Evidence of A Negative Feedback Network Between TDP-43 and miRNAs Dependent on TDP-43 Nuclear Localization

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Abstract

TAR DNA-binding protein 43 (TDP-43) is a DNA/RNA-binding protein that is integral to RNA processing. Among these functions is a critical role in microRNA (miRNA) biogenesis through interactions with the DROSHA and DICER complexes. It has been previously shown that there is a general reduction in miRNA levels within the spinal cord and spinal motor neurons of amyotrophic lateral sclerosis (ALS) patients. In addition, the most common pathological feature of ALS is re-distribution of TDP-43 from the nucleus to the cytoplasm where it forms cytoplasmic inclusions. Among miRNAs dysregulated in ALS, several are known to regulate TDP-43 expression. In this study, we demonstrate that TDP-43 is in a regulatory negative feedback network with miR-181c-5p and miR-27b-3p that is dependent on its nuclear localization within HEK293T cells. Further, we show that cellular stress which induces a redistribution of TDP-43 from the nucleus to the cytoplasm correlates with the reduced production of miR-27b-3p and miR-181c-5p. This suggests that reduced nuclear TDP-43 disrupts a negative feedback network between itself and miRNAs. These findings provide a further understanding of altered miRNA biogenesis as a key pathogenic process in ALS.

Introduction

TAR DNA-binding Protein 43 (TDP-43) is a primarily nuclear DNA/RNA-binding protein that is ubiquitously expressed in every cell and has a diverse set of functions which include: mRNA transcription, mRNA splicing, mRNA transport, microRNA (miRNA) and long non-coding RNA (lncRNA) processing, and mRNA translation.[1] Several of these functions have been identified as dysregulated within motor neurons of patients with amyotrophic lateral sclerosis (ALS) where TDP-43 re-locates from the nucleus to the cytoplasm resulting in the subsequent formation of pathological aggregates. This phenomenon occurs in 97% of all ALS cases.[2–5]

In vitro and *in vivo* models have shown that an induced cellular stress can cause TDP-43 to re-locate from the nucleus to the cytoplasm and enter stress granule structures.[6–8] Proteins with low-complexity domains (LCD), like TDP-43, have been shown to phase separate into liquid droplets;

however, if left to accumulate, LCD-containing proteins can form irreversible insoluble fibrils which are believed to be the precursors of pathological inclusions in ALS motor neurons.[9,10] Evidence that stress granules may act as a seed for pathological cytoplasmic aggregates of TDP-43 in ALS is that TIA-1, an essential stress granule component, co-localizes with TDP-43 aggregates in ALS motor neurons.[11,12] Further, increased mRNA and protein levels of TDP-43 have been observed in both the spinal cord and motor neurons of ALS patients,[13,14] suggesting that TDP-43 levels are being aberrantly regulated which may contribute to its accumulation into cytoplasmic aggregates.

Our lab, and others, have shown that a large pool of miRNAs, small RNA molecules (20, 22 nucleotides) generally responsible for post-transcriptional gene suppression[15]—have reduced levels within ALS spinal cord and motor neurons.[3,16] Further, we have shown that ALS-linked miRNAs suppress TDP-43 expression suggesting that the loss of miRNA function could result in increased levels of TDP-43 in ALS motor neurons.[17] Given the observation that TDP-43 is involved in the miRNA biogenesis pathway through interactions with both the DROSHA and DICER complex,[18] we postulated that TDP-43 regulates the production of miRNAs that in turn will suppress its expression. We further hypothesize that TDP-43 is in a negative feedback loop with a specific group of miRNAs and that this function is dependent on its nuclear localization.

In this manuscript, we explore the negative feedback network between TDP-43 and two ALS-related miRNAs, miR-27b-3p and miR-181c-5p, in HEK293T cells and demonstrate that this regulation is dependent on TDP-43 nuclear localization. Further, we also demonstrate that TDP-43 affects the processing of these two miRNAs at different levels within the miRNA biogenesis pathway.

Results

Knockdown of TDP-43 alters small RNA profile in HEK293T cells

Knockdown of TDP-43 in HEK293T cells was accomplished using an siRNA pool targeting *TARDBP* (siTDP #1) mRNA (Figure 1(A)). MiRNA expression was then analyzed by microarray. We observed that more than 370 small RNAs (scaRNAs, snoRNAs, and miRNAs) were significantly dysregulated, most of which were miRNAs (Figure 1(B), Table S1). Interestingly, several miRNAs that were downregulated following knockdown of TDP-43 also contained a miRNA recognition element (MRE) within the 3′untranslated region (UTR) of *TARDBP* (Figure 1(C)) according to miRanda software. We selected five miRNA candidates with MREs in the *TARDBP* 3′UTR that had the greatest reduced fold-change following knockdown of TDP-43, and confirmed with real-time PCR that four of the five selected miRNAs (miR-27b-3p, miR-30a-5p, miR-181c-5p and miR-425-3p) were significantly downregulated after knockdown of TDP-43, while miR-1260b showed a non-significant reduction (Figure 1(D)).

In previous work, we had shown that of the 4 miRNA candidates identified here, miR-27b-3p, miR-181c-5p and miR-425-3p, but not miR-30a-5p, were significantly reduced in sALS spinal cord.[3] However, using TargetScan 7.2,[19] only miR-27b-3p and miR-181c-5p had conserved MREs in the *TARDBP* 3′UTR giving them a high probability that they would affect TDP-43 expression. Given this information, we decided to check whether miR-27b-3p and miR-181c-5p were expressed in spinal motor neurons. Indeed, using fluorescent *in situ* hybridization, we observed that both miRNAs are expressed in human spinal motor neurons (Figure S1), and therefore, we decided to explore their relationship with TDP-43 expression.

MiR-27b-3p and miR-181c-5p reduce TDP-43 expression

To determine whether miR-27b-3p and/or miR-181c-5p regulate TDP-43 expression, miRNA mimics of both miR-27b-3p and miR-181c-5p were individually transfected into HEK293T cells. This led to a reduction of both endogenous TDP-43 protein and mRNA levels 48 hours after transfection (Figure 2(A)–(C)). Further, we individually transfected antagomirs to suppress the endogenous function of either miR-27b-3p, miR-181c-5p, or both. This led to a significant increase in TDP-43 protein and mRNA expression when the antagomir targeting miR-181c-5p (Anti-181c) was transfected, but no effect was observed in transfecting the antagomir targeting miR-27b-3p (Anti-27b) alone. Transfecting both Anti-181c and Anti-27b had no greater impact on TDP-43 expression than observed when transfecting Anti-181c alone (Figure 2(D)–(F)). Previous research has shown that miR-27b-3p, and its miRNA family member miR-27a-3p, work together to suppress gene expression as they have the same MREs.[20–22] Therefore, since miR-27b-3p inhibition alone is insufficient to induce increased TDP-43 expression, we examined whether miR-27a-3p could suppress TDP-43 levels in the absence of miR-27b-3p. As observed with miR-27b-3p, the inhibition of miR-27a-3p alone does not affect TDP-43 protein levels. However, the inhibition of both miR-27a-3p and miR-27b-3p simultaneously induces a significant increase in TDP-43 protein levels (Figure S2). Therefore, this data indicates that while miR-181c-5p and miR-27b-3p are in a negative feedback loop with TDP-43, miR-27a-3p can replace the function of miR-27b-3p in suppressing TDP-43 when miR-27b-3p is absent and explains our failure to observe an

