HHS Public Access

Author manuscript

Neurobiol Dis. Author manuscript; available in PMC 2020 December 01.

Published in final edited form as:

Neurobiol Dis. 2019 December ; 132: 104514. doi:10.1016/j.nbd.2019.104514.

TDP-43 knockdown causes innate immune activation via protein kinase R in astrocytes

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Abstract

TAR-DNA binding protein 43 (TDP-43) is a multifunctional RNA binding protein directly implicated in the etiology of amyotrophic lateral sclerosis (ALS). Previous studies have demonstrated that loss of TDP-43 function leads to intracellular accumulation of non-coding repetitive element transcripts and double-stranded RNA (dsRNA). These events could cause immune activation and contribute to the neuroinflammation observed in ALS, but this possibility has not been investigated. Here, we knock down TDP-43 in primary rat astrocytes via siRNA, and we use RNA-seq, immunofluorescence, and immunoblotting to show that this results in: 1) accumulation of repetitive element transcripts and dsRNA; and 2) pro-inflammatory gene and protein expression consistent with innate immune signaling and astrocyte activation. We also show that both chemical inhibition and siRNA knockdown of PKR, a dsRNA-activated kinase implicated in the innate immune response, block the expression of all activation markers assayed. Based on these findings, we suggest that intracellular accumulation of endogenous dsRNA may be a novel and important mechanism underlying the pathogenesis of ALS (and perhaps other neurodegenerative diseases), and that PKR inhibitors may have the potential to prevent reactive astrocytosis in ALS.

Keywords

TDP-43; amyotrophic lateral sclerosis; reactive astrocytes; protein kinase R

INTRODUCTION

TAR-DNA binding protein 43 (TDP-43) is a multifunctional RNA/DNA binding protein directly implicated in amyotrophic lateral sclerosis (ALS) and other neurodegenerative

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

diseases (1,2). Specific mutations affecting TDP-43 function have been linked with familial ALS, and general TDP-43 pathology also appears in most cases of sporadic ALS and up to 50% of Alzheimer's disease patients (i.e., in the absence of mutations) (1, 3). In all cases, TDP-43 dysfunction is characterized by its nuclear depletion and cytoplasmic accumulation/deposition, and studies suggest that both loss and gain of TDP-43 function can contribute to this pathology (4, 5). TDP-43 modulates transcription, mRNA splicing and stability, micro-RNA biogenesis and gene expression (6), and disruption of any of these processes could play a role in pathogenesis. However, the specific events by which altered TDP-43 function leads to neuropathology remain unclear.

Although ALS is characterized by pathology and altered TDP-43 function in motor neurons, TDP-43 inclusions have also been reported in glial cells (7-9), and there is good evidence of a non-cell autonomous role for glia in the disease (10-12). For example, astrocytes from transgenic mouse models of ALS (including TDP-43 mutants) are toxic to motor neurons in culture (13). Similarly, astrocytes derived from ALS patient cells do not support motor neurons, and those from sporadic ALS cases are just as toxic as those with specific, ALS-linked mutations (14). These and other related studies are reviewed well elsewhere (15, 16). These observations might suggest a common, astrocyte-specific mechanism for ALS pathology, and there is good reason to investigate this (17). Astrocytes are highly abundant glial cells that provide metabolic/functional support for neurons and play a key role in central nervous system homeostasis (18). However, in response to injury and other adverse stimuli, astrocytes may assume a "reactive" state. Reactive astrocytes are marked by increased transcription of specific pro-inflammatory proteins (15, 19); they are generally neurotoxic, and have been implicated in numerous diseases characterized by neuroinflammation (18). Some evidence suggests that altered TDP-43 function may be associated with reactive astrocytosis (20, 21), but again, the underlying mechanisms are unknown.

One novel possibility is that altered TDP-43 function leads to impaired RNA homeostasis that somehow causes astrocyte activation. In support of this idea, we recently showed that tdp-1, the *C. elegans* ortholog of TDP-43, plays an important role in limiting the accumulation of cellular double-stranded RNA (dsRNA) (22). The underlying mechanism is unknown, but it could involve repetitive element (RE) transcripts, as these are over-represented in dsRNA from tdp-1 mutants. RE transcripts are normally suppressed RNAs derived from non-coding, repetitive DNA sequences such as long terminal repeats, long interspersed nuclear elements, and alu elements—all of which are predisposed to form dsRNA (23, 24). Previous reports indicate that altered TDP-43 function is associated with an increase in RE transcripts (21, 25), and we recently demonstrated that RE transcripts are increased in ALS patient brains (26). These findings could be disease relevant, because dsRNA is a potent activator of the innate immune response (and subsequent inflammatory signaling) (27, 28). Moreover, increased expression/activation of dsRNA-activated protein kinase (PKR) has been reported in the context of neurodegenerative diseases, including ALS (29-31). Still, the significance of these observations is unclear. Therefore, in the present study we tested the hypothesis that altered TDP-43 function leads to an accumulation of RE transcripts and dsRNA, resulting in a pro-inflammatory, reactive astrocyte phenotype. Our

results support this hypothesis, and we also show that inhibiting PKR can block this astrocyte activation.

RESULTS

TDP-43 knockdown activates astrocytes.

