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G_2C_4 targeting antisense oligonucleotides potently mitigate TDP-43 dysfunction in human C9orf72 ALS/FTD induced pluripotent stem cell derived neurons

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Abstract

The G_4C_2 repeat expansion in the C9orf72 gene is the most common genetic cause of Amyotrophic Lateral Sclerosis and Frontotemporal Dementia. Many studies suggest that dipeptide repeat proteins produced from this repeat are toxic, yet, the contribution of repeat RNA toxicity is under investigated and even less is known regarding the pathogenicity of antisense repeat RNA. Recently, two clinical trials targeting G_4C_2 (sense) repeat RNA via antisense oligonucleotide failed despite a robust decrease in sense-encoded dipeptide repeat proteins demonstrating target engagement. Here, in this brief report, we show that G_2C_4 antisense, but not G_4C_2 sense, repeat RNA is sufficient to induce TDP-43 dysfunction in induced pluripotent stem cell (iPSC) derived neurons (iPSNs). Unexpectedly, only G_2C_4, but not G_4C_2 sense strand targeting, ASOs mitigate

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deficits in TDP-43 function in authentic C9orf72 ALS/FTD patient iPSNs. Collectively, our data suggest that the G_2C_4 antisense repeat RNA may be an important therapeutic target and provide insights into a possible explanation for the recent G_4C_2 ASO clinical trial failure.

Keywords

C9orf72; Repeat RNA; TDP-43; Antisense oligonucleotides; Induced pluripotent stem cell derived neurons; ALS

Introduction

An intronic GGGGCC (G_4C_2) hexanucleotide repeat expansion (HRE) in the C9orf72 gene is the most common cause of familial ALS and FTD, collectively referred to as C9orf72 ALS/FTD, and also causes ~ 8% of apparently sporadic ALS or FTD [12, 39]. The HRE is bidirectionally transcribed to form G_4C_2 (sense) and G_2C_4 (antisense) RNA species both of which have been documented to pathologically accumulate into RNA foci in human autopsy tissue and induced pluripotent stem cell (iPSC) lines, as well as in mouse and fly models of C9orf72 ALS/FTD. In addition, repeat-associated non-ATG (RAN) translation of G_4C_2 and G_2C_4 RNA produces five distinct dipeptide repeat (DPR) proteins: sense encoded Poly(GA), Poly(GP), Poly(GR) and antisense encoded Poly(PR), Poly(PA), Poly(GP). Together, these RNA species and DPR proteins are thought to contribute to disease through gain of toxicity mechanisms [18]. While much research has concluded that specific DPRs, including Poly(PR) and Poly(GR), translated from the G_2C_4 and G_4C_2 repeat RNA strands respectively, are toxic when highly overexpressed in multiple model systems [15, 20, 27, 46, 49], little is known regarding the pathogenicity of repeat RNAs themselves especially in endogenous expression models where it has been technically challenging to dissociate repeat RNA and DPR mediated toxicity.

It is thought that expression of C9orf72 repeat RNA species can sequester RNA binding proteins (RBPs) into pathologic RNA foci and impede their function [6, 48] and disrupt nuclear pore complexes and nucleocytoplasmic transport [9, 48]. Importantly, 97% of ALS, ~ 50% of FTD, and all C9orf72 ALS/FTD cases exhibit a nuclear reduction and/or clearance along with associated loss of nuclear function and subsequent cytoplasmic mislocalization of the RNA binding protein TDP-43 [44, 45]. However, histology studies failed to evaluate the functional consequences of repeat RNA – RBP interactions as well as the role of soluble repeat RNA species in disease pathogenesis. In fact, the vast number of studies published in the last decade have focused solely on the DPR species. Notably, prior work from our labs and others have shown that the G_4C_2 sense repeat RNA can be itself toxic in human neurons [9].

Little is known regarding the pathobiology of the G_2C_4 antisense RNA species in human neurons. Although antisense pathology is detectable in CNS regions at autopsy, multiple reports suggest that the expression of G_2C_4 repeat RNA and the presence of G_2C_4 foci is low in human tissue, as compared to G_4C_2 RNA. However, the exact length and proportion of these different transcripts remains unclear. Furthermore, histological pathology, by itself, is not a measure of physiological toxicity. Nonetheless, the reliable presence of translated

DPR products from the antisense strand (e.g. Poly(PR) and Poly(PA)) provides clear in vivo evidence of protein products from antisense RNA in human brain and spinal cord [7, 10–12, 16, 26, 31, 34, 39]. Interestingly, histological analyses in postmortem C9orf72 ALS/FTD patient tissues have suggested that nuclear depletion and the mislocalization of the RNA binding protein TDP-43 from the nucleus to the cytoplasm is more frequently observed in cells containing abundant G_2C_4 antisense repeat RNA foci [1, 7]. However, it is important to note that few neurons harbor TDP-43 aggregates at end-stage disease [25, 36] and nuclear depletion of TDP-43 has been observed prior to TDP-43 aggregation in C9orf72 disease [45]. Nonetheless, these studies highlight relationship between G_2C_4 antisense repeat RNA and TDP-43 pathology in C9orf72 disease which may be therapeutically relevant and worthy of functional studies.

Due to the vast literature largely focused on sense strand RNA abundance and sense strand DPR products, antisense oligonucleotide (ASO) therapies targeting the G_4C_2 sense strand repeat RNA were identified [14, 22, 41]. Unfortunately, a subsequent international clinical trial of the G_4C_2 (sense) targeting ASO BIIB078 (NCT04288856) in over 100 C9orf72 ALS patients was terminated (https://investors.biogen.com/news-releases/news-release-details/biogen-and-ionis-announce-topline-phase-1-study-results) due to neurotoxicity and a lack of clinical efficacy, despite a robust BIIB078 initiated reduction in G_4C_2 sense encoded DPRs PolyGA and PolyGP in patient CSF. Similarly, Wave Pharmaceuticals terminated their C9orf72 ASO trial, which also targeted the sense strand, due to lack of efficacy (https://www.thepharmaletter.com/article/wave-life-sciences-ends-wve-004-program). Importantly, these G_4C_2 sense strand targeting ASO therapies have no known effect on the G_2C_4 antisense RNA or its DPR products. While ASOs targeting G_4C_2 sense repeat RNA can partially reduce G_4C_2 linked DPR pathology [9, 14, 17, 24], the therapeutic potential of targeting the G_2C_4 antisense repeat RNA is completely unknown in human systems. As such, an understanding of the G_2C_4 repeat RNA elicited toxicity is critically needed.

In this brief report, we utilize iPSNs to demonstrate that G_2C_4 but not G_4C_2 repeat RNA expression is sufficient to induce a loss of nuclear TDP-43 function in human spinal neurons. Moreover, using antisense oligonucleotides that specifically target G_4C_2 or G_2C_4 repeat RNAs, we find that reduction of antisense but not sense repeat RNA restores TDP-43 function in authentic C9orf72 ALS/FTD patient iPSNs expressing endogenous levels of the C9orf72 repeat expansion. Together, our data suggest that therapeutic targeting of G_2C_4 antisense repeat RNA may be beneficial for restoring TDP-43 function in C9orf72 ALS/FTD.