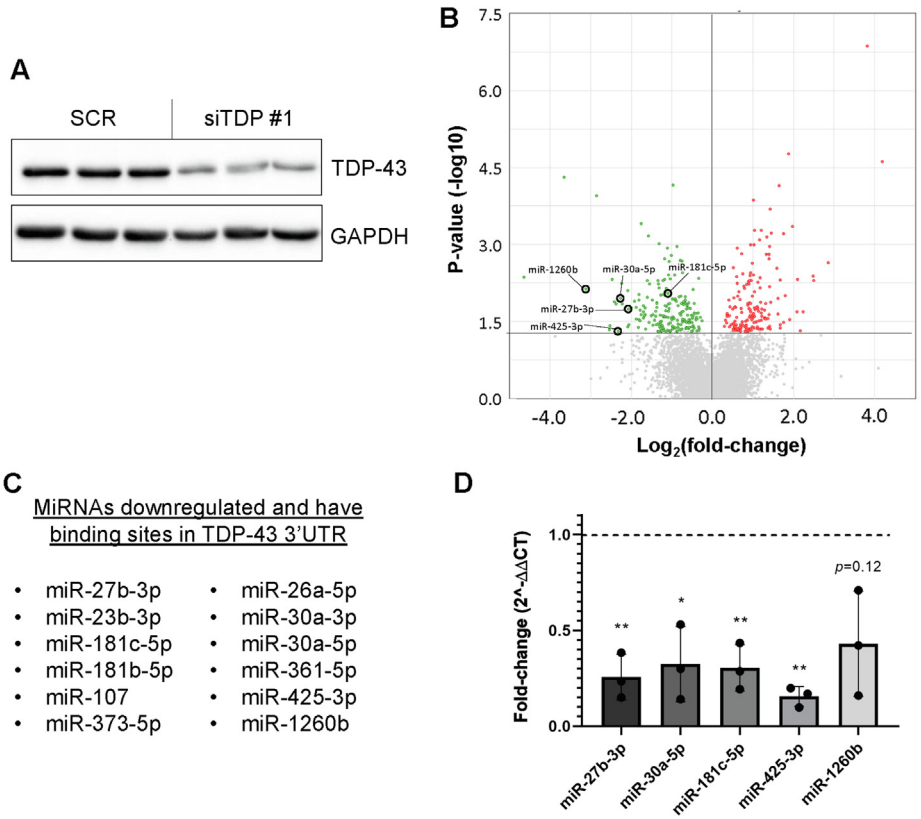

Figure 1. Change in small RNA expression following knockdown of TDP-43 in HEK293T cells. (A) Western blot showing knockdown of TDP-43 via siRNA (siTDP #1). (B) Volcano plot indicating change in global small RNA expression following knockdown of TDP-43 (n = 3). Green and red represent downregulated and upregulated small RNAs, respectively. Microarray data was normalized to spike-in control probe sets. (C) List of miRNAs that are downregulated following knockdown of TDP-43 and that have MREs within the *TARDBP* 3′UTR according to miRanda software. (D) Real-time PCR was used to confirm the change in candidate miRNA expression following TDP-43 knockdown (n = 3). Real-time PCR data was normalized to an endogenous control (miR-1296-5p). Bars represent mean fold-change (2^-ΔΔCT) ± SD in real-time PCR analysis. Student's t-test was used to determine significance in microarray and real-time PCR analyses (*$p < 0.05$, **$p < 0.01$).

alteration in TDP-43 expression in the presence of the antagomir targeting miR-27b-3p alone.

We then examined whether miR-27b-3p and miR-181c-5p regulated *TARDBP* expression via direct interactions with the 3′UTR of *TARDBP*. Wild-type *TARDBP* 3′UTR or *TARDBP* 3′UTR containing mutations in the +2 and +3 position of either the miR-27b-3p or miR-181c-5p MRE were linked to a firefly luciferase gene (Figure 2(G)). Reporter gene assays revealed that when the firefly gene contains the wild-type *TARDBP* 3′UTR there is a reduction in luciferase activity in the presence of either exogenous miR-27b-3p or miR-181c-5p. However, these miRNAs have no effect on the luciferase activity when the *TARDBP* 3′UTR contains mutations in their respective MREs (Figure 2(H)). This indicates that both miR-27b-3p and miR-181c-5p can interact directly with the 3′UTR of *TARDBP* in order to reduce gene expression.

Nuclear localization of TDP-43 is required to regulate miR-27b-3p and miR-181c-5p expression

Since cellular stress is known to alter TDP-43 localization from the nucleus to the cytoplasm, we wanted to determine whether stress would lead to a reduction in the levels of miR-27b-3p and miR-181c-5p similar to that observed following knockdown of TDP-43. After an induced cellular stress, levels of both miR-27b-3p and miR-181c-5p were significantly reduced concomitant with the expected reduction of nuclear localization of TDP-43 (Figure 3).

Based on this information, we next determined if the nuclear localization of TDP-43 is a requirement for the regulation of miR-27b-3p and miR-181c-5p expression. To do this, we used plasmids (siRES) resistant to a specific siRNA (siTDP #2) which contained either FLAG-tagged

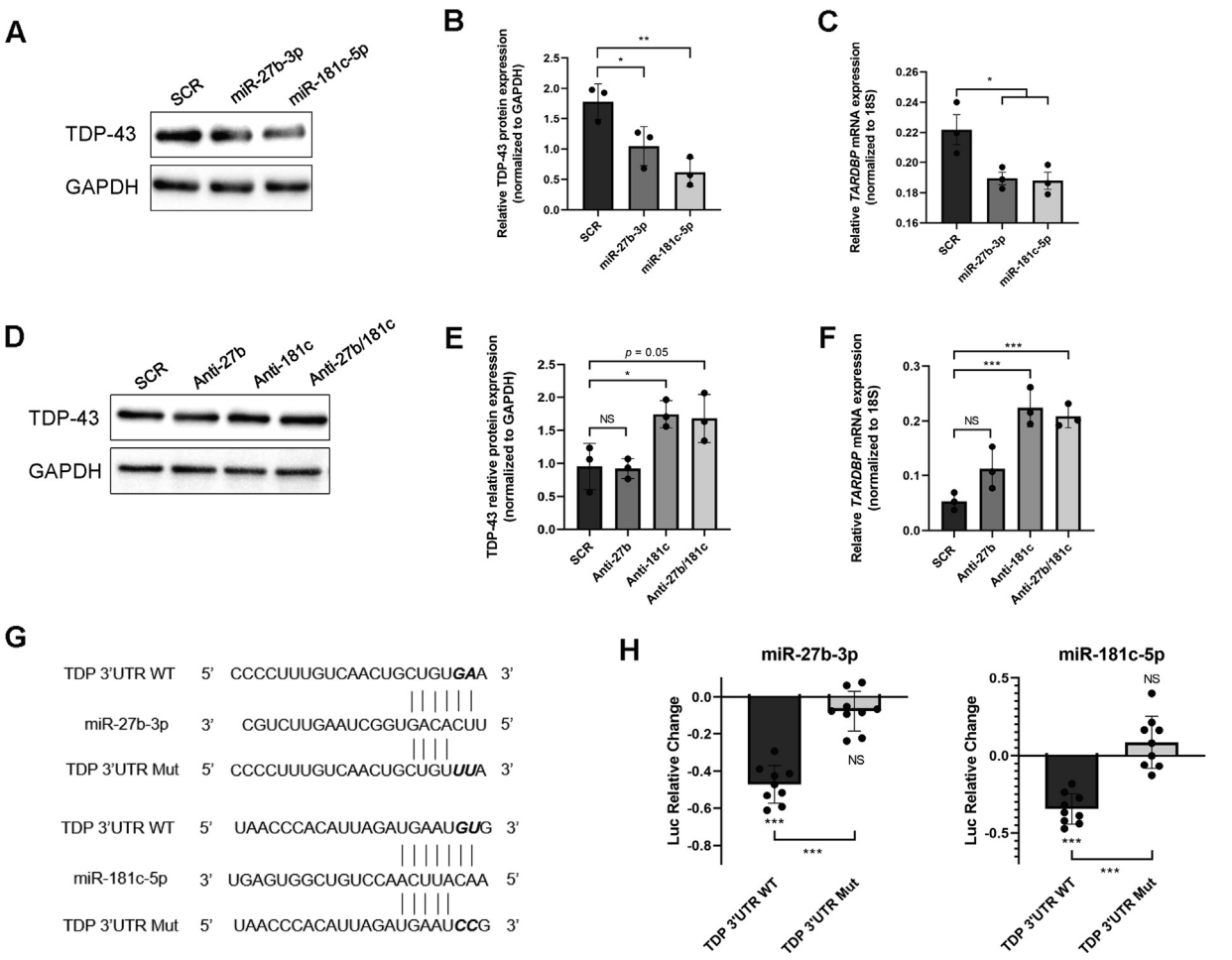

Figure 2. MiR-27b-3p and miR-181c-5p decrease TDP-43 protein and mRNA expression by interacting with the *TARDBP* 3′UTR. (A) Western blot showing TDP-43 and GAPDH protein levels following transfection of either scramble control (SCR), miR-27b-3p and miR-181c-5p. (B) Quantification of relative TDP-43 protein expression when normalized to GAPDH ($n = 3$). (C) Change in mRNA expression of *TARDBP* when normalized to 18S rRNA following transfection of either SCR, miR-27b-3p, or miR-181c-5p ($n = 3$). (D) Western blot showing TDP-43 and GAPDH protein following transfection of SCR, Anti-27b, Anti-181c, or both Anti-27b and Anti-181c. (E) Quantification of TDP-43 protein when normalized to GAPDH. (F) *TARDBP* mRNA when normalized to 18S following transfection of SCR or antagomirs ($n = 3$). (G) Plasmids containing a reporter gene linked to wild-type or mutant *TARDBP* 3′UTRs were co-transfected in HEK293T cells with either SCR (control), miR-27b-3p or miR-181c-5p to determine changes in reporter gene expression. (H) Reporter gene assays showing the change in expression of the reporter gene when linked to either the wild-type (TDP WT) or mutated (TDP Mut) *TARDBP* 3′UTR when in the presence of either miR-27b-3p, or miR-181c-5p ($n = 9$). Data was normalized to renilla expression prior to comparison and was further normalized to account for the individual miRNAs effect on the reporter plasmid itself. Bars represent mean ± SD. Significance was determined using a one-way ANOVA followed by a Tukey's post-hoc for multiple comparisons ($^{NS}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

wild-type TDP-43 (siRES pC32-FLAG-TDP-43 WT) or FLAG-tagged TDP-43 without its nuclear localization signal (siRES pC32-FLAG-TDP-43 ΔNLS), as previously described.[23] Both plasmids had a transfection efficiency of ~70% (Figure S3 (A) and (B)). SiTDP #2 was able to knockdown endogenous TDP-43 (Figure 4(A)) without affecting the expression of the siRES plasmids (Figure S3(C)–€). The siRES pC32-FLAG-TDP-43 WT plasmid was primarily nuclear, while siRES pC32-FLAG-TDP-43 ΔNLS was primarily cytoplasmic (Figure 4(B)) as expected. 24 hours after transfection of either SCR (negative control) or siTDP #2, either pcDNA 3.1 (negative control), siRES pC32-FLAG-TDP-43 WT, or siRES pC32-FLAG-TDP-43 ΔNLS were transfected into