Almost all ALS cases display TDP-43 pathology and altered TDP-43 function (1). Therefore, to determine the effects of altered/dysfunctional TDP-43 in astrocytes, we knocked down TDP-43 in primary rat astrocytes using siRNA. We also performed additional knockdown experiments in the presence of ATP (i.e., a "second hit" which is often implicated in neuroinflammation) (32). In general, we achieved a knockdown efficiency of ~80% (Fig. 1A). Interestingly, we found that this reduction in TDP-43 was associated with phosphorylation (activation) of the major pro-inflammatory transcription factor nuclear factor kappa B (NF-kB, detected by western blotting for Ser536 phosphorylation), which appeared to be exacerbated by co-treatment with ATP (Fig. 1A). Therefore, we next performed RNA-seq (strand-specific Illumina sequencing, >40M single-end reads/sample) and differential expression analyses to characterize gene expression changes with TDP-43 knockdown, and with knockdown plus ATP (Fig. 1B). We found that TDP-43 knockdown alone differentially affected transcript levels of several thousand genes, and that most of these were further increased/decreased with the addition of ATP. In fact, TDP-43 knockdown plus ATP increased the abundance of 2451 transcripts and decreased 2805 transcripts vs. scrambled siRNA treatment (FDR <0.05, mapping performed via Tophat/Bowtie2 software, and differential expression analyses using Deseq2; detailed gene expression data in Supplementary Material). We then performed standard gene ontology analyses (33) and found that, in addition to numerous transcriptional modules related to cellular stimulus sensing, TDP-43 knockdown was associated with a major increase in transcription of pro-inflammatory genes, indicated by enrichment in several gene ontology terms related to immune/inflammatory activation and even innate immune signaling, especially with the addition of ATP (Supplementary Material). These findings are consistent with previous reports that TDP-43 dysfunction activates astrocytes and causes upregulation of pro-inflammatory proteins (20, 34). Moreover, significantly upregulated genes in our dataset included LCN2, FKBP5, GBP2 and CD44, all of which have been identified as reactive astrocyte markers (19, 35, 36), as well as CXCL1, which is associated with ALS in clinical samples (37). We also observed a major increase in transcripts for plasminogen activator inhibitor-1 (PAI-1, encoded by the SERPINE1 gene), which has been implicated in injury-related astrogliosis and neurodegenerative diseases, including ALS (38, 39). Genes downregulated in response to TDP-43 knockdown did not cluster with a clear pattern in gene ontology terms, but were generally associated with changes in cellular morphology. A number of these reflected reduced actin-related signaling, which has been reported as a feature of astrocyte activation (40). Indeed, we found that cells treated with TDP-43 siRNA typically contracted and assumed a more stellate morphology characteristic of activated astrocytes (Fig. 1C).

We confirmed changes in gene expression by performing RT-PCR and examining protein expression of key reactive astrocyte markers, including CD44, LCN2, FKBP5, and PAI-1

(Fig. 2A,B). Most of these increased 1-2 fold with TDP-43 knockdown, and in some cases this was associated with changes in subcellular localization (e.g., vesicle-like staining in the case of PAI-1, which could be associated with cellular packaging/secretion). We did not observe an obvious increase in cell death with TDP-43 knockdown, and the number of pyknotic nuclei (~4%) was similar in controls and treated cells. Taken together, these observations suggest that altered TDP-43 function in astrocytes leads to a reactive, pro-inflammatory state.

TDP-43 knockdown increases repetitive element transcripts.

Our previous work suggests that RE transcript levels are increased in the brains of ALS patients (26). Therefore, in addition to analyzing changes in gene expression, we examined the effects of TDP-43 knockdown in astrocytes on RE transcript levels in our RNA-seq data using the Repenrich algorithm (41). We found that TDP-43 knockdown alone caused a significant increase in many RE transcripts, and that this effect was exacerbated by the addition of ATP (Fig. 3A). This suggests that: 1) TDP-43 is necessary for RE homeostasis; and 2) RE transcription/accumulation may play a role in immune activation. No particular class or family of RE was significantly enriched compared to control siRNA-treated cells. However, we did find overlap between RE that increased with TDP-43 knockdown in astrocytes and those that are increased in ALS patient brains. In particular, a long terminal repeat known as MER21B was one of the most highly upregulated RE with TDP-43 knockdown in our experiments, and it is also increased in ALS patient brains (26). In our RNA-seq data, MER21B increased ~2-fold with TDP-43 knockdown, and ~3-fold with the addition of ATP (both compared to scrambled siRNA). We confirmed these observations using both RT-PCR as described above (data not shown) and single molecule RNA fluorescence *in-situ* hybridization (smRNA-FISH) with a probe specific for MER21B. Using this latter approach, we found that TDP-43 knockdown caused a distinct increase in perinuclear accumulation of this RE transcript (Fig. 3B). Although many other RE found in the human genome are absent in mice/rats, we did find additional RE that both: 1) followed the same pattern as MER21B in our RNA-seq data, and 2) are increased in ALS patient brains, as we previously reported (e.g., L1MA9). Taken together with our previous work, these findings may indicate that suppressing the accumulation of RE transcripts in glial cells such as astrocytes is a conserved and disease relevant function of TDP-43.

TDP-43 knockdown increases dsRNA.

One possible mechanism by which altered TDP-43 function could lead to astrocyte activation (and neuroinflammation) is via the formation of dsRNA. Indeed, transcripts with antisense overlap and those containing inverted repeats (e.g., RE transcripts) are predisposed to form inter- or intra-molecular dsRNA (24), and we have reported that TDP-43 knockdown increases both nuclear and cytoplasmic dsRNA in human cells (22). To determine if TDP-43 knockdown increases dsRNA in astrocytes, we first looked for evidence of RNA editing by A-to-I dsRNA-specific deaminases (ADARs) in our RNA-seq data using the SPRINT algorithm (42), which identifies clusters of ADAR editing sites. We found that astrocytes knocked down for TDP-43 had a ~2-fold increase in edited transcripts, which was accounted for by editing in both genes and RE (Fig. 4A). We did note a slight increase in ADAR1 transcript levels in our RNA-seq data. Therefore, to confirm that this increase in editing was

associated with increased dsRNA, we performed an immuno-dot blot using the dsRNA-specific antibody J2, which binds specifically to dsRNA duplexes (43); we found a similar ~2.5-fold increase in dsRNA staining using this approach (Fig. 4B). We also used the J2 antibody to perform RNA immunoprecipitation and, subsequently, RNA-seq to characterize the dsRNA induced by TDP-43 knockdown. As we have reported previously, the J2 antibody preferentially recovers double-stranded transcripts (22). Here, we found that J2-IP RNA from astrocytes treated with TDP-43 siRNA was not enriched for any particular set of genes, but did contain a significant number of RE transcripts. In fact, most RE showed increased dsRNA structure/stability (i.e., greater expression in J2-IP RNA-seq) in astrocytes treated with TDP-43 siRNA as compared to scrambled/control siRNA (Fig. 4C). The major classes of enriched RE transcripts we identified included DNA transposons, long interspersed nuclear elements, long terminal repeats, and short interspersed nuclear elements. Collectively, these data demonstrate that the accumulation of RE transcripts observed with altered TDP-43 function in this and other studies is associated with an increase in endogenous dsRNA.

PKR inhibition suppresses astrocyte activation in response to TDP-43 knockdown.

