP-43 proteins 2 and 4 prevent TDP-43 aggregation observed in two mechanistic models: cell stress challenge and GFAP R239H expression (Alexander disease model). De novo TDP-43 protein 4 was found to be most amenable for oligo design. Additional sites not shown were evaluated and deprioritized.

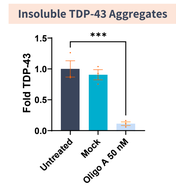

Figure 3. Oligonucleotides designed to introduce the *TARDBP* edit site corresponding to de novo TDP-43 protein 4 were transfected into the neuronal line SK-N-AS. Under cell stress challenge, oligo treatment reduced the extent of insoluble TDP-43 aggregate formation compared to untreated and mock (transfection agent alone) conditions.

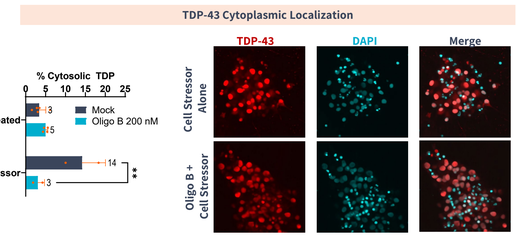

Figure 4. In differentiated iPSC motor neurons under cell stress, Oligo B (optimized from Oligo A) treatment maintains nuclear TDP-43. TDP-43 signal was quantified from confocal microscopy images using thresholds defined from untreated controls to exclude background signal.

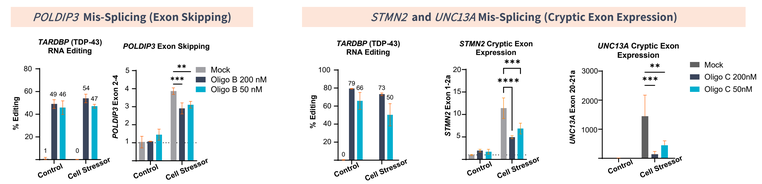

Figure 5. Oligos B and C lead to specific and sustained RNA editing up to 79% *in vitro* through day 7 in iPSC motor neurons. In a cell stress model that typically causes TDP-43 loss of function, RNA editing with oligo treatment leads to reduced mis-splicing of TDP-43 targets *POLDIP3*, *STMN2,* and *UNC13A.*

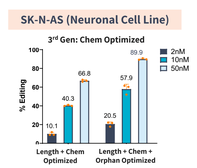

Figure 6. Oligonucleotide design improvements such as edit site "orphan" base and chemistry optimization can be applied to shorter lengths predicted to enhance CNS delivery.

Conclusions: A single A>I edit in *TARDBP* uniquely addresses both loss and gain of function impacts of TDP-43 dysregulation and is highly differentiated compared to alternative ALS therapeutic approaches: Korro Bio identified a *de novo* protein TDP-43 protein that (1) reduces TDP-43 aggregates, (2) restores nuclear localization, and (3) maintains normal splicing. RNA editing oligos to introduce this edit demonstrate >50% editing in neuronal cells, accompanied by reductions in mis-splicing and aggregation as well as enhanced nuclear localization. Korro Bio has progressed oligo designs for CNS delivery to be evaluated *in vivo*.