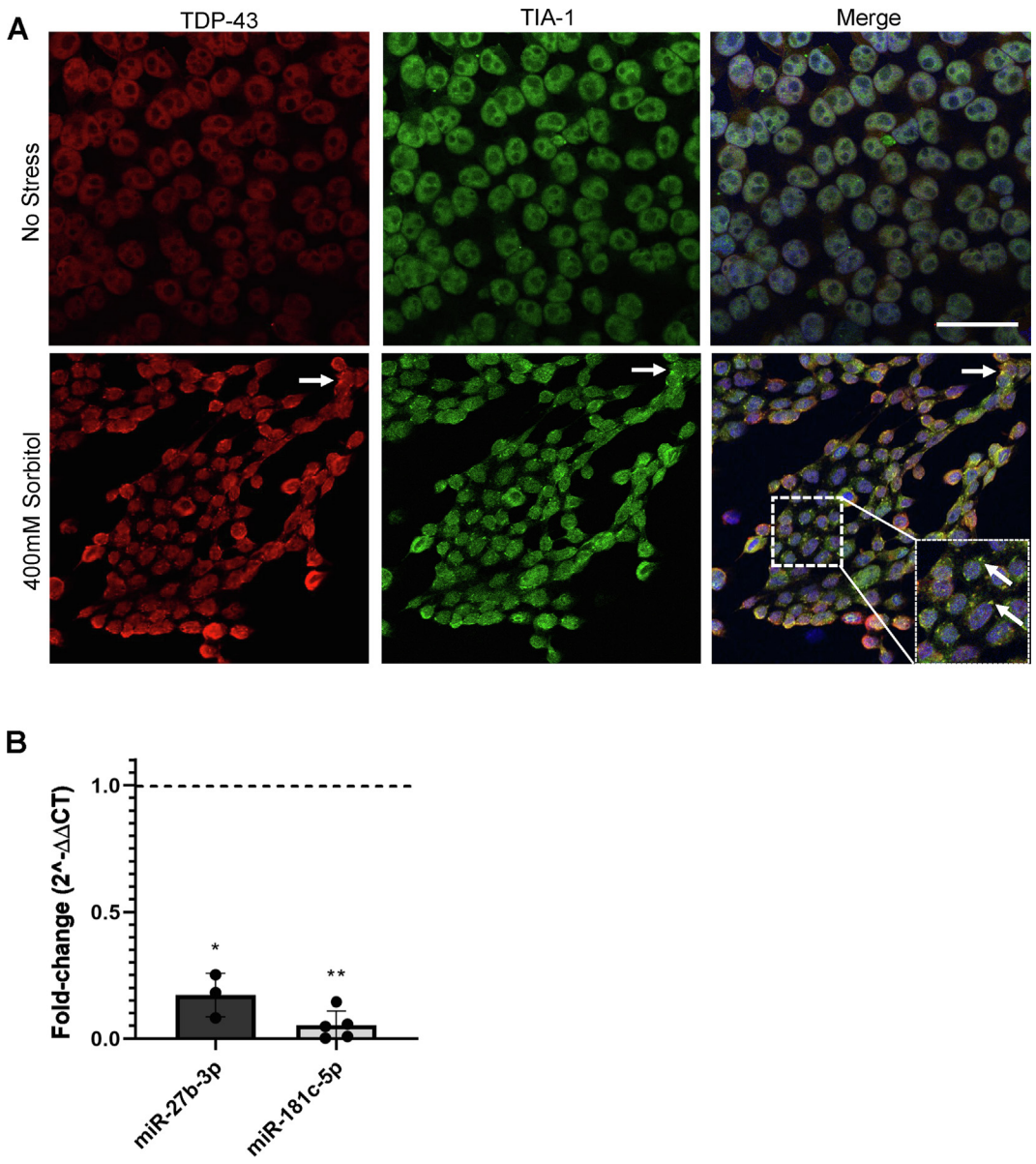

Figure 3. Reduced expression of miR-27b-3p and miR-181c-5p and reduced nuclear localization of TDP-43 five hours post-osmotic stress (A) HEK293T cells were either not treated (control) or treated with 400 mM of sorbitol to induce an osmotic stress. Cells were fixed and stained for TDP-43 (red) and TIA-1 (green) immunofluorescence. Scale bar represents 50 μm. (B) MiR-27b-3p ($n = 3$) and miR-181c-5p ($n = 5$) expression between non-stressed and stressed cells was measured via real-time PCR. Expression levels were normalized to an exogenous control (cel-miR-39b-5p) prior to comparison. Bar represents mean fold-change (2^-ΔΔCT) ± SD. Student's t-test was used to determine significance (*$p < 0.05$, **$p < 0.01$).

HEK293T cells. Recovery of both miR-27b-3p and miR-181c-5p to normal levels 48 hours post-knockdown of TDP-43 was seen only when cells were transfected with siRES pC32-FLAG-TDP-43 WT and not when either pcDNA 3.1 or siRES pC32-FLAG-TDP-43 ΔNLS plasmids were transfected (Figure 4(C)), indicating that TDP-43 nuclear localization is necessary for the expression of miR-27b-3p and miR-181c-5p.

Knockdown of TDP-43 or cellular stress affect primary miRNA processing

Based on the reduction of mature miRNA levels of miR-27b-3p and miR-181c-5p observed following knockdown of TDP-43, we wanted to determine at what level in the biogenesis pathway of these two miRNAs that TDP-43 was acting. After total RNA extraction, we designed primers