PKR (also known as eukaryotic translation initiation factor 2-alpha kinase 2, EIF2AK2) is an intracellular stress sensor that responds to a variety of stimuli, but it is most potently activated by dsRNA (44, 45). This leads to autophosphorylation and subsequent pro-inflammatory signaling via NF-kB, p38 and c-Jun N-terminal kinase (JNK) as well as modulation/suppression of translation—all part of the classic interferon response to viral infection (46). Therefore, we reasoned that inhibiting PKR might reduce the pro-inflammatory effects of TDP-43 knockdown in astrocytes. To test this possibility, we repeated the siRNA experiments described above and co-treated astrocytes with C16, a small molecule inhibitor of PKR (47). We then examined CD44, FKBP5 and LCN2 expression via immunofluorescence, as these reactive astrocyte markers were the most elevated in our initial knockdown experiments and did not redistribute in the cytoplasm. We found that C16 suppressed the effects of TDP-43 knockdown on all three of these markers (Fig. 5), and that cells treated with C16 had a less stellate appearance.

To confirm the role of PKR activation/inhibition in our experiments, we used siRNA to simultaneously knock down TDP-43 and PKR, followed by immunoblotting for proteins of interest. Importantly, we found that TDP-43 knockdown causes a robust increase in phosphorylation (Ser-51) of eukaryotic translation initiation factor 2A (eIF2α), the major target of activated PKR(Fig. 6A). However, PKR knockdown (~90% efficiency) blocked this effect and also suppressed the increase in CD44 (the most robust marker of astrocyte activation in our experiments) that occurs with TDP-43 knockdown (Fig. 6A). We also noted a trend for greater expression of GFAP (a classic marker of astrocyte reactivity) with TDP-43 knockdown, which again was prevented by PKR inhibition, and we confirmed these findings by immunofluorescence (Fig. 6B). Additionally, in these experiments we noted that TDP-43 and/or PKR knockdown did not cause an increase in the PKR activator PACT, which has been reported to be involved in neurodegenerative diseases (48), further indicating that PKR itself transduces the effects of TDP-43 loss-of-function in astrocytes. Thus, these observations suggest that the dsRNA sensor PKR may play a causal role in astrocyte

activation when TDP-43 function is altered/impaired, and that inhibiting PKR and/or other cellular dsRNA sensors may be a viable strategy for preventing or reducing reactive astrocyte-driven inflammation in this context.

DISCUSSION

Our major finding is that knockdown of the RNA-binding protein TDP-43 results in an accumulation of RE transcripts, increased dsRNA, and pro-inflammatory activation in astrocytes. We also showed that reactive/pro-inflammatory protein expression in response to TDP-43 knockdown can be blocked by inhibiting PKR, which implies that dsRNA may play a causal role in astrocyte activation. These findings are clinically relevant, because neuroinflammation and TDP-43 abnormalities (e.g., mutations, dysfunction, aggregates) are observed in most types of ALS and several other neurodegenerative diseases, and have been documented in patient glial cells specifically (7-9)—but the mechanisms connecting these phenomena have remained unclear. Based on our data, we speculate that: 1) TDP-43 may limit dsRNA accumulation in astrocytes; 2) RE transcript levels may be an ALS-relevant transcriptome biomarker of dsRNA; and 3) altered TDP-43 may lead to PKR activation via dsRNA accumulation and subsequent neuroinflammation. This would be an entirely novel, intracellular mechanism by which astrocytes (and perhaps other CNS cells) may become activated/pro-inflammatory in ALS and other neurodegenerative diseases. It could also be an important therapeutic target, because pharmacological agents like C16 have the potential to suppress PKR activity.

Inflammation and astrocyte activation

One definitive feature of ALS and other neurodegenerative diseases associated with TDP-43 dysfunction is neuroinflammation, characterized by microglial and macrophage activation, monocyte recruitment, and dysregulation of immune-related transcription (49). Growing evidence also suggests that astrocytes contribute significantly to neuroinflammation, in part by modulating innate immunity (18, 50). However, the role of TDP-43 dysfunction in astrocytes/neuroinflammation has remained unclear. Through its DNA and RNA binding activity, TDP-43 modulates the expression of many genes, and it is possible that these genes are involved in specific biological processes that explain how altered TDP-43 function leads to pathological events (e.g., glial cell inflammation) in different cell types. Others have reported that altered TDP-43 function causes astrocyte activation and increases pro-inflammatory signaling (20, 34), but our study is the first to address this question by profiling transcriptome changes via RNA-seq in this context. Using this approach, we found that TDP-43 knockdown increased the transcription of many known markers of astrocyte activation (e.g., LCN2, FKBP5, CD44, GBP2, CXCL1 and others). Importantly, we note that genes identified as direct targets of TDP-43 in UV cross-linking and immunoprecipitation studies (51, 52) do not overlap with the inflammatory genes we have identified, and the overall lists of direct TDP-43 target genes in these same studies are not enriched in inflammatory function. This suggests that the immune activation we observed in the present study is not a result of TDP-43 directly controlling these genes, but rather a consequence of cellular events that activate inflammation when TDP-43 function is altered. This possibility is also consistent with our observation that the addition of ATP exacerbates inflammatory

gene expression changes in the when TDP-43 is knocked down, because ATP also synergistically activates inflammatory signaling when combined with classic innate immune system activators like LPS (32). Moreover, many of the genes upregulated in response to TDP-43 knockdown in our experiments are commonly associated with LPS/ATP-induced immune activation as well. In the present study, we focused on the role of altered TDP-43 function alone in activating innate immune signaling (i.e., without ATP), because TDP-43 dysfunction *per se* plays a central role in ALS. However, activation of the innate immune system is also clearly involved in ALS (53), and based on our observations, future studies might investigate the role of ATP-related innate immune signaling in the disease.

Interestingly, some of the most significantly knockdown-enriched genes in our data are associated with both astrocyte activation/injury and the cellular pathogen response. For example, increased LCN2 expression is observed in both TDP-43-induced reactive astrocytes and the bacterial defense response (20, 54), and PAI-1 has been implicated in both injury-driven astrogliosis and the cellular anti-viral response (55, 56). Thus, our novel observations may indicate that the neuroinflammation associated with altered TDP-43 function results from intracellular events that activate innate immune signaling in astrocytes, rather than anything related to TDP-43 s role in modulating gene expression. We cannot exclude the possibility that similar events may occur in other cell types, such as microglia, which play a key role in central nervous system immunity and neuroinflammation, but this possibility requires further investigation.