Materials and methods

iPSC derived neuronal differentiation

C9orf72 and non-neurological control iPSC lines were obtained from the Answer ALS repository [3] at Cedars-Sinai (see Supplemental Table 1 for demographics) and maintained and differentiated into spinal neurons using a modified version of the direct induced motor neuron (diMNs) protocol recently described in detail [2]. All cells were maintained at 37 °C with 5% CO_2. iPSCs and iPSNs routinely tested negative for mycoplasma.

ASO treatment of iPSNs

Scrambled ASO (676,630): CCTATAGGACTATCCAGGAA, G_4C_2 ASO (619,251): CAGGCTGCGGTTGTTTCCCT, and G_2C_4 ASO (813,214): TCTCATTTCTCTGACCGAAG were generously provided by Ionis Pharmaceuticals. On day 46 of differentiation, ASOs were added to iPSN culture media at a final concentration of 5 μM. On day 49, media was exchanged for fresh stage 3 iPSN culture media and media was subsequently exchanged every 2–3 additional days without ASO until experiments performed at indicated time points.

DNA constructs

pcDNA3.1(+) containing 108 sense G_4C_2 RNA only repeats was a kind gift from Adrian Isaacs [33]. To generate an antisense G_2C_4 RNA only repeat construct, 108 sense G_4C_2 RNA only repeats were digested out of the pcDNA3.1(+) 108 RNA only repeat construct with BamHI and XbaI and inserted in an antisense orientation into empty pcDNA3.1(+) that had been digested with NheI and BamHI. One repeat unit was lost during cloning, resulting in a 107 antisense G_2C_4 RNA only repeat construct. Repeat size was confirmed using sequencing with dGTP (Source Bioscience). Sequences are available on request.

Expression of repeat RNAs

On day 18 of differentiation, control iPSNs were dissociated with accutase and 5×10^6 iPSNs were transfected with 4 μg G_4C_2 and G_2C_4 repeat RNA only plasmids in suspension with the Lonza 4D nucleofection system as previously described [9]. qRT-PCR and immunostaining and confocal imaging were carried out on day 32 as detailed below.

qRT-PCR

iPSNs were harvested in 1 × DPBS with calcium and magnesium and pelleted using a microcentrifuge. 350 μL RLT Buffer was added to iPSN pellets and RNA was isolated using the RNeasy kit (QIAGEN). RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). For detection of G_4C_2 and G_2C_4 repeat containing transcripts, 1 μg RNA was used for cDNA synthesis using gene specific primers and the Superscript IV First-Strand cDNA Synthesis System (Thermo Fisher Scientific). For detection of TDP-43 mRNA targets, 1 μg RNA was used for cDNA synthesis using random hexamers and the Superscript IV First-Strand cDNA Synthesis System (Thermo Fisher Scientific). All qRT-qPCR reactions were carried out using SYBR Green Master Mix or TaqMan Gene Expression Master Mix (Thermo Fisher) and an Applied Biosystems QuantStudio 3 (Applied Biosystems). Previously described primer/probe sets (see Supplemental Table 2 for sequences) [24] were used to detect G_4C_2 and G_2C_4 repeat containing transcripts. Previously described primer sets (see Supplemental Table 2 for sequences) [30, 32, 40, 43] were used to detect truncated STMN2 and cryptic exon containing mRNA transcripts. TaqMan Gene Expression Assays (see Supplemental Table 2 for probe information) were used to detect mRNA targets. GAPDH was used for normalization of gene expression.

Immunostaining and confocal imaging

iPSNs were re-plated using accutase dissociation in Matrigel coated 24 well optical bottom dishes (Cellvis) 3 days prior to fixation. iPSNs were fixed in 4% PFA for 15 min, washed 3X 10 min with 1X PBS, permeabilized for 15 min with 1X PBST containing 0.1% Triton X-100, blocked for 30 min in 10% normal goat serum diluted in 1X PBS, and incubated in primary antibody solution (Rabbit Anti-TDP-43 (Proteintech 10,782–2-AP), Guinea Pig Anti-Map2 (Synaptic Systems 188,004) for 2 h at room temperature. iPSNs were then washed 3X 10 min with 1X PBS, incubated in secondary antibody (Thermo Fisher Scientific; Goat Anti-Rabbit Alexa 488; Goat Anti-Guinea Pig 647) for 1 h at room temperature, washed 2X 10 min in 1X PBS, incubated for 10 min with Hoescht diluted 1:1000 in 1X PBS, and washed 2X 10 min in 1X PBS. iPSNs were mounted using Prolong Gold Antifade Reagent. iPSNs were imaged using a Zeiss LSM 980 confocal microscope using identical imaging parameters for each well and image. Images presented are maximum intensity projections generated in Zeiss Zen Blue 2.3. Nuclear to cytoplasmic ratios of TDP-43 were calculated as previously described [2, 8, 9].

Immunoprecipitation

On day 32 of differentiation, iPSNs were rinsed in 1 × PBS and lysed in 1 mL IP buffer (IP lysis buffer (Thermo Fisher Scientific), 1 × protease inhibitor cocktail (Roche), 0.4 U/μL RNasin Plus (Promega)) by scraping with a cell scraper, transferring to an Eppendorf tube, and vortexing for 30–45 s. iPSN lysates were centrifuged for 10 min at 2500 rpm and 4 °C to remove debris. Following centrifugation, the supernatant was transferred to a clean Eppendorf tube. 25 μL of the supernatant was added to 25 μL 2X Laemmli buffer (BioRad) and set aside as protein input and an additional 25 μL supernatant was set aside on ice as RNA input. The remainder of the lysate was split equally between Eppendorf tubes containing 10 μg Rabbit IgG Isotype Control (Thermo Fisher) and Rabbit Anti-TDP-43 (ProteinTech) antibody. Lysates were incubated with antibody with end over end rotation for 2 h at 4 °C. After 2 h, Magnetic Dyna-beads Protein G were washed with IP lysis buffer and 50 μL resuspended bead solution was added to each tube of antibody/lysate solution. Lysate-antibody-bead solutions were incubated with end over end rotation for 2 h at 4 °C. 25 μL of the unbound supernatant was collected and added to 25 μL 2 × Laemmli buffer for western blot analysis. The remainder of the unbound supernatant was discarded and immunoprecipitated complexes were washed 3X 5 min in 200 μL IP buffer with end over end rotation at 4 °C. Bead—IP complexes were resuspended in 200 μL IP buffer. 100 μL of each bead – IP complex sample was set aside for RNA analysis. For the remaining 100 μL, the supernatant was discarded and 50 μL 1X Laemmli buffer was added to bead – IP complexes. All RNA samples (including input) were added to 500 μL RLT Buffer (QIAGEN) and RNA isolation, cDNA synthesis, and qRT-PCR were performed as described above.

Western blot

Following immunoprecipitation, samples were heated at 100 °C for 5 min, and equal volumes of each sample (12.5 μL) were loaded in 4–20% acrylamide gels (BioRad). Gels were run until the dye front reached the bottom. Protein was transferred onto a nitrocellulose

membrane using the Trans-Blot Turbo Transfer System (BioRad). Blots were blocked for 30 min with 5% nonfat milk in 1 × TBST (0.1% Tween-20) and incubated overnight at 4 °C with Mouse Anti-TDP-43 (ProteinTech) primary antibody diluted in block. The next day, blots were washed 3 × 10 min with 1 × TBST and probed with Horse Anti-Mouse IgG HRP (Cell Signaling) secondary antibody diluted in block for 1 h at room temperature. Blots were then washed 3 × 10 min with 1 × TBST and ECL substrate (Thermo Fisher Scientific) was applied for 30 s. Chemiluminescent images were acquired with the GE Healthcare ImageQuant LAS 4000 system.