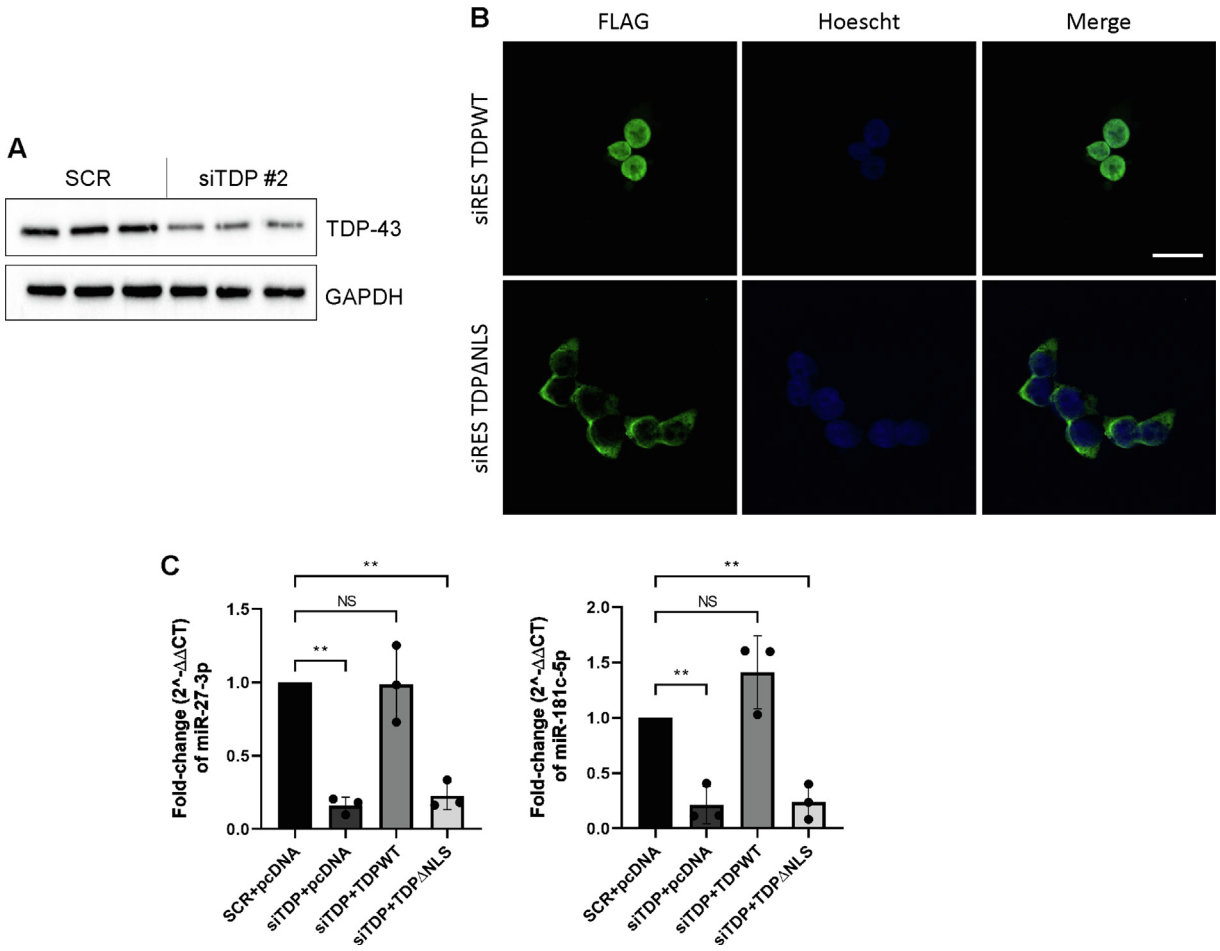

Figure 4. Nuclear localization signal of TDP-43 is required for the regulation of miR-27b-3p and miR-181c-5p expression. (A) Western blot showing knockdown of TDP-43 following transfection of siRNA (siTDP #2). (B) Immunofluorescence showing localization of either pC32-FLAG-TDP-43 wild-type (TDP WT) or pC32-FLAG-TDP-43 ΔNLS (TDP ΔNLS in which the nuclear localization signal was deleted) siRES plasmids. Scale bar represents 25 μm. (C) HEK293T cells were transfected with either SCR (control) or siTDP #2, and then transfected again 24 hours later with either pcDNA (control), TDP WT or TDP ΔNLS plasmids. MiR-27b-3p and miR-181c-5p levels were measured 24 hours after transfection of plasmids ($n = 3$). Expression levels were normalized to an exogenous control (cel-miR-39b-5p) prior to comparison. Bar represents mean fold-change (2^-ΔΔCT) ± SD. SCR + pcDNA samples were negative controls in which the bar representing their expression levels was set at one for comparison purposes. Significance was determined using (A) Student's t-test, or (D) a one-way ANOVA followed by a Tukey's post-hoc for multiple comparisons ($^{NS}p > 0.05$, $^{*}p < 0.05^{**}p < 0.01$).

that would capture both primary and precursor miRNA molecules (Figure S4(A)). Amplification of primary (pri-miRNA) and precursor miRNAs (pre-miRNA) together for both miR-27b and miR-181c showed no change in expression following knockdown of TDP-43 (Figure S4(B)–(C)). In order to examine primary miRNA structures separately from precursor molecules, we designed primers outside the region of the precursor sequence (Figure 5(A)). When examining pri-miRNAs separately, we observed that there was a significant increase in the amount of pri-miR-181c, while there was no change in pri-miR-27b levels (Figure 5(B) and (C)) showing that reduced TDP-43 affects the levels of pri-miR-181c.

We next examined whether cellular stress would have a similar effect on the primary and precursor miRNAs as observed with knockdown of TDP-43. We observed a significant increase in pri/pre-miR-27b and pri/pre-miR-181c after stress (Figure S4 (D) and (E)). When looking at the primary miRNA molecules specifically, there was a significant increase in pri-miR-27b and pri-miR-181c following a cellular stress (Figure 5(D)–(E)). Taken together, the data suggests that reduced levels of mature miRNA levels either by knockdown of TDP-43 or cellular stress is not due to reduced primary miRNA levels, but rather, reduced processing of these miRNAs. Given this, we next further investigated precursor structures of miR-27b and miR-181c.

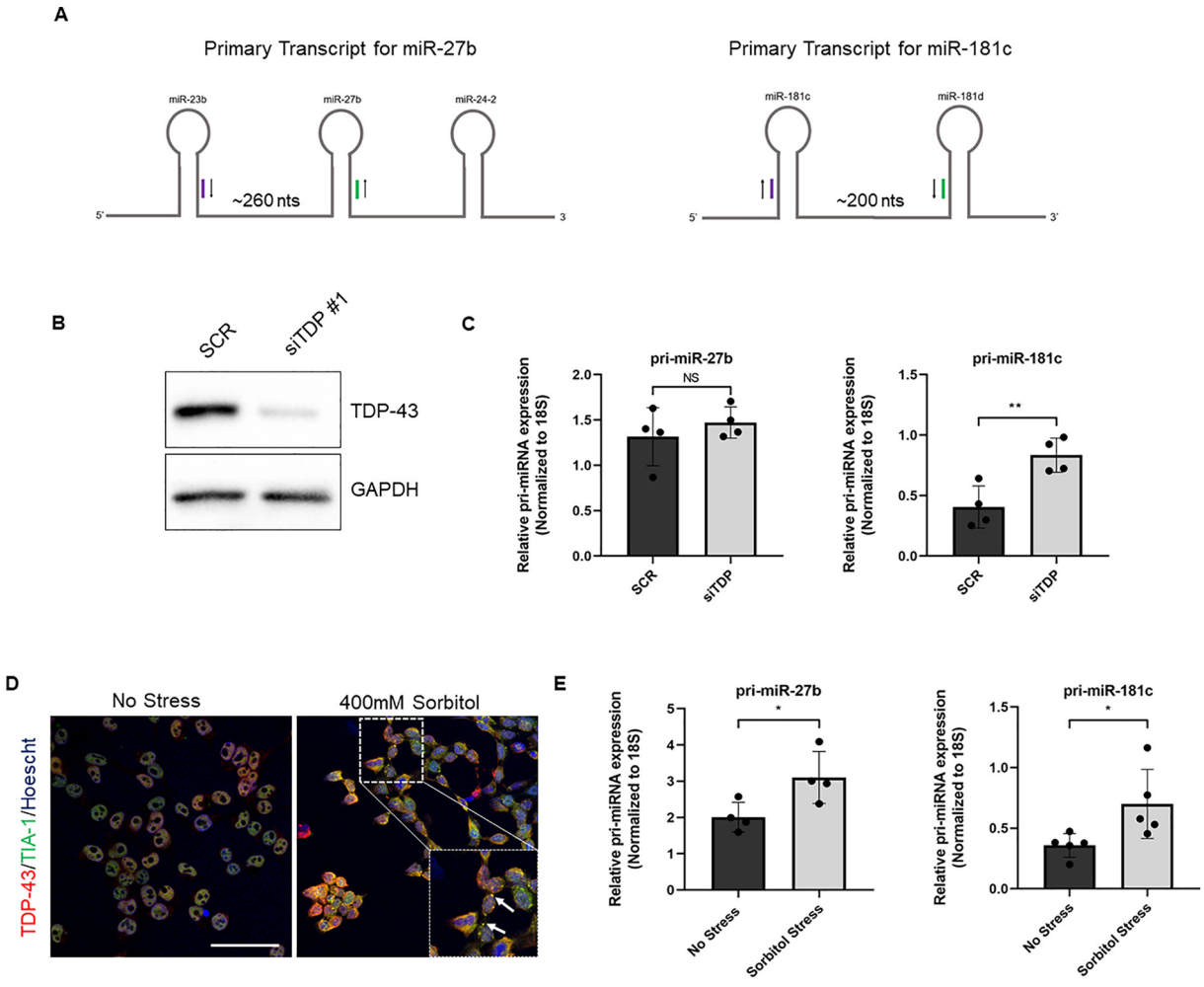

Figure 5. Knockdown of TDP-43 increases levels of pri-miR-181c, while cellular stress increases levels of both pri-miR-27b and pri-miR-181c. (A) Representative imagine of primary transcripts showing location of forward (purple line) and reverse primers (green line). Primers were designed only to capture pri-miRNA structure. HEK293T cells were either (B) transfected with siTDP #1, or (D) exposed to an osmotic stress (Scale bar represents 50 μm). RT-qPCR of pri-miR-27b (n = 4) and pri-miR-181c (n = 4/5) following (C) knockdown of TDP-43, or (E) an osmotic stress. Expression levels were measured using densitometry and were normalized to 18S rRNA prior to comparison. Bars represent mean ± SD. Significance was determined using a Student's t-test ($^{NS}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$).

Knockdown of TDP-43 and cellular stress affects precursor miRNA processing

Knockdown of TDP-43 showed no change in the overall levels of pre-miR-27b or pre-miR-181c (Figure 6(A) and (B)), while cellular stress led to a significant increase in pre-miR-27b but had no effect on pre-miR-181c levels (Figure 6(C) and (D)). Since precursor miRNA molecules are present in the nucleus and cytoplasm, we wanted to determine the levels of pre-miR-27b and pre-miR-181c within both cellular compartments.

We successfully fractionated samples into nuclear and cytosolic fractions (Figure S5). Both knockdown of TDP-43 and an induced cellular stress caused a significant reduction in cytosolic pre-miR-181c, but no significant change in its nuclear levels, whereas there was no significant change in nuclear or cytosolic levels of pre-miR-27b in both conditions (Figure 7). This suggests that the knockdown of TDP-43 and cellular stress can affect the levels of certain pre-miRNA molecules within the cytosolic compartment, specifically pre-miR-181c, while having no effect on other pre-miRNA molecules, such as pre-miR-27b.

Despite showing that knocking down TDP-43 causes deficits at several levels of miRNA processing, there was no effect on the expression of the major proteins involved in the miRNA biogenesis pathway at the time we see changes to miRNA levels (Figure S6). This suggests in this time frame that TDP-43 plays a critical role in the function of proteins within the miRNA biogenesis pathway when processing miR-27b-3p and miR-181c-5p.