RE transcripts and TDP-43/ALS

Evidence indicates that TDP-43 preferentially binds certain types of transcripts, particularly those with long introns (51). The reason for this is unknown, but it may be due to an inherent characteristic of the RNA itself (rather than any specific functional paradigm). In support of this concept, we previously reported that a significant number of transcripts modulated by TDP-43 contain either antisense overlap with another gene or multiple inverted repeats (22). RE fit this description well and may, therefore, serve as a good transcriptomic biomarker for the general dysregulation of this transcript type—especially in the context of altered TDP-43 function. Indeed, we and others have observed an association between RE transcript levels and TDP-43 dysfunction (21, 25). Furthermore, others have shown that RE transcripts derived from certain endogenous retroviruses are increased in ALS patient samples (57), and we recently reported that RE transcripts *in general* are increased in ALS brains (26). Here, we demonstrated that TDP-43 knockdown causes RE transcript accumulation in astrocytes, one of the most abundant cell types in the brain. We also showed that ATP in addition to TDP-43 knockdown further increases RE transcript levels, suggesting that RE (or at least the transcript type they reflect) may play a role in the immunomodulatory effects of TDP-43 dysfunction. Consistent with this possibility, others have shown that RE transcripts, such as alu elements and long interspersed nuclear elements, have the potential to activate innate immune/inflammatory signaling (58, 59). In the context of altered TDP-43 function, it is unclear whether RE transcripts accumulate as a result of increased transcription or reduced turnover, but the granular staining pattern we observed for the MER21B transcript may suggest the latter (i.e., a change in RE metabolism or downstream/cytoplasmic processing). Interestingly, MER21B is a long terminal repeat with few inverted repeats; its transcript may

form intra-strand dsRNA, but to the best of our knowledge this has not been investigated. Thus, future studies are needed to determine the mechanisms by which altered TDP-43 function leads to the accumulation of RE transcripts like MER21B, and where/how these and other similarly structured transcripts may cause pathological cellular events.

dsRNA, TDP-43 function and PKR

Interestingly, both long-intron gene and RE transcripts are predisposed to form dsRNA (60). In the case of long-intron transcripts, this is because longer length increases the probability of repeated sequences capable of forming intra-strand dsRNA. RE may form dsRNA for the same reason, but they also are commonly oriented antisense to each other in the genome, which increases the probability of forming inter-strand dsRNA as well. Yet another potential source of endogenous dsRNA is antisense transcription (i.e., co-expression of complementary sense and anti-sense transcripts). We did not observe this in our data, which may imply that these transcripts accumulate in the absence of TDP-43's binding/chaperone functions. In any case, dsRNA is a well-characterized activator of the innate immune system (27). It is produced during RNA virus replication and, as such, is a classic pathogen-associated molecular pattern (PAMP). Thus, it is at least plausible that the neuroinflammation associated with altered TDP-43 function is partly due to dsRNA-initiated innate immune signaling. Evidence for this possibility in the context of ALS is sparse, but others have shown that Geographic Atrophy, an age-related form of macular degeneration, is associated with increased dsRNA-driven pathology in retinal cells as a result of reduced activity of DICER (a major cellular dsRNase) (61). Previous reports also indicate that deletion of DICER in astrocytes accelerates neurodegeneration in mice (62), and knocking down the NLRP3 inflammasome (a downstream mediator of the proinflammatory viral response) reduces astrogliosis with aging in mice (63). Here, we extended on these observations by demonstrating that TDP-43 knockdown in astrocytes is associated with an increase in dsRNA, which we showed both directly by immunoblot and RNA immunoprecipitation, and indirectly by quantifying A-to-I editing (a process that requires dsRNA) in our RNA-seq data. Interestingly, and consistent with this latter observation, others have shown that the primary cellular dsRNA editing enzyme ADAR1 protects against innate immune and stress response signaling in mammalian cells (64). However, our study provides the first evidence for increased dsRNA and innate immune signaling as a result of TDP-43 knockdown in glial cells, and it suggests the intriguing possibility that *endogenous* (rather than pathogen-derived) dsRNA may be an upstream cause of neuroinflammation during TDP-43 loss of function.

Cytosolic dsRNA can activate several intracellular sensors, including PKR, melanoma differentiation-associated protein 5 (MDA5), and retinoic acid-inducible gene I (RIG-I)—all of which lead to a pro-inflammatory interferon response (28). Importantly, some evidence indicates that PKR is required for subsequent signaling in all cases (65, 66). PKR is a central cellular stress sensor that is also activated by bacterial infection and even metabolic stress, and it commonly leads to adverse/pro-inflammatory cellular signaling, in part by activating NF-kB (27). Limited evidence exists for the role of PKR in ALS, but one report has shown that PKR phosphorylation (activation) is increased in ALS patient samples (29). Additionally, others have shown that inhibiting PKR-like endoplasmic reticulum kinase

(PERK), which likely also inhibits PKR due to structural similarities (67), reduces TDP-43 toxicity in *C. elegans* and *D. rerio* (68), as well as flies and mammalian neurons (69). Interestingly, there also is evidence of a role for PKR in Alzheimer's disease (AD), as increased phospho-PKR has been reported in AD brains and CSF (31, 70, 71), and PKR knockout mice, as well as cells isolated from these animals, seem to be resistant to various AD-related stresses, such as amyloid beta (72, 73). Here, we extend on these observations and provide evidence for a novel *intracellular mechanism* by which PKR may become activated in ALS and/or other neurodegenerative diseases (via endogenous dsRNA). Moreover, we show that both pharmacological inhibition and direct siRNA knockdown of PKR prevent the pro-inflammatory effects of TDP-43 loss-of-function (knockdown) in astrocytes, lending further support to the idea that PKR may play a central role in the neuroinflammation observed in ALS and other neurodegenerative diseases. The exact events downstream of PKR that lead to innate immune activation in astrocytes require further investigation, but we did detect an increase in phosphorylation (activation) of NF-kB upon TDP-43 knockdown, suggesting that this pathway may be involved. Consistent with this possibility, we note that our transcriptome data show that TDP-43 knockdown increases mRNA for numerous cytokines, cytokine receptors, and chemokines that are transcriptionally regulated by NF-kB (e.g., IL-1α, TNF-α related pathways; CXCL1, CXCL10). These pro-inflammatory signals are involved in propagating immune reactivity in astrocytes, and in activating microglia that may further promote neuroinflammation (74, 75). Others have shown that these and other related cytokines can drive astrocytes to a reactive state that is toxic to neurons (15, 19). Furthermore, it is clear that astrocytes derived from transgenic TDP-43 mutant mice and patients with sporadic ALS (commonly associated with TDP-43 dysfunction) are similarly toxic to cultured neurons (13, 14). Therefore, based on our present findings, we speculate that activation of PKR by intracellular dsRNA may be involved in these events. If so, PKR itself may be an important therapeutic target in both ALS and other neurodegenerative diseases characterized by neuroinflammation.