Glutamate toxicity

Glutamate induced neuronal death assays were carried out as recently described [2]. Briefly, iPSN media was exchanged for artificial CSF (ASCF, Tocris) containing 0 or 10 μM glutamate. For Alamar Blue cell viability assays, Alamar Blue reagent was added at this time. For propidium iodide (PI) based cell death assays, 1 μM PI and one drop of NucBlue live ready probes was added to ACSF/glutamate for the final 30 min of the 4 h incubation. As previously described [2, 8, 9], after 4 h of incubation with ACSF/glutamate, PI assays were imaged on a Zeiss LSM 980 confocal microscope in an environmentally controlled chamber. 10 images per well were captured with a 20X objective and the number of PI and DAPI spots were counted in FIJI. For Alamar Blue assays, plates were evaluated according to manufacturer protocol.

Statistical analysis

All image analysis was either completely automated or blinded. Statistical analyses were performed using GraphPad Prism version 9 (GraphPad). For imaging-based experiments (e.g. TDP-43 immunostaining), the average values for all cells analyzed per iPSC line represent n = 1. The total number of cells evaluated per experiment is indicated in the figure legends. One-way or Two-way ANOVA with Tukey's multiple comparison test was used as described in figure legends. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Violin plots are used to display the full spread and variability of data within imaging analysis of iPSNs. The center dotted line indicates the median value. Two additional dotted lines indicate the 25th and 75th percentiles. Bar graphs with individual data points representing each iPSC line are used to display data obtained from all other assays and as detailed in figure legends.

Results

Expression of G_2C_4 (antisense), but not G_4C_2 (sense) repeat RNA is sufficient to induce molecular hallmarks of TDP-43 loss of function in iPSNs

Nuclear clearance and cytoplasmic mislocalization and aggregation of the RNA binding protein TDP-43 are pathological hallmarks of ALS and related neurodegenerative diseases [44, 45]. It is thought that nuclear loss of TDP-43 function is an early and significant contributor to neurodegenerative disease pathogenesis [4, 21, 23, 28, 30, 32, 37, 43] perhaps even prior to overt detection of cytoplasmic mislocalization. Moreover, histology studies in postmortem human CNS tissues have suggested that nuclear loss of TDP-43 precedes cytoplasmic aggregation [45]. Consistent with the hypothesis that nuclear loss of TDP-43

function precedes cytoplasmic aggregation, studies in C9orf72 iPSNs have demonstrated the emergence of a molecular signature of TDP-43 dysfunction prior to overt nuclear clearance and cytoplasmic mislocalization [8, 9]. Interestingly, prior analyses have reported that TDP-43 pathology is more prevalent in CNS cells with G_2C_4 repeat RNA foci compared to those with G_4C_2 foci [1, 7]. Thus, we hypothesized that G_2C_4 repeat RNA itself may contribute to TDP-43 loss of function observed in C9orf72 ALS/FTD.

To address the contribution of G_2C_4 repeat RNA to TDP-43 dysfunction and mislocalization in human neurons, we first utilized repeat RNA-only constructs previously demonstrated to produce G_4C_2 and G_2C_4 repeat RNA but not DPRs [33, 35] and generated an additional long G_2C_4 repeat construct capable of expression in mammalian cells. Recent studies have identified a number of reliable gene expression and RNA processing changes that occur following artificial TDP-43 depletion in human neurons [4, 23, 43]. We have recently demonstrated that a panel of these RNA targets and associated splicing alterations can be employed as a robust measure of TDP-43 functionality in C9orf72 ALS/FTD and sALS iPSNs [2, 8]. Using qRT-PCR to evaluate the expression of multiple TDP-43 mRNA targets, we observe a significant molecular hallmark of TDP-43 dysfunction, including altered gene expression (Fig. 1) and the emergence of multiple cryptic exon containing mRNA species (Fig. 2), following the expression of G_4C_2 and G_2C_4 repeat RNA-only constructs in otherwise wildtype iPSNs. In addition, immunostaining and confocal imaging revealed a small but statistically significant number of G_2C_4, but not G_4C_2 expressing neurons that contained observable new cytoplasmic TDP-43 immunoreactivity (Supplemental Fig. 1). However, we note that this cytoplasmic signal was minimal suggesting that TDP-43 nuclear function may be impacted prior to overt histological mislocalization. Although only detectable in a small percentage of cells, this is consistent with prior studies where TDP-43 pathology (e.g. nuclear depletion and/or cytoplasmic aggregation) is only observed in a handful of cells in CNS autopsy tissues [25, 36]. Notably, we did not detect TDP-43 aggregation in G_2C_4 or G_4C_2 expressing iPSNs (Supplemental Fig. 1) consistent with prior reports from our group and others that cytoplasmic aggregation of TDP-43 does not occur in the majority of iPSN models of C9orf72 disease despite subtle cytoplasmic mislocalization [9, 14, 19, 47, 48]. Although expression of either G_4C_2 and G_2C_4 led to increased susceptibility to glutamate induced neuronal death (Supplemental Fig. 2), our data demonstrate that G_2C_4 repeat RNA expression is sufficient to induce TDP-43 dysfunction in human neurons and suggest that perhaps our prior observations of molecular hallmarks of TDP-43 loss of function in endogenous C9orf72 iPSNs [8] could be predominantly linked to endogenous antisense G_2C_4 and not G_4C_2 repeat RNA expression.

Treatment with G_2C_4, but not G_4C_2, targeting ASOs reverses molecular hallmarks of TDP-43 loss of function in C9orf72 patient iPSNs

In C9orf72 neurodegeneration, ASOs have previously been employed to induce the highly selective degradation of *C9orf72* mRNA or G_4C_2 repeat RNA [14, 22, 24]. To facilitate the study of sense vs antisense repeat RNA toxicity in iPSNs expressing endogenous levels of the C9orf72 HRE, ASOs were designed to selectively target G_4C_2 (S-ASO) or G_2C_4 (AS-ASO) repeat containing RNAs. To test the efficacy of these ASOs in the selective degradation of G_4C_2 or G_2C_4 repeat RNA, we performed qRT-PCR for G_4C_2 or G_2C_4 repeat

RNA in C9orf72 iPSC derived spinal neurons following 5, 10, 15, and 20 days of treatment with ASOs. We observed a significant strand specific decrease in G_4C_2 and G_2C_4 repeat RNA expression as early as 5 days after treatment (Supplemental Fig. 3), an observation that is supported by prior reports that G_4C_2 repeat RNA knockdown happens significantly faster than DPR reduction [9, 14, 17]. Consistent with this notion, we recently established that 5 day treatment with ASOs targeting G_4C_2 sense repeat RNA has no impact on Poly(GP) levels [9]. Important for evaluation of biological specificity, the G_4C_2 targeting ASO had no impact on G_2C_4 expression and the ASO targeting G_2C_4 containing RNA had no impact on G_4C_2 expression (Supplemental Fig. 3).