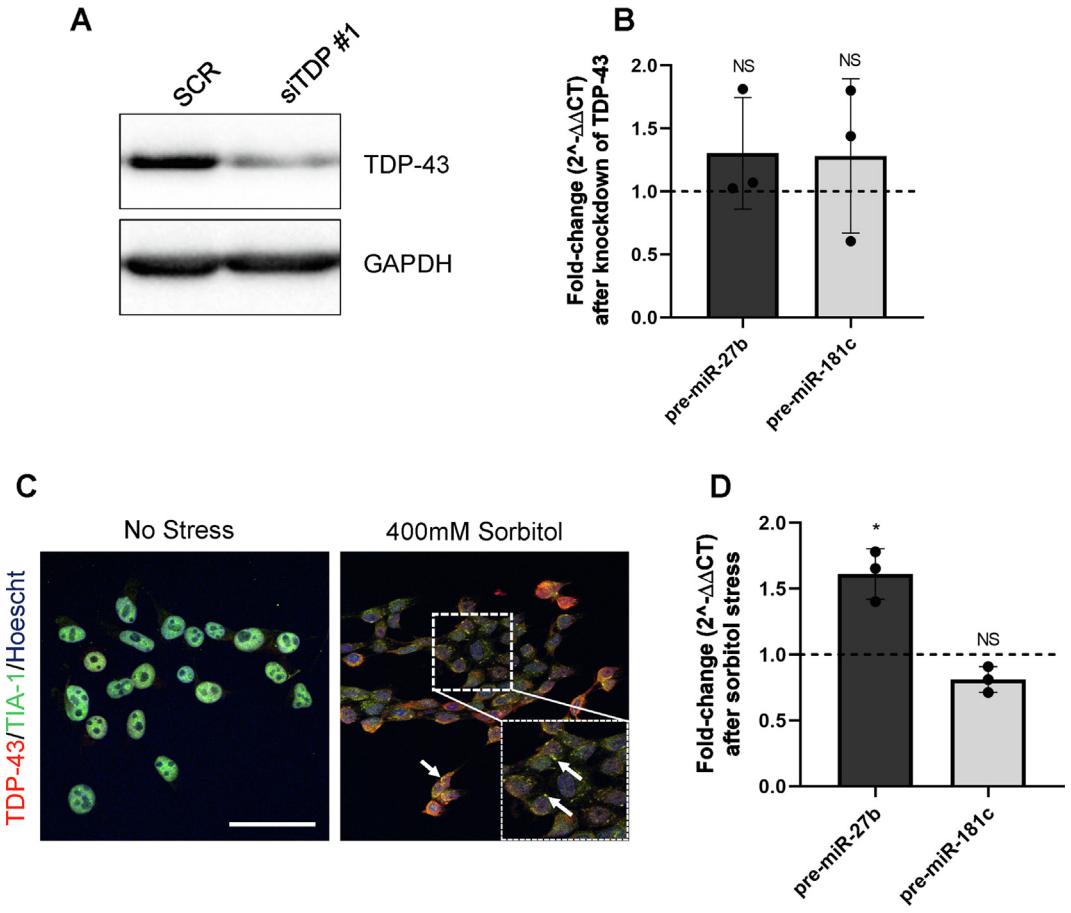

Figure 6. Knockdown of TDP-43 has no effect on overall pre-miR-27b or pre-miR-181c levels, while cellular stress increases levels of pre-miR-27b. HEK293T cells were either (A) transfected with siTDP #1, or (C) exposed to an osmotic stress (Scale bar represents 50 μm). Real time PCR of pre-miR-27b ($n = 3$) and pre-miR-181c ($n = 3$) levels following (B) knockdown of TDP-43, or (D) an osmotic stress. Expression levels were normalized to an exogenous control (cel-miR-39b-5p) prior to comparison. Bar represents mean fold-change (2^-ΔΔCT) ± SD. Significance was determined using a Student's t-test ($^{NS}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$).

Discussion

We have provided evidence of a negative feedback regulatory loop in which TDP-43, miR-27b-3p and miR-181c-5p interact in a manner that is dependent on the nuclear localization of TDP-43. We show that TDP-43 regulates these two miRNAs differently within the miRNA biogenesis pathway, and that cellular stress which reduces TDP-43 nuclear localization had similar, but not the exact same effects on the processing of these two miRNAs as when levels of TDP-43 are reduced via an siRNA.

In our data, we showed that inhibition of miR-27b-3p did not result in the upregulation of endogenous TDP-43 expression, despite the fact that we showed that overexpression of miR-27b-3p can suppress endogenous TDP-43 expression and that miR-27b-3p can bind to the 3′UTR of TDP-43 to regulate this suppression. Since miR-27a-3p has the same MRE as miR-27b-3p within the *TARDBP* 3′UTR, we decided to test if these two miRNAs were working together to suppress TDP-43 expression, and thus, inhibition of one miRNA would not be enough to see an effect on TDP-43 levels. We showed that in fact you need both inhibition of miR-27a-3p and miR-27b-3p to effectively de-repress TDP-43. This matches previous research as miR-27a-3p and miR-27b-3p have been shown to work together to suppress the expression of several genes including, DPD, TFPIα and PINK1.[20–22] Therefore, inhibition of miR-27b-3p alone is not be enough to de-repress TDP-43 expression as miR-27a-3p is able to complement its function.

TDP-43 has previously been shown to be involved in pri-miRNA and pre-miRNA processing within the DROSHA and DICER complex, respectively.[18] Interestingly, TDP-43 regulates miRNAs in different ways, including selectively promoting the processing of certain primary miRNAs, or promoting or preventing the processing of

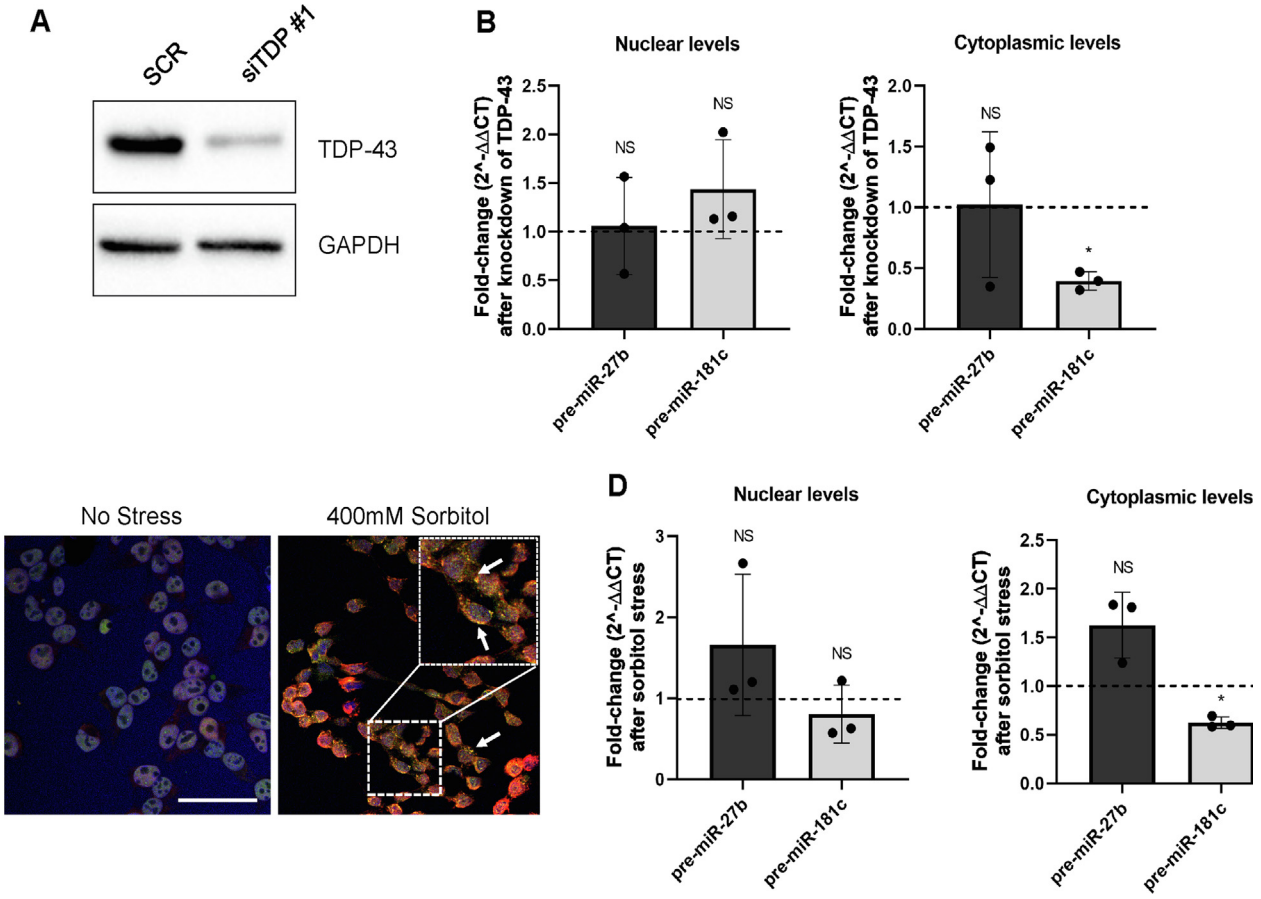

Figure 7. Nuclear/cytosolic fractionation indicated reduced cytoplasmic levels of pre-miR-181c following knockdown of TDP-43 or cellular stress. HEK293T cells were either (A) transfected with siTDP #1, or (C) exposed to an osmotic stress (Scale bar represents 50 μm). Real-time PCR of pre-miR-27b ($n = 3$) or pre-miR-181c ($n = 3$) levels within either the nucleus or cytoplasm of HEK293T cells following (B) knockdown of TDP-43, or (D) osmotic stress. Expression levels were normalized to an exogenous control (cel-miR-39b-5p) prior to comparison. Bar represents mean fold-change (2^-ΔΔCT) ± SD. Significance was determined using a Student's t-test ($^{NS}p > 0.05$, $^{*}p < 0.05$).