Conclusions

Collectively, our findings suggest that dsRNA accumulation as a result of altered TDP-43 function may be relevant to ALS pathology, in part because it could underlie PKR-driven neuroinflammation in glial cells like astrocytes. It is clear that both neuroinflammation and TDP-43 aberrations occur in ALS and other neuropathologies, and there also is growing evidence that: 1) RE transcripts (which likely reflect dysregulation of RNA homeostasis and increased dsRNA) accumulate in neurodegenerative diseases and are linked to neurotoxicity; and 2) PKR, the major cellular dsRNA sensor, is involved in multiple neurodegenerative diseases. However, our study provides the first evidence for mechanistic connections between these phenomena in the context of glial cell inflammation. The exact transcripts that may activate PKR when TDP-43 function is altered are unknown, as are where and how these events occur in astrocytes (and perhaps other cells, such as neurons and microglia). Still, our novel findings may serve as a platform for future studies investigating these and other related questions, such as the efficacy of PKR inhibitors for treating ALS-relevant neuroinflammation.

MATERIALS AND METHODS

Cell culture

Primary rat astrocytes were used for all experiments. Cells were harvested from neo-natal pups as previously described (76). All procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23) and were approved by the University of Colorado Boulder Animal Care and Use Committee. Briefly, cerebral cortices were dissected from neo-natal Sprague-Dawley rats (Charles River Laboratories) and subjected to enzymatic digestion in buffer with 2.0 mg/mL trypsin and 0.005% DNase I (ThermoFisher). The tissue was then mechanically disrupted using 23 gauge needles, and the suspension was centrifuged and resuspended in medium (DMEM, 10% FBS and penicillin/streptomycin). Cells were then filtered through a 50-micron strainer, transferred to 75 cm^2 polystyrene tissue culture flasks, and incubated in fresh medium at 37°C and 5% CO_2 for 7– 10 days until confluent. Contaminating non-astrocyte cells were removed by shaking cells on an orbital shaker overnight, and astrocytes were harvested using trypsin/EDTA in DPBS and plated at 2×10^4 cells per cm^2. Cells were tested for positive GFAP staining before use, and routinely grown in medium containing DMEM/F12 medium (Gibco) with 10% FBS and penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. All experiments were performed on passage 2-3 cells, treatments (aside from siRNA) were applied by adding fresh medium containing reagents, and cells were harvested at 90% confluency. All experiments were repeated 3-5 times.

siRNA transfections

Cells were transfected when 40-60% confluent. Transfections were performed using: ThermoFisher Silencer siRNAs for TDP-43 knockdown (20 nM, target sequence: AAGAGTTGTCATTGTTGGAAA) and PKR knockdown (20 nM, target sequence: CGACAGAAGGUUUACAUUU); Qiagen negative control siRNAs (20 nM, target sequence: AATTCTCCGAACGTGTCACGT); Lipofectamine 3000 or RNAiMAX (for double transfection) reagents (ThermoFisher); and Opti-MEM (ThermoFisher), all according to manufacturer's instructions. Cells were then incubated at 37°C, 5% CO_2, and 90% humidity for 48 h without additional medium changes, and either: 1) rinsed with DPBS and flash frozen for immunoblotting; 2) rinsed with DBPS and then lysed in Trizol (ThermoFisher) for RNA isolation; or 3) fixed in 4% paraformaldehyde for immunofluorescence staining.

Immunoblotting

Western blot analyses were performed on whole-cell lysates using standard techniques. Cells were lysed in ice-cold radio-immunoprecipitation assay lysis buffer with protease and phosphatase inhibitors, and ten micrograms of protein was loaded onto 4–12% poly-acrylamide gels, separated by electrophoresis and transferred to nitrocellulose membranes. Blots were then blocked and incubated overnight with primary antibodies: TDP-43 (Protein Tech, 1:1000 dilution); phosphorylated NF-kB (Cell Signaling, 1:500 dilution); PKR (Santa Cruz Biosciences, 1:1000); phosphorylated eif2α (ThermoFisher, 1:500); CD44 (Genetex, 1:1000); PACT (Abcam, 1:1000). Proteins were then detected using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) and ECL chemiluminescent

substrate (Pierce). Protein expression is presented normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling, 1:1000), and data are expressed relative to the control mean. Dot blotting for dsRNA was performed using similar techniques, and according to previously published methods (77, 78). Cells were rinsed with DPBS and lysed in Trizol, and RNA was isolated using an RNA-specific spin column kit as described below. RNA concentration was determined using a NanoDrop spectrometer, and 4 μg of each sample was spotted onto a nitrocellulose membrane and crosslinked by UV irradiation using a Stratalinker crosslinker (Stratagene). Membranes were blocked in milk overnight at 4°C, followed by overnight incubation with the J2 antibody (English and Scientific; 1:500 dilution), and subsequent secondary antibody and imaging as described above for Western blots.