Having observed specificity for G_4C_2 and G_2C_4 repeat RNA targeting ASOs, we next asked whether repeat RNA strand specific ASO treatment could alleviate disease pathophysiology, namely TDP-43 dysfunction, in human iPSNs derived from multiple C9orf72 patients. Although we have previously reported that G_4C_2 repeat RNA ASO treatment can *prevent* cytotoxicity in C9orf72 iPSNs [9, 14], importantly for this study we elected to initiate ASO treatment *after* the emergence of TDP-43 dysfunction, reminiscent of clinical treatment paradigms. To determine whether G_4C_2 and G_2C_4 targeting ASO treatment could restore TDP-43 function, we performed qRT-PCR for a panel of TDP-43 mRNA targets in C9orf72 iPSNs treated with strand specific ASOs for 5, 10, 15, and 20 days. In doing so, we found that 5 and 10 day treatment with either G_4C_2 or G_2C_4 repeat RNA targeting ASOs was not sufficient to repair TDP-43 function (Supplemental Figs. 4–7). In contrast, consistent with a time-dependent therapeutic efficacy, 15 and 20 day treatment with G_2C_4 antisense, *but not* G_4C_2 sense, repeat RNA targeting ASOs was sufficient to restore the expression and splicing of TDP-43 mRNA targets (Figs. 3–4, Supplemental Figs. 8–9) thereby alleviating TDP-43 dysfunction. We also observed a significant reduction in the percentage of C9orf72 iPSNs with observable cytoplasmic TDP-43 immunoreactivity following 20 days of treatment with G_2C_4 but not G_4C_2 repeat RNA targeting ASOs (Supplemental Fig. 10). Again, consistent with prior reports [9, 14, 19, 25, 36, 47, 48], the percentage of neurons with detectable cytoplasmic TDP-43 immunoreactivity was low and we did not observe cytoplasmic TDP-43 puncta of aggregates (Supplemental Fig. 10). Moreover, despite our data that only treatment with G_2C_4 repeat RNA targeting ASOs repairs TDP-43 function (Figs. 3–4, Supplemental Figs. 8–9), both G_4C_2 and G_2C_4 repeat RNA targeting ASOs alleviated C9orf72 neuronal death in response to glutamate stress (Supplemental Fig. 11). Interestingly, using immunoprecipitations for TDP-43 followed by qRT-PCR for repeat RNAs, we found that that G_2C_4 antisense, but not G_4C_2 sense, repeat RNA is enriched in TDP-43 complexes in C9orf72 patient iPSNs (Supplemental Fig. 12) further underscoring the relationship between G_2C_4 antisense repeat RNA and TDP-43 in C9orf72 ALS/FTD pathogenesis.

We next tested the hypothesis that combined treatment with G_4C_2 and G_2C_4 repeat RNA targeting ASOs might enhance the therapeutic benefit of G_2C_4 repeat RNA targeting ASO treatment alone, at least in regard to TDP-43 functionality. Importantly, the efficacy of repeat RNA knockdown was essentially unchanged following simultaneous treatment with both repeat RNA targeting ASOs (Supplemental Fig. 13). Using qRT-PCR, we did not observe an increased benefit of TDP-43 function restoration upon simultaneous treatment with G_4C_2 and G_2C_4 targeting ASOs compared to G_2C_4 targeting ASOs alone (Figs. 5–6).

Additionally, simultaneous treatment with G_4C_2 and G_2C_4 targeting ASOs provided no additional protection against glutamate-induced neuronal death in C9orf72 patient iPSNs compared to either ASO alone (Supplemental Fig. 14). These data suggest that there is no added benefit of targeting both sense and antisense C9orf72 repeat RNAs to alleviate TDP-43 dysfunction in human neurons.

Discussion

Reduced nuclear localization of TDP-43 and corresponding loss of nuclear TDP-43 function is widely regarded as a prominent pathophysiological event contributing to neuronal demise in ALS, FTD, and related neurodegenerative diseases [4, 23, 28, 32, 43]. Current therapeutic strategies in the clinic are aimed at restoring the expression of singular TDP-43 mRNA targets such as STMN2 [23, 32] as opposed to broadly alleviating TDP-43 dysfunction. Further, in the case of C9orf72 ALS/FTD, although G_2C_4 antisense repeat RNA foci correlate with TDP-43 pathology at end-stage disease [1, 7], little is known regarding the contribution of pathologic C9orf72 repeat RNA species to TDP-43 dysfunction in authentic human neurons. In this brief report, we have demonstrated that G_2C_4 antisense, but not G_4C_2 sense, repeat RNA compromises TDP-43 functionality in endogenous C9orf72 human neurons. Although the mechanism by which G_2C_4 antisense repeat RNA specifically elicits TDP-43 dysfunction remains unknown and will require further future studies, this pathophysiological consequence is consistent with postmortem histological analyses indicating a correlation between G_2C_4 repeat RNA foci and TDP-43 pathology [1, 7]. Our RNA immunoprecipitation experiments (Supplemental Fig. 12) suggest that TDP-43 may be at least partially sequestered away from it's multiple RNA targets by G_2C_4 repeat RNA. Further, our data highlight the possibility that G_2C_4 repeat RNA induced TDP-43 dysfunction occurs in the absence of and perhaps prior to overt TDP-43 nuclear clearance and mislocalization to the cytoplasm (Figs. 1–2, Supplemental Fig. 1). In fact, complete nuclear clearance and cytoplasmic aggregation of TDP-43 is only observed in a small percentage of CNS cells in C9orf72 patients at autopsy [8, 25, 36], suggesting that more histologically subtle nuclear depletion may be sufficient to induce robust alterations in TDP-43 function as has been demonstrated for STMN2 in patient CNS tissues [32, 37]. We note that we and others have been unable to detect the most well-characterized mouse TDP-43 associated RNA misprocessing event, altered sortilin splicing [38], in C9orf72 BAC [29] or AAV9-$(G_4C_2)_{149}$ transgenic mice ([5], unpublished data). These mouse models also do not show histological evidence of actual nuclear TDP-43 clearing typical of sporadic ALS or C9orf72 ALS/FTD ([5, 29], unpublished data). Thus, at the current time, this highlights authentic C9orf72 patient iPSNs as an essential model for evaluating the efficacy of therapeutic strategies in the repair of TDP-43 functionality in the absence of TDP-43 aggregation and accumulation in the cytoplasm in ALS/FTD.

ASOs are now a commonly used therapeutic modality to alter the expression of target genes in neurodegeneration [13, 42]. Prior pre-clinical research in iPSNs and mice has suggested that selective degradation of G_4C_2 (sense) repeat RNA via ASO may elicit neuroprotection [14, 17, 22, 24, 41]. This led to an international clinical trial. However, this trial, as well as a second using an independent ASO molecule, recently failed for reasons which remain unknown. Notably, in preclinical studies, exposure to ASOs is often short and treatment

is often initiated prior to the onset of many disease associated cellular and molecular changes. Additionally, these efforts focused solely on degradation of the G_4C_2 repeat RNA species. Thus, the failure of these molecules in clinical trials raises the intriguing possibility that the antisense G_2C_4 repeat RNA species may be pathologically more important than previously appreciated. In this manuscript, we demonstrate that treatment with G_2C_4, but not G_4C_2, ASOs after the onset of TDP-43 loss of function associated molecular changes, is sufficient to restore molecular signatures of TDP-43 function (Figs. 3–4, Supplemental Figs. 8–9). Importantly, this is a time dependent phenomenon (Figs. 3–4, Supplemental Figs. 4–9) suggesting that a neuronal "recovery" period is needed following ASO initiated degradation of antisense repeat RNA species. Nonetheless, treatment with either G_4C_2 or G_2C_4 repeat RNA targeting ASOs was sufficient to alleviate neuronal death in response to exogenous glutamate stress (Supplemental Fig. 11) suggesting that both repeat RNA species can disrupt neuronal survival perhaps via distinct mechanisms. Therefore, while our molecular studies show that simultaneous treatment with both G_4C_2 and G_2C_4 repeat RNA targeting ASOs does not provide additional benefits for TDP-43 function (Figs. 5–6), it may still be important to consider a muti-pronged therapeutic approach in humans.