certain precursor miRNAs.[18,24] However, the molecular reason for this selectivity is still unclear. In line with previous work, our data showed this diverse role of TDP-43 within the miRNA biogenesis pathway as knockdown of TDP-43 led to reduced levels of cytoplasmic pre-miR-181c and mature miR-181c-5p levels, but increased pri-miR-181c levels. Low levels of TDP-43 function likely reduces DROSHA processing as has been previously shown and could explain the accumulation of pri-miR-181c.[18] The reduction of cytoplasmic pre-miR-181c means that reduced TDP-43 levels either impedes pre-miR-181c transport or promotes the degradation of this pre-miRNA molecule in the cytoplasm. Regardless, it is clear that TDP-43 promotes the production of miR-181c-5p at several levels within the miRNA biogenesis pathway. Further, knockdown of TDP-43 only caused a reduction of mature miR-27b-3p with no effect on the pri- and pre-miRNAs, indicating that TDP-43 likely promotes DICER processing of pre-miR-27b.

Since TDP-43 interacts with DROSHA and DICER in both an RNA-dependent and independent manner,[18] it is difficult to know whether TDP-43 needs to bind directly to either pri- and/or pre-miRNAs, DROSHA and/or DICER complexes, or both to assist in the processing of these two miRNAs. Further, in the presented data, it cannot be concluded that TDP-43 is directly involved in the biogenesis of these two miRNAs which could be why TDP-43 affects cytoplasmic DICER processing of pre-miR-27b even though the regulation of miR-27b-3p levels is dependent on TDP-43 nuclear localization. Of note, TDP-43 has been shown via CLIP-Seq data to bind to pre-miR-181c in human brain samples, but not pre-miR-27b.[25] This could suggest that TDP-43 has more of an indirect effect on miR-27b-3p levels, while having more of a direct effect on the expression of pre-miR-181c. This may explain why TDP-43 affects these two miRNAs differently in their biogenesis. However, this needs further investigation.

While TDP-43 has been shown to regulate several different miRNAs in multiple cell lines, this is the first study, to our knowledge, that shows TDP-43 in a negative feedback network with miRNAs. Similar networks have been identified between other RNA-binding proteins and miRNAs, such as FUS and miR-141/200a, and hnRNPA1 and miR-18a.[26–28] These negative feedback networks are likely critical biological mechanisms that regulate homeostatic levels of RNA-binding proteins and miRNAs. The observation that ALS-associated mutations in the *FUS* 3′UTR can disrupt the negative feedback loop between miR-141/200a and FUS, highlights the potential importance of these networks in motor neurons.[28] While there is currently no genetic connection with miRNAs and TDP-43 MREs, as there is with *FUS*, genetic studies examining non-coding regions in ALS patient samples is still very limited and further research in needed.

Our data suggests that the regulation of miR-27b-3p and miR-181c-5p, and hence the negative feedback network, is dependent on the nuclear localization of TDP-43. This observation has relevance for several neurodegenerative diseases in which TDP-43 forms neuronal cytoplasmic inclusions, including ALS, Frontotemporal Dementia and Alzheimer's disease.[29–31] Both miR-27b-3p and miR-181c-5p have been shown to be reduced in the spinal cord of patients with ALS.[3] We show here that these miRNAs are expressed in motor neurons, providing a correlation between TDP-43 cytoplasmic re-localization and the dysregulation of these two miRNAs in ALS. MiR-181c-5p has also been shown to be reduced in the anterior temporal cortex and parietal lobe cortex of patients with Alzheimer's disease, and in cultures of primary hippocampal neurons from mouse Alzheimer models, indicating that miR-181c-5p may be critical for overall central nervous system and neuronal function.[32,33] It has been shown that TDP-43 can regulate the mature levels of miR-27b-3p and miR-181c-5p, and other members of the miR-181 family (miR-181a-d) in several cell lines.[18,34,35] Thus, TDP-43 regulation on the biogenesis of miR-27b-3p and the miR-181a-d family likely represents a broad phenomenon rather than a cell-specific one.

Further, we showed an induced cellular stress which causes reduced TDP-43 nuclear localization alters miRNA processing leading to a reduction in miR-27b-3p and miR-181c-5p, similar to what was seen after knockdown of TDP-43. This is a critical finding as cellular stress has been shown to drive TDP-43 from the nucleus to the cytoplasm in both *in vitro* and *in vivo* neuronal models,[6–8] indicating that cellular stress, while not the only effect, results in the reduction of nuclear TDP-43 levels to regulate miRNA biogenesis. The differences observed between the results for the cellular stress and siRNA experiments is likely due to the fact that stress is not a targeted approach and that there are other *cis*- or *trans*-acting factors besides TDP-43 that regulate the biogenesis of these miRNAs that are also affected by cellular stress. However, these experiments provide evidence that stress may be a major component to the pathogenesis of ALS where an extensive reduction in miRNA levels in spinal cord and motor neurons is observed.[3,16]

While we identified a regulatory network that could have major significance to ALS, we also recognize that this study has limitations. For example, studying molecular pathways in HEK293T cells may not be fully representative of the molecular pathways observed in motor neurons. Therefore, this study offers a foundation of understanding miRNA regulatory networks and how they may be disrupted in ALS. Future studies should extend these observations into both *in vitro* and *in vivo* models of motor neuron dysfunction to fully understand how this pathway may contribute to ALS pathogenesis.

In conclusion, we propose a biological mechanism by which under normal conditions TDP-43 is in a negative feedback network with miRNAs (i.e. miR-27b-3p and miR-181c-5p), and that this is perturbed during cell stress due to reduced TDP-43 nuclear localization. Thus, reduction of nuclear TDP-43 reduces miRNAs levels resulting in a de-repression of the *TARDBP* transcript, and ultimately, increased TDP-43 levels. This biological mechanism could have important implications for the pathogenesis of neurodegenerative diseases, such as ALS, where the reduction of miRNA levels coincides with the upregulation and mis-localization of TDP-43.[3,13,14,16,31] Further, enhancement of miRNA processing at the level of DICER has been shown to improve motor function of rodent models that carry ALS-related mutations in TDP-43.[16] Since we and others have shown that TDP-43 affects miRNA processing at multiple levels within the miRNA biogenesis pathway,[18] an enhancement of both the DROSHA and DICER complexes may be necessary to have the greatest impact to slow ALS disease progression.

Methods and Materials

Plasmid constructs

SiRNA resistant plasmids (siRES) were either pC32-FLAG-TDP-43 wild-type or pC32-FLAG-TDP-43 ΔNLS and were resistant to an siRNA that targets endogenous *TARDBP* (siTDP #2).[23]

TARDBP 3′UTR that is expressed in human spinal cord was linked to the firefly luciferase gene within the pmirGLO plasmid (Promega Cat. # E1330) as described previously.[17] Mutations were put into the +2 and +3 position of the miRNA recognition element (MRE) of either miR-27b-3p or

miR-181c-5p of the *TARDBP* 3′UTR using the QuikChange Site-Directed Mutagenesis Kit (Agilent Cat. # 210219). Cloned sequences and mutants were confirmed using Sanger sequencing.

Cell culture and transfection

HEK293T cells were maintained in Dulbecco's Modified Eagles Media (DMEM; Gibco Cat. # 11965084) which contained 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Sorbitol was added to cell media at a concentration of 400 mM to induce an osmotic stress. Media with sorbitol was then added to HEK293T cells with an 80% confluency. The osmotic stress was maintained for 5 hours prior to experimentation.

HEK293T cells were seeded into a 6-well plate (150,000 cells/well) and incubated for 48 hours. Cells were then either transfected with 100 nM of SCR (Sigma-Aldrich Cat. # SIC001) or a custom siRNA targeting endogenous *TARDBP* (siTDP #2; Dharmacon) but not siRES plasmids as previously described.[23] 24 hours post-transfection of siTDP #2, cells were transfected again with 2.5 μg of either pcDNA 3.1 (negative control), pC32-FLAG-TDP-43 wild-type or pC32-FLAG-TDP-43 ΔNLS siRES plasmids. Mature miRNA levels were examined 24 hours post-transfection of plasmids. Transfections were done using the Lipofectamine 2000 Reagent (Invitrogen Cat. # 11668019) protocol following the manufacturer's instructions.

HEK293T cells were seeded into a 6-well plate (150,000 cells/well) and incubated for 48 hours. Cells were either transfected with a SCR (Sigma-Aldrich Cat. # SIC001), miR-27b-3p, or miR-181c-5p human *mir*Vana miRNA mimic (chemically modified double stranded RNA) or miR-27a-3p, miR-27b-3p, or miR-181c-5p inhibitor (chemically modified single-stranded RNA; Invitrogen, Cat. # 4464066 and Cat. #4464084, respectively) to measure changes to endogenous TDP-43 protein or *TARDBP* mRNA levels.