RNA isolation, RNA-seq, gene expression and repetitive elements analysis

RNA isolation, sequencing and gene expression analyses were performed using standard techniques. For basic RNA-seq analyses, cells were rinsed with DPBS, and gently lysed directly in Trizol reagent. For RNA immunoprecipitations, RNA was isolated as previously described (22, 79). Briefly, cells were lysed in 10 mM KCl/1.5 mM MgC12/1 mM DTT/10 mM Tris HCl (pH 8.0)/50 mM Sucrose/0.05% Nonidet P-40/1 mM PMSF with protease inhibitors. Half of each sample was collected in Trizol (input), and the remainder was used for immunoprecipitation reactions. Immunoprecipitation beads were prepared by washing in buffer (20 mM Tris (pH 8.0)/100 mM KC1/0.2 mM EDTA/0.1% Nonidet P-40/0.1 mM DTT with protease inhibitors), blocking with 0.1% nonfat dry milk, and incubating with J2 antibody for 1 h at room temperature. Lysate was added to beads with RNase inhibitors and rocked at 15°C for 2 h. The supernatant was then collected, beads were washed with buffer, and Trizol was added to isolate RNA. For all preparations, RNA was recovered using an RNA-specific spin column kit (Direct-Zol, Zymo Research), which included DNase I treatment to remove genomic DNA. To generate poly(A)-selected libraries for standard gene expression and RE analyses, RNA was selected using sera-mag magnetic oligo dT beads (Therma Scientific), and libraries were created using Illumina TruSeq kits. For J2 immunoprecipitated RNA, samples and corresponding input material (as a loading control) were converted into strand-specific total RNA libraries using V2 Scriptseq (Epicenter) kits following manufacturer's instructions, but reverse transcription was performed using Super-Script III (Invitrogen) and progressivley increasing temperature from 42 to 59°C to allow for transcription though structured RNAs. rRNA was not removed from immunoprecipitated RNA samples. All libraries were sequenced on Illumina HiSeq 4000 platforms, producing >40 M 150-bp strand-specific single-end (poly(A)-selected) or paired-end (immunoprecipitated) FASTQ reads. These reads were trimmed and filtered using Trimmomatic and FastQC programs, then aligned to the rn6 *Rattus norvegicus* genome using Tophat2. Reads that failed to map were remapped using Bowtie2. Raw read/gene counts for alignment files were obtained using HTseq-count, and differential gene expression was analyzed using Deseq2 software with a significance cut-off of $P < 0.05$ and FDR < 0.1. To quantify repetitive element transcripts, a repeat-masker file for rn6 was created, and reads mapping to repeat regions were identified in each FASTQ file using Repenrich (Bowtie mapping followed by Python-based aggregating of multi-mapped reads) as previously described (41). Differences in repetitive element expression were analyzed

using Deseq2 software as described above, including sample-specific size factors to account for differences in library size. Size factors for J2-immunoprecipitated RNA samples were calculated based on Deseq2 output for input libraries.

RT-PCR

Total RNA was extracted from astrocyte cultures by Trizol extraction. RNA was purified using spin columns as described above, and DNA was degraded via DNase I treatment. cDNA was generated from 1ug RNA per condition with random hexamers using Super Script IV First-Strand cDNA Synthesis Reaction (Invitrogen) according to manufacturer's instructions. A cDNA linearity curve was generated for all treatment and control condition replicates, and 5ng cDNA was used for each PCR reaction using PerfeCTa SYBR Green FastMix Low ROX (Quanta Biosciences) on an ABI Prism 7000 real-time PCR instrument. Data are presented normalized to GAPDH. Primers included: GAPDH forward:5'-GTTTGTGATGGGTGTGAACC-3', reverse: 5' -TCTTCTGAGTGGCAGTGATG-3'; TDP-43 forward:5'-CATCTGACCTCATAGTGTTGGG-3', reverse:5'-TCTGCTTCTCAAAGGCTCG-3'; FKBP5 forward:5'-ACTCTGGGAGGTTTAAAGCAG-3', reverse:5'- CTGGAGACTTTGGCTGATGTAG-3'; PAI-1 forward:5'-GATTTGATTCTCGTGGCAAGC-3', reverse:5'-TCTTTGACAGTTCCCCAGTTG-3'; CD44 forward:5'-AAGTGGGAATCAAGACAGTGG-3', reverse:5'-CCACACCTTCTCCTACTGTTG-3'; MER21B forward:5'- GCCCTATGAATCTCTTCATCTG-3', reverse:5' -CTCCTTGGGTTCGATTAATTTGC-3'.

Immunofluorescence staining

At 48 h post-transfection, medium was removed and cells were washed once in DPBS and fixed in 4% paraformaldehyde. Cells were then washed and permeabilized in 0.25% Triton-X, washed again, and then blocked in 3% fetal bovine serum and 3% normal goat serum. Primary antibody solution was then added (diluted in 5% normal goat serum), and Cells were incubated at 4°C overnight. Primary antibodies included: CD44 (Novus Biologicals, 1:500 dilution), FKBP5 (ThermoFisher, 1:500), LCN2 (Abcam, 1:100), PAI-1 (Novus Biologicals, 1:500); GFAP (Abcam, 1:500). After primary antibodies, cells were washed in DPBS with 0.1% tween, and subsequently incubated with secondary antibody solution (1:500-1:1000 dilutions 5% normal goat serum) for 1 hr. DAPI was added (1μg/mL DPBS solution) in the dark for 10 min at room temperature, and cells were washed again before mounting with Prolong mounting medium for imaging.

Fluorescence *in-situ* hybridization

Fluorescence *in-situ* hybridization (FISH) probes for repetitive element MER21B were designed using LGC Biosearch Technologies Stellaris Probe Designer Version 4.2 and used according to manufacturer's instructions (probe sequences in Supplementary Methods). Briefly: 5nmol dried oligonucleotide probe blend was reconstituted in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) to produce a 12.5 μM stock solution. Hybridization buffer was prepared as 90% Stellaris RNA FISH Hybridization buffer and 10% deionized formamide. At 48 h post-transfection cells were washed once in DPBS and fixed with 4% paraformaldehyde in RNase-free DPBS for 10 min at room temperature. Cells were then

washed twice in DPBS and permeabilized in 70% ethanol for 2 h at 4°C. Ethanol was removed, and cells were washed with Stellaris RNA FISH wash buffer A prepared in nuclease free water with 10% formamide 5 min at room temperature. Hybridization buffer with probe was then added, and cells were incubated in the dark at 37°C for 4 h. Cells were then incubated in fresh wash buffer in the dark at 37°C for 30 min, followed by 5 ng/mL DAPI diluted in wash buffer for an additional 30 min. Finally, cells were incubated in Stellaris secondary wash buffer for 5 min at room temperature, and slides were mounted with Prolong mounting medium for imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by NIH grant NS063964.