Importantly, our data illuminate new insights into repeat RNA pathophysiology in C9orf72 ALS/FTD and provide human relevant data suggesting that G_2C_4 repeat RNA and downstream cellular interactions could be a significant contributor to disease pathogenesis. Collectively, these data suggest that targeting G_2C_4 antisense repeat RNA may be a viable therapeutic strategy for the restoration of TDP-43 function in ALS/FTD. Further, our data may provide insights into one reason that G_4C_2 sense stand targeting C9orf72 therapy failed to have clinical efficacy. However, at least for these studies in authentic human C9orf72 ALS/FTD iPSNs, do not support the possibility of a combined sense and antisense repeat RNA targeting ASO therapy at least for the alleviation of TDP-43 dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

All data and materials are detailed within the main text or the supplementary materials. Requests for materials should be addressed to Alyssa N. Coyne, PhD email: acoyne3@jhmi.edu. ASOs used in this study can be obtained from Ionis Pharmaceuticals (Frank Bennett, PhD email: fbennett@ionisph.com) under a standard research MTA.

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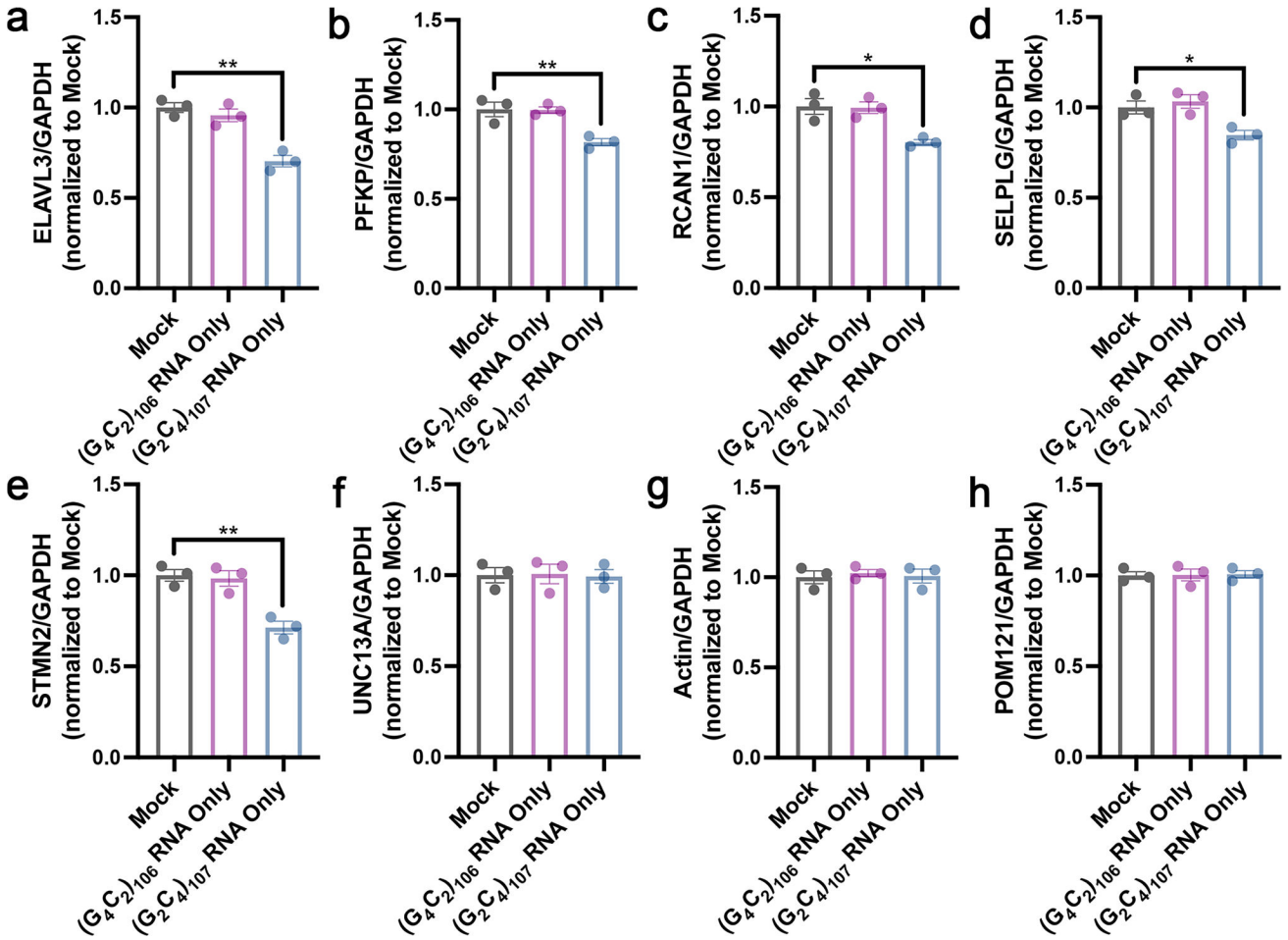

Fig. 1.

G_2C_4 antisense repeat RNA expression triggers gene expression changes linked to TDP-43 dysfunction in iPSNs. **a**–**h** qRT-PCR for *ELAVL3* (**a**), *PFKP* (**b**), *RCAN1* (**c**), and *SELPLG* (**d**), *STMN2* (**e**), *UNC13A* (**f**), *ACTIN* (**g**), and *POM121* (**h**) mRNA in control iPSNs 2 weeks following expression of G_4C_2 or G_2C_4 repeat RNA only plasmids. GAPDH was used for normalization. *ACTIN* and *POM121* were used as negative control mRNAs not known to be regulated by TDP-43. $n = 3$ control iPSC lines. One-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. *$p < 0.05$, **$p < 0.01$