Microarray analysis

HEK293T cells were seeded into a 6-well plate (150,000 cells/well) and incubated for 48 hours. Cells were either transfected with an 100 nM of siRNA SMARTpool targeting *TARDBP* (siTDP #1; Dharmacon Cat. # L-012394-00-0005), or a scramble negative control (SCR; Sigma-Aldrich Cat. # SIC001) at a 60% confluency using the Lipofectamine 2000 Reagent (Invitrogen Cat. # 11668019) protocol. Total RNA extraction was then performed 48 hours post-transfection using the RNeasy Mini Kit (Qiagen Cat. # 74106). RNA samples were tested for integrity using bioanalyzer analysis. Total RNA extracts were sent to the Center of Applied Genomics – TCAG Facilities at the University of Toronto for microarray analysis using the GeneChip miRNA 4.0 Array (Applied Biosystems Cat. # 902412). Quality control and experimental data from the microarray was analyzed using the Transcriptome Analysis Console Software (ThermoFisher Scientific).

Real-time PCR

Samples from HEK293T cells went under a small RNA extraction using the mirVana miRNA Isolation Kit (Invitrogen Cat. # AM1560) to obtain RNA molecules < 200nts which allowed us to separate pri-miRNAs (>200nts) from pre-miRNAs (<200nts). Yield and purity of small RNAs was determined using spectrophotometry. When measuring mature miRNA levels, small RNA extracts went under cDNA synthesis using the TaqMan Advanced cDNA Synthesis Kit (Applied Biosystems Cat. # A28007), but when measuring the levels of pre-miRNAs, small RNA extracts went under cDNA synthesis using the SuperScript IV VILO Master Mix with DNease Enzyme (Invitrogen Cat. # 11766050) protocol. All cDNA synthesis protocols were done according to the manufacturers' instructions. Real-time PCR for mature miRNAs and pre-miRNAs was done either using the TaqMan Advanced miRNA Assay (Applied Biosystems Cat. # A25576) or the TaqMan Pre-miRNA Assay (Applied Biosystems Cat. # 4331182), respectively, and the Fast TaqMan Advanced Master Mix (Applied Biosystems Cat. # 4444965) in accordance to the manufactures protocol. Since the microarray was normalized to an exogenous control, confirmation of changes to miRNA levels was done using an endogenous control miRNA (miR-1296-5p) which showed no change in expression following TDP-43 knockdown (Figure S7). Candidate miRNA expression for the rest of the experiments was normalized to an RNA spike-in (cel-miR-39b-5p). All real-time PCR data was quantified according to the 2^-ΔΔCT method.

Reverse Transcriptase relative quantitative PCR (RT-qPCR)

HEK293T cells were either transfected with a SCR (Sigma-Aldrich Cat. # SIC001), miR-27b-3p, or miR-181c-5p human *mir*Vana miRNA mimic (chemically modified double stranded RNA) or inhibitor (chemically modified single-stranded RNA; Invitrogen, Cat. # 4464066 and Cat. #4464084, respectively) to measure changes to endogenous *TARDBP* mRNA levels, or cells were transfected with either SCR (Sigma-Aldrich Cat. # SIC001) or siTDP #1 (Dharmacon Cat. # L-012394-00-0005) to measure changes in pre-and pri-miRNA together levels, or pri-miRNA levels alone using the Lipofectamine 2000 Reagent (Invitrogen Cat. # 11668019) protocol. Total RNA extraction using the RNeasy Mini Kit (Qiagen Cat. # 74106) was performed 48 hours post-transfection. Yield and purity of small RNAs was

determined using spectrophotometry. RNA extracts went under cDNA synthesis using the SuperScript II Reverse Transcriptase (Invitrogen Cat. # 18064014) in accordance with the manufactures instructions which was followed by quantitative PCR using the Platinum Taq DNA Polymerase (Invitrogen Cat. # 10966018) protocol for amplification via primers outlined in Table S1. Expression levels were normalized to 18S rRNA levels prior to comparison.

Luciferase assay

HEK293T cells were co-transfected with either SCR (Sigma-Aldrich Cat. # SIC001), or human *mir*Vana miRNA mimics (miR-27b-3p or miR-181c-5p; Invitrogen Cat. # 4464066) and either an empty pmirGLO plasmid or a pmirGLO plasmid containing the 3′UTR of *TARDBP* using the Lipofectamine 2000 Reagent (Invitrogen Cat. # 11668019) protocol. Luciferase activity was measured 24 hours post-transfection using the Dual-GLO Luciferase Assay System (Promega Cat. # E2920). Firefly luciferase levels were normalized to renilla luciferase levels, and data was further normalized to account for the effect the miRNAs have on the plasmid itself prior to comparison as previously described.[36]

Western blot

All protein extractions were done from HEK293T cells using the NP40 lysis buffer with proteinase inhibitors followed by sonication. Protein lysates were resuspended in loading buffer containing 5% β-mercaptoethanol and denatured at 90 °C for 5 min. Samples ran on an 10% SDS-page gel and then were transferred onto a nitrocellulose membrane. Blots were probed with either rabbit anti-TDP-43 (1:5000; Proteintech Cat. # 10782-2-AP), mouse anti-FLAG (1:2500; Cedarlane Cat. # CLANT146-2), rabbit anti-DROSHA (1:1000; abcam Cat. # ab12286), rabbit anti-DCGR8 (1:1000; abcam Cat. # ab191875), mouse anti-XPO5 (1:1000; abcam Cat. # ab57491), mouse anti-DICER (1:1000; abcam Cat. # ab14601), mouse anti-AGO2 (1:1000; abcam Cat. # ab57113), rabbit anti-TRBP (1:1000; Proteintech Cat. # 15753-1-AP), or rabbit anti-GAPDH (1:5000; abcam Cat. # ab9485) and later with an HRP secondary antibody (goat anti-mouse 1:3000; Biorad Cat. # STAR207P, or goat anti-rabbit 1:5000; Invitrogen Cat. # 65-6120). Data was quantified using densitometry measured by ImageJ software. Relative protein levels were normalized to GAPDH expression prior to comparison.

Fluorescent in situ hybridization (FISH)

Neuropathologically intact human spinal cord tissue was used to examine whether candidate miRNAs were expressed in human spinal motor neurons. Tissue was cut into 7 μm sections. FISH was done as previously described.[37] MiRCURY LNA miRNA Detection Probes that targeted miRNA candidates contained 5′ and 3′ DIG labels (Qiagen Cat. # 339111), which were further targeted by an HRP secondary antibody (Sigma-Aldrich Ca. # 11633716001) and a Tyramide Signal Amplification tagged with a Cy3 fluorophore (PerkinElmer Cat. # NEL744001KT). Spinal cord samples were examined for positive expression of candidate miRNAs using the Leica TSC SP8 confocal microscope.

Immunocytochemistry

Cells were fixed using 4% paraformaldehyde (PFA) and blocked with 8% Bovine Serum Albumin (BSA). Proteins of interest were targeted by either rabbit anti-TDP-43 (1:250; Proteintech Cat. # 10782-2-AP), goat anti-TIA-1 (1:100; Santa Cruz Cat. # sc-166247), or rabbit anti-FLAG (1:100; abcam Cat. # ab49763) primary antibodies, which were targeted by Alexa 488 goat anti-rabbit, Alexa 555 donkey anti-goat, and Alexa 488 goat anti-mouse, respectively, secondary antibodies (1:200; Life Technologies Cat. # A32721, A21432, and A32721, respectively). Samples were imaged using the Leica TSC SP8 confocal microscope.

Fractionation

Nuclear and cytosolic protein and RNA fractionations was performed using the PARIS kit (Invitrogen Cat. # AM1921) in accordance to the manufacturer's instructions. 100 U/ml of SUPERase IN RNase Inhibitor (Invitrogen Cat. # AM2694) was added to the cell fractionation buffer and cell disruption buffer within the PARIS kit to prevent RNA degradation.

Statistics

Significance was determined either using a Students t-test when comparing two conditions, or One-Way ANOVA followed by a Tukey's post-hoc when comparing multiple conditions. Data was considered significant if $p < 0.05$.

Author contributions

ZCEH designed and performed the experiments and data analysis. DCM assisted with the experimental design and interpretation of the data. MJS supervised the project with input from DCM. ZCEH, DCM, and MJS all contributed to the writing of the manuscript. All authors have read and approved the final manuscript.

Disclosure statement

The authors report no conflict of interest.

Data availability

Datasets used in this study are available from the corresponding author upon reasonable request.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2020.10.029.