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Highlights

- The RNA binding protein TDP-43 is implicated in amyotrophic lateral sclerosis.
- TDP-43 knockdown in astrocytes increases repetitive and double-stranded RNA levels
- Knocking down TDP-43 also causes astrocytes to become reactive/pro-inflammatory
- Inhibition of protein kinase R prevents astrocyte activation with TDP-43 knockdown

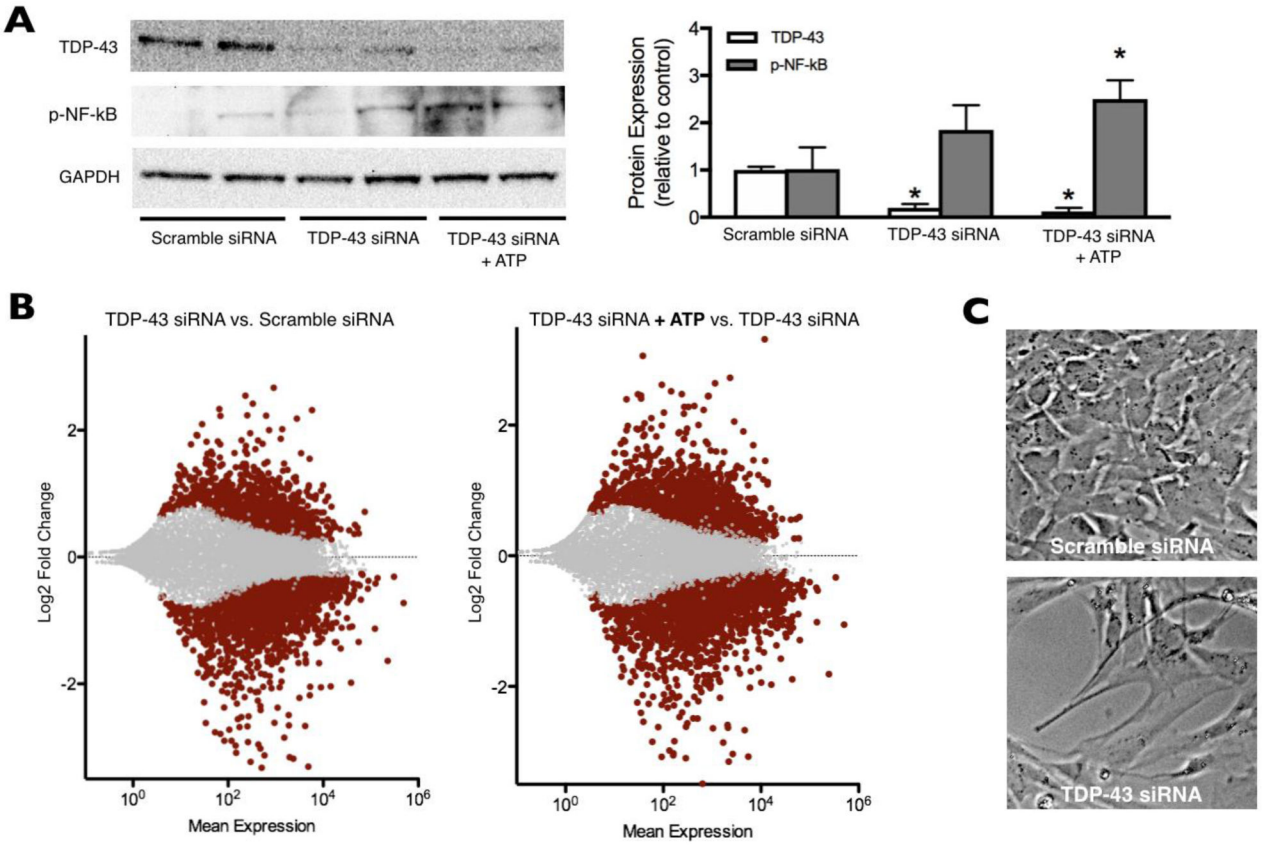

Figure 1. TDP-43 knockdown activates astrocytes.

(A) Representative immunoblots showing efficient siRNA knockdown of TDP-43 and an associated increase in phosphorylation (activation, Ser-536) of the pro-inflammatory transcription factor NF-kB, which is exacerbated by ATP. Quanitification of protein expression at right. (B) MA plots showing Log2 fold change and mean gene expression levels based on RNA-seq data collected in astrocytes knocked down for TDP-43. Note >5000 significantly increased/decreased genes shown in red (FDR<0.1), many of which are further increased/decreased with the addition of ATP. (C) Representative images of stellation in astrocytes treated with TDP-43 siRNA vs. scrambled siRNA (less confluent regions of slide shown to make morphological differences clearer). All experiments based on three or more biological replicates collected 48 h after siRNA treatment.

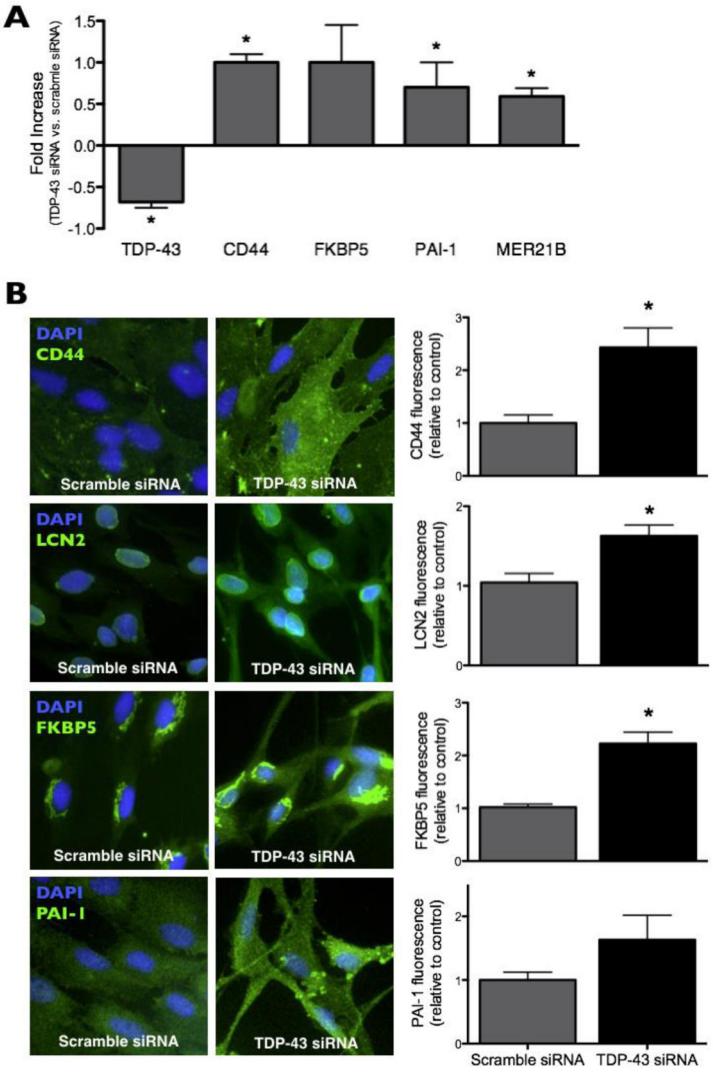

Figure 2. TDP-43 knockdown increases expression of inflammatory/reactive astrocyte markers.