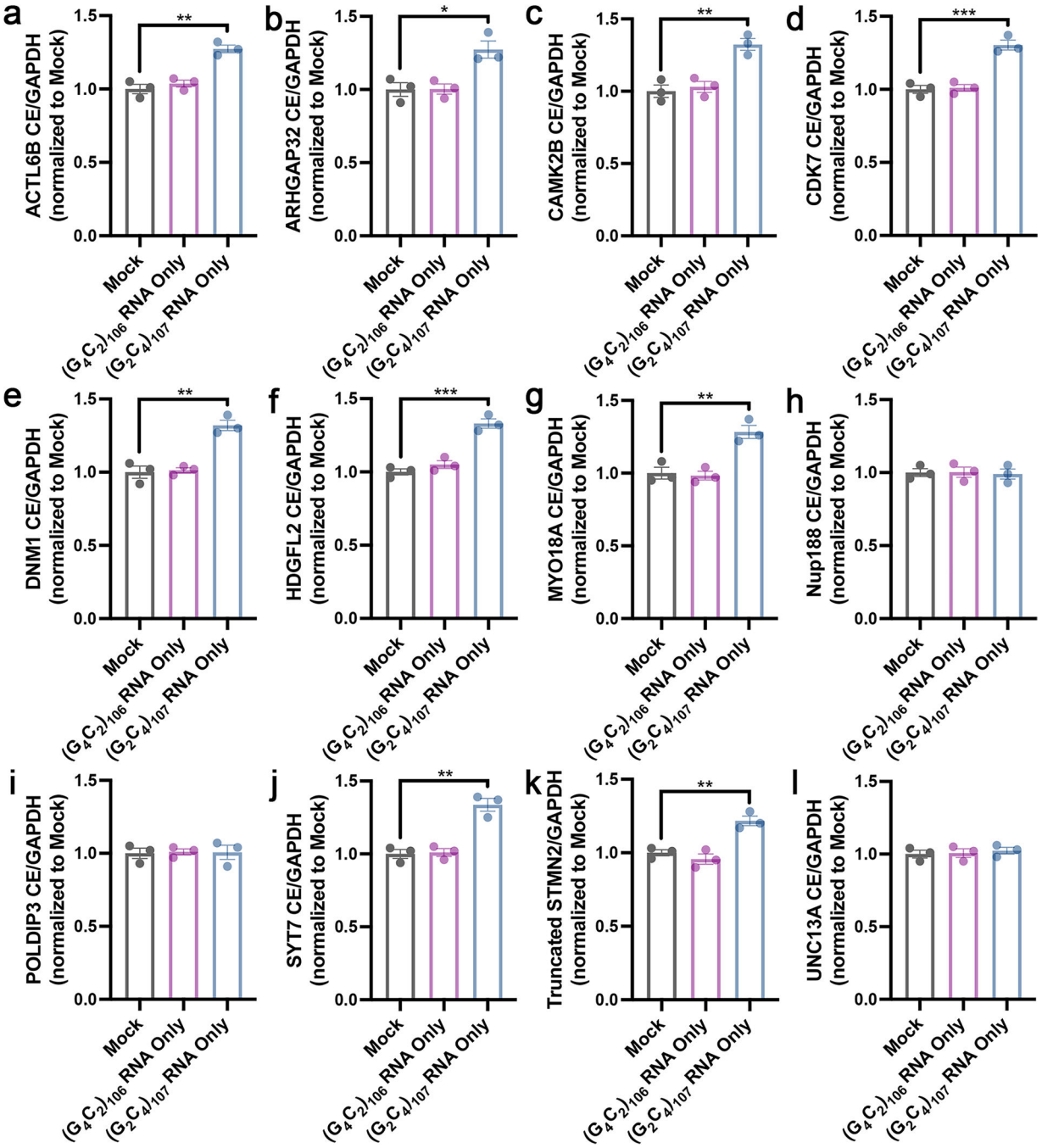

Fig. 2.

G_2C_4 antisense repeat RNA expression triggers splicing alterations linked to TDP-43 dysfunction in iPSNs. **a–l** qRT-PCR for *ACTL6B* cryptic exon containing (**a**), *ARHGAP32* cryptic exon containing (**b**), *CAMK2B* cryptic exon containing (**c**), *CDK7* cryptic exon containing (**d**), *DNM1* cryptic exon containing (**e**), *HDGFL2* cryptic exon containing (**f**), *MYO18A* cryptic exon containing (**g**), *NUP188* cryptic exon containing (**h**), *POLDIP3* cryptic exon containing (**i**), *SYT7* cryptic exon containing (**j**), truncated *STMN2* (**k**), and *UNC13A* cryptic exon containing mRNA in control iPSNs 2 weeks following expression

of G_4C_2 or G_2C_4 repeat RNA only plasmids. GAPDH was used for normalization. $n = 3$ control iPSC lines. One-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

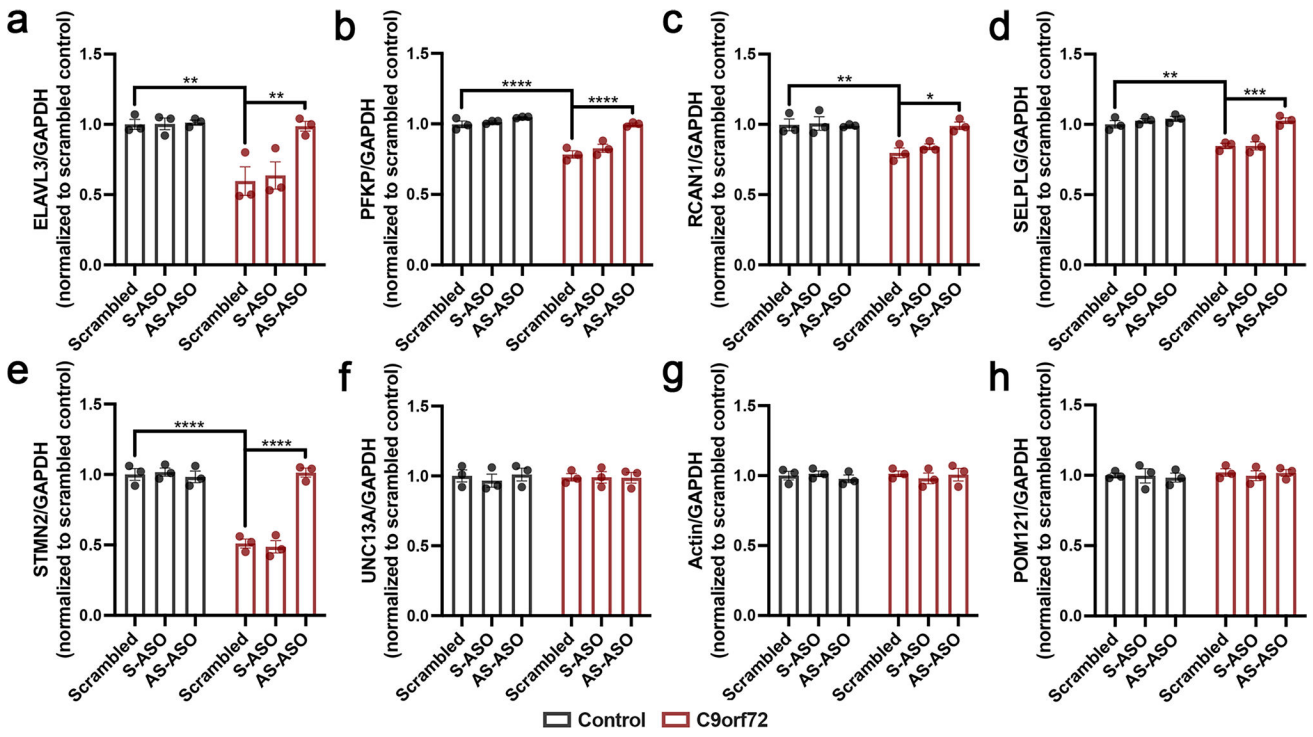

Fig. 3.