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References

1. Ratti, A., Buratti, E., (2016). Physiological functions and pathobiology of TDP-43 and FUS/TLS proteins. *J Neurochem.*, **138** (Suppl 1), 95–111.
2. Weskamp, K., Barmada, S.J., (2018). TDP43 and RNA instability in amyotrophic lateral sclerosis. *Brain Res.*, **1693**, 67–74.
3. Campos-Melo, D., Droppelmann, C.A., He, Z., Volkening, K., Strong, M.J., (2013). Altered microRNA expression profile in Amyotrophic Lateral Sclerosis: a role in the regulation of NFL mRNA levels. *Mol. Brain.*, **6**, 26.
4. Fratta, P., Sivakumar, P., Humphrey, J., Lo, K., Ricketts, T., Oliveira, H., et al., (2018). Mice with endogenous TDP-43 mutations exhibit gain of splicing function and characteristics of amyotrophic lateral sclerosis. *EMBO J.*, **37**
5. Ling, S.C., Polymenidou, M., Cleveland, D.W., (2013). Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron*, **79**, 416–438.
6. Khalfallah, Y., Kuta, R., Grasmuck, C., Prat, A., Durham, H. D., Vande, V.C., (2018). TDP-43 regulation of stress granule dynamics in neurodegenerative disease-relevant cell types. *Sci. Rep.*, **8**, 7551.
7. McDonald, K.K., Aulas, A., Destroismaisons, L., Pickles, S., Beleac, E., Camu, W., et al., (2011). TAR DNA-binding protein 43 (TDP-43) regulates stress granule dynamics via differential regulation of G3BP and TIA-1. *Hum. Mol. Genet.*, **20**, 1400–1410.
8. Moisse, K., Mepham, J., Volkening, K., Welch, I., Hill, T., Strong, M.J., (2009). Cytosolic TDP-43 expression following axotomy is associated with caspase 3 activation in NFL-/- mice: support for a role for TDP-43 in the physiological response to neuronal injury. *Brain Res.*, **1296**, 176–186.
9. Murakami, T., Qamar, S., Lin, J.Q., Schierle, G.S., Rees, E., Miyashita, A., et al., (2015). ALS/FTD mutation-induced phase transition of FUS liquid droplets and reversible hydrogels into irreversible hydrogels impairs RNP Granule function. *Neuron*, **88**, 678–690.
10. Molliex, A., Temirov, J., Lee, J., Coughlin, M., Kanagaraj, A.P., Kim, H.J., et al., (2015). Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell*, **163**, 123–133.
11. Volkening, K., Leystra-Lantz, C., Yang, W., Jaffee, H., Strong, M.J., (2009). Tar DNA binding protein of 43 kDa (TDP-43), 14-3-3 proteins and copper/zinc superoxide dismutase (SOD1) interact to modulate NFL mRNA stability. Implications for altered RNA processing in amyotrophic lateral sclerosis (ALS). *Brain Res.*, **1305**, 168–182.
12. Liu-Yesucevitz, L., Bilgutay, A., Zhang, Y.J., Vanderweyde, T., Citro, A., Mehta, T., et al., (2010). Tar DNA binding protein-43 (TDP-43) associates with stress granules: analysis of cultured cells and pathological brain tissue. *PLoS One*, **5**, e13250
13. Koyama, A., Sugai, A., Kato, T., Ishihara, T., Shiga, A., Toyoshima, Y., et al., (2016). Increased cytoplasmic TARDBP mRNA in affected spinal motor neurons in ALS caused by abnormal autoregulation of TDP-43. *Nucleic Acids Res.*, **44**, 5820–5836.
14. Swarup, V., Phaneuf, D., Dupre, N., Petri, S., Strong, M., Kriz, J., et al., (2011). Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor kappaB-mediated pathogenic pathways. *J. Exp. Med.*, **208**, 2429–2447.
15. Bartel, D.P., (2018). Metazoan MicroRNAs. *Cell*, **173**, 20–51.
16. Emde, A., Eitan, C., Liou, L.L., Libby, R.T., Rivkin, N., Magen, I., et al., (2015). Dysregulated miRNA biogenesis downstream of cellular stress and ALS-causing mutations: a new mechanism for ALS. *EMBO J.*, **34**, 2633–2651.
17. Hawley, Z.C.E., Campos-Melo, D., Strong, M.J., (2017). Novel miR-b2122 regulates several ALS-related RNA-binding proteins. *Mol. Brain*, **10**, 46.
18. Kawahara, Y., Mieda-Sato, A., (2012). TDP-43 promotes microRNA biogenesis as a component of the Drosha and Dicer complexes. *Proc. Natl. Acad. Sci. USA*, **109**, 3347–3352.
19. Agarwal, V., Bell, G.W., Nam, J.W., Bartel, D.P., (2015). Predicting effective microRNA target sites in mammalian mRNAs. *Elife*, **4**

20. Arroyo, A.B., Salloum-Asfar, S., Perez-Sanchez, C., Teruel-Montoya, R., Navarro, S., Garcia-Barbera, N., et al., (2017). Regulation of TFPIalpha expression by miR-27a/b-3p in human endothelial cells under normal conditions and in response to androgens. *Sci. Rep.*, **7**, 43500.
21. Kim, J., Fiesel, F.C., Belmonte, K.C., Hudec, R., Wang, W. X., Kim, C., et al., (2016). miR-27a and miR-27b regulate autophagic clearance of damaged mitochondria by targeting PTEN-induced putative kinase 1 (PINK1). *Mol. Neurodegener.*, **11**, 55.
22. Offer, S.M., Butterfield, G.L., Jerde, C.R., Fossum, C.C., Wegner, N.J., Diasio, R.B., (2014). microRNAs miR-27a and miR-27b directly regulate liver dihydropyrimidine dehydrogenase expression through two conserved binding sites. *Mol. Cancer Ther.*, **13**, 742–751.
23. Deshaies, J.E., Shkreta, L., Moszczynski, A.J., Sidibe, H., Semmler, S., Fouillen, A., et al., (2018). TDP-43 regulates the alternative splicing of hnRNP A1 to yield an aggregation-prone variant in amyotrophic lateral sclerosis. *Brain*, **141**, 1320–1333.
24. King, I.N., Yartseva, V., Salas, D., Kumar, A., Heidersbach, A., Ando, D.M., et al., (2014). The RNA-binding protein TDP-43 selectively disrupts microRNA-1/206 incorporation into the RNA-induced silencing complex. *J. Biol. Chem.*, **289**, 14263–14271.
25. Fan, Z., Chen, X., Chen, R., (2014). Transcriptome-wide analysis of TDP-43 binding small RNAs identifies miR-NID1 (miR-8485), a novel miRNA that represses NRXN1 expression. *Genomics*, **103**, 76–82.
26. Fujiya, M., Konishi, H., Mohamed Kamel, M.K., Ueno, N., Inaba, Y., Moriichi, K., et al., (2014). microRNA-18a induces apoptosis in colon cancer cells via the autophagolysosomal degradation of oncogenic heterogeneous nuclear ribonucleoprotein A1. *Oncogene*, **33**, 4847–4856.
27. Guil, S., Caceres, J.F., (2007). The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. *Nature Struct. Mol. Biol.*, **14**, 591–596.
28. Dini Modigliani, S., Morlando, M., Errichelli, L., Sabatelli, M., Bozzoni, I., (2014). An ALS-associated mutation in the FUS 3'-UTR disrupts a microRNA-FUS regulatory circuitry. *Nature Commun.*, **5**, 4335.
29. Amador-Ortiz, C., Lin, W.L., Ahmed, Z., Personett, D., Davies, P., Duara, R., et al., (2007). TDP-43 immunoreactivity in hippocampal sclerosis and Alzheimer's disease. *Ann. Neurol.*, **61**, 435–445.
30. Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., et al., (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem. Biophys. Res. Commun.*, **351**, 602–611.
31. Tan, C.F., Eguchi, H., Tagawa, A., Onodera, O., Iwasaki, T., Tsujino, A., et al., (2007). TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. *Acta Neuropathol.*, **113**, 535–542.
32. Schonrock, N., Humphreys, D.T., Preiss, T., Gotz, J., (2012). Target gene repression mediated by miRNAs miR-181c and miR-9 both of which are down-regulated by amyloid-beta. *J. Mol. Neurosci.*, **46**, 324–335.
33. Schonrock, N., Ke, Y.D., Humphreys, D., Staufenbiel, M., Ittner, L.M., Preiss, T., et al., (2010). Neuronal microRNA deregulation in response to Alzheimer's disease amyloid-beta. *PLoS One*, **5**, e11070
34. Di Carlo, V., Grossi, E., Laneve, P., Morlando, M., Dini Modigliani, S., Ballarino, M., et al., (2013). TDP-43 regulates the microprocessor complex activity during in vitro neuronal differentiation. *Mol. Neurobiol.*, **48**, 952–963.
35. Chen, X., Fan, Z., McGee, W., Chen, M., Kong, R., Wen, P., et al., (2018). TDP-43 regulates cancer-associated microRNAs. *Protein Cell*, **9**, 848–866.
36. Campos-Melo, D., Droppelmann, C.A., Volkening, K., Strong, M.J., (2014). Comprehensive luciferase-based reporter gene assay reveals previously masked up-regulatory effects of miRNAs. *Int. J. Mol. Sci.*, **15**, 15592–15602.
37. Hawley, Z.C.E., Campos-Melo, D., Strong, M.J., (2019). MiR-105 and miR-9 regulate the mRNA stability of neuronal intermediate filaments. Implications for the pathogenesis of amyotrophic lateral sclerosis (ALS). *Brain Res.*, **1706**, 93–100.