(A) Quantitative RT-PCR confirmation of selected transcripts associated with astrocyte activation that were identified as increased with TDP-43 knockdown in RNA-seq data. Two biological replicates per condition, collected 48 h after siRNA transfections. (B) Immunofluorescence confirmation of increased protein expression for CD44, LCN2, FKBP5 and PAI-1, genes/proteins linked with astrocyte activation that were identified as increased with TDP-43 knockdown via RNA-seq. All immunofluorescence experiments based on three or more biological replicates collected 48 h after siRNA treatment. *P<0.05, TDP-43 siRNA vs. scramble siRNA.

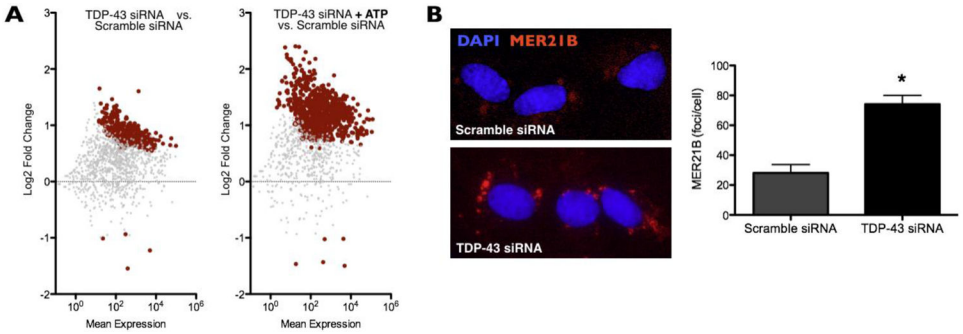

Figure 3. Accumulation of RE transcripts with TDP-43 knockdown in astrocytes.

(A) MA plots showing Log2 fold change and mean RE transcript levels based on RNA-seq data collected in astrocytes knocked down for TDP-43 vs. cells treated with scramble (control) siRNA. Note >300 significantly increased RE with TDP-43 knockdown and >700 significantly increased RE with knockdown plus ATP, all shown in red (FDR<0.1). Data derived from the same RNA-seq experiments plotted in Figure 1. (B) Fluorescence *in-situ* hybridization confirmation of the MER21B RE transcript showing perinuclear accumulation and a ~3-fold increase in foci with TDP-43 knockdown. All experiments based on three or more biological replicates collected 48 h after siRNA treatment. *P<0.05, TDP-43 siRNA vs. scramble siRNA.

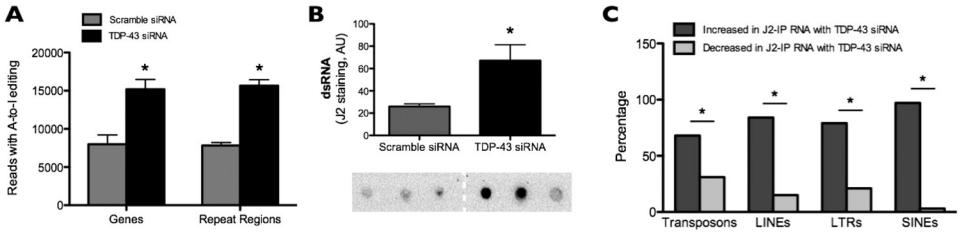

Figure 4. Increased dsRNA with TDP-43 knockdown in astrocytes.

(A) Number of RNA-seq reads with A-to-I editing, a marker of dsRNA, in astrocytes treated with TDP-43 siRNA or scramble (control) siRNA. (B) dsRNA quantified by dot blot using the dsRNA-specific antibody J2 in astrocytes treated with TDP-43 siRNA or scramble (control) siRNA. (C) Percentage of all expressed repetitive elements by class/type that are significantly increased and decreased in J2 immuno-precipitated RNA isolated from astrocytes knocked down for TDP-43. Major enriched RE classes included DNA transposons, long interspersed nuclear elements (LINEs), long terminal repeats (LTRs), and short interspersed nuclear elements (SINEs). All experiments based on three or more biological replicates collected 48 h after siRNA treatment. *P<0.05, TDP-43 siRNA vs. scramble siRNA.

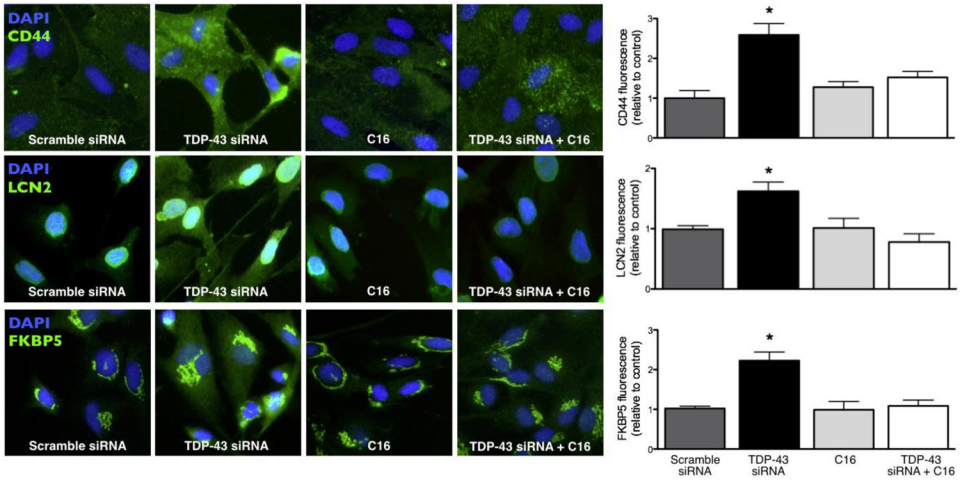

Figure 5. Inhibition of PKR blocks the pro-inflammatory effects of TDP-43 knockdown.

Representative immunofluorescence images and quantification showing that TDP-43 knockdown increases expression of the reactive astrocyte markers CD44, LCN2 and FKBP5 in astrocytes, and that these changes are blocked by the PKR inhibitor C16 (300 nM). All experiments based on three or more biological replicates collected 48 h after siRNA and/or C16 treatment. *P<0.05 vs. scramble siRNA and other conditions.