Treatment with G_2C_4, but not G_4C_2, targeting ASO for 20 days reverses gene expression changes associated with TDP-43 dysfunction in C9orf72 patient iPSNs. **a**–**h** qRT-PCR for *ELAVL3* (**a**), *PFKP* (**b**), *RCAN1* (**c**), and *SELPLG* (**d**), *STMN2* (**e**), *UNC13A* (**f**), *ACTIN* (**g**), and *POM121* (**h**) mRNA in control and C9orf72 iPSNs 20 days following a single dose of scrambled or repeat targeting ASO. GAPDH was used for normalization. *ACTIN* and *POM121* were used as negative control mRNAs not known to be regulated by TDP-43. $n = 3$ control and 3 C9orf72 iPSC lines. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

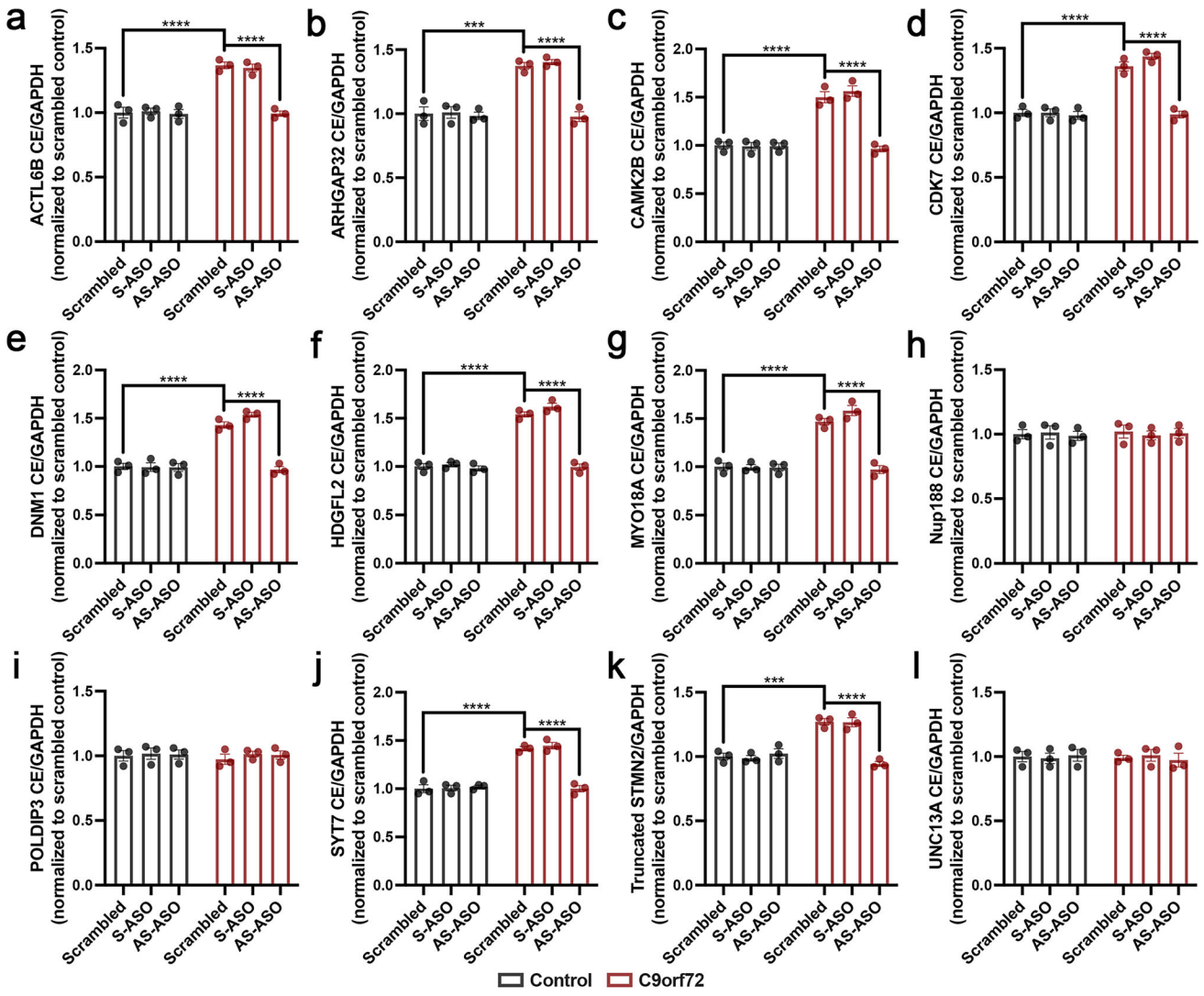

Fig. 4.

Treatment with G_2C_4, but not G_4C_2, targeting ASO for 20 days reverses splicing alterations associated with TDP-43 dysfunction in C9orf72 patient iPSNs. **a**–**l** qRT-PCR for *ACTL6B* cryptic exon containing (**a**), *ARHGAP32* cryptic exon containing (**b**), *CAMK2B* cryptic exon containing (**c**), *CDK7* cryptic exon containing (**d**), *DNM1* cryptic exon containing (**e**), *HDGFL2* cryptic exon containing (**f**), *MYO18A* cryptic exon containing (**g**), *NUP188* cryptic exon containing (**h**), *POLDIP3* cryptic exon containing (**i**), *SYT7* cryptic exon containing (**j**), truncated *STMN2* (**k**), and *UNC13A* cryptic exon containing mRNA in control and C9orf72 iPSNs 20 days following a single dose of scrambled or repeat targeting ASO. GAPDH was used for normalization. $n = 3$ control and 3 C9orf72 iPSC lines. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. ***$p < 0.001$, ****$p < 0.0001$

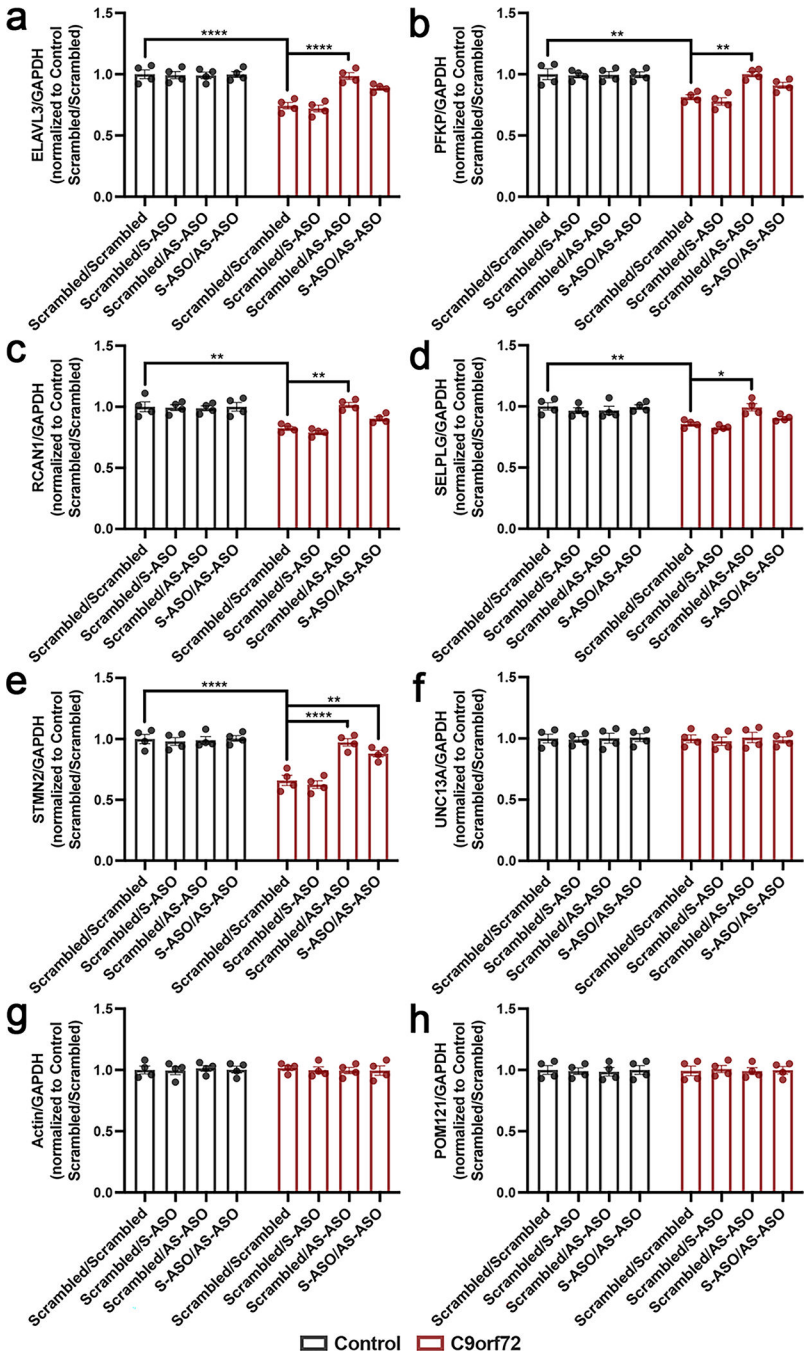

Fig. 5.

Simultaneous treatment with G_4C_2 and G_2C_4 repeat RNA targeting ASOs does not provide additional reversal of gene expression changes associated with TDP-43 dysfunction. **a**–**h** qRT-PCR for *ELAVL3* (**a**), *PFKP* (**b**), *RCAN1* (**c**), and *SELPLG* (**d**), *STMN2* (**e**), *UNC13A* (**f**), *ACTIN* (**g**), and *POM121* (**h**) mRNA in in control and C9orf72 iPSNs 15 days following 5 μM dosing with a combination of scrambled, G_4C_2, and G_2C_4 repeat RNA targeting ASOs. GAPDH was used for normalization. *ACTIN* and *POM121* were used as negative control mRNAs not known to be regulated by TDP-43. $n = 4$ control and 4 C9orf72

iPSC lines. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

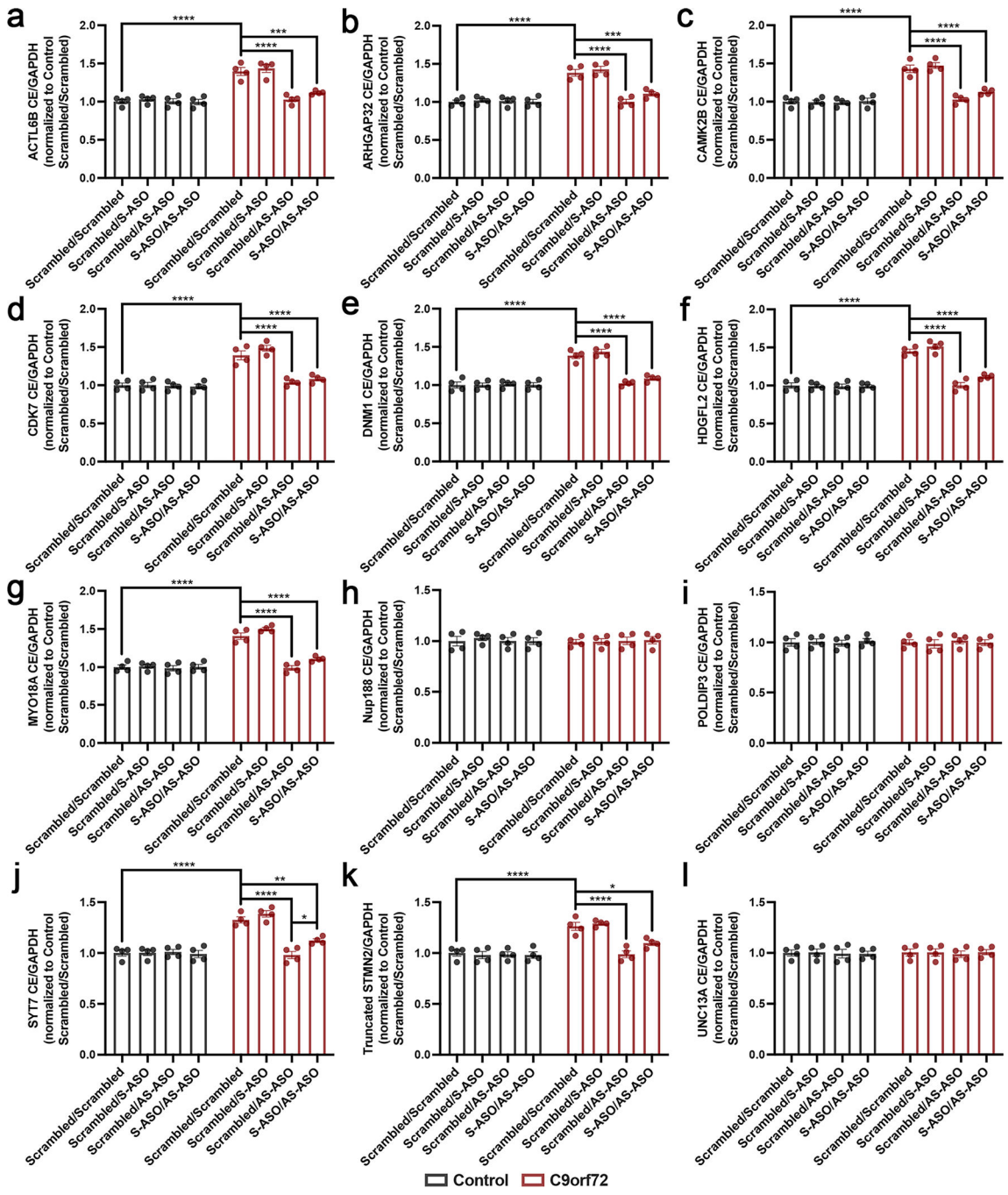

Fig. 6.

Simultaneous treatment with G_4C_2 and G_2C_4 repeat RNA targeting ASOs does not provide additional reversal of splicing alterations associated with TDP-43 dysfunction. **a**–**l** qRT-PCR for *ACTL6B* cryptic exon containing (**a**), *ARHGAP32* cryptic exon containing (**b**), *CAMK2B* cryptic exon containing (**c**), *CDK7* cryptic exon containing (**d**), *DNM1* cryptic exon containing (**e**), *HDGFL2* cryptic exon containing (**f**), *MYO18A* cryptic exon containing (**g**), *NUP188* cryptic exon containing (**h**), *POLDIP3* cryptic exon containing (**i**), *SYT7* cryptic exon containing (**j**), truncated *STMN2* (**k**), and *UNC13A* cryptic exon

containing mRNA in control and C9orf72 iPSNs 15 days following 5 μM dosing with a combination of scrambled, G_4C_2, and G_2C_4 repeat RNA targeting ASOs. GAPDH was used for normalization. $n = 4$ control and 4 C9orf72 iPSC lines. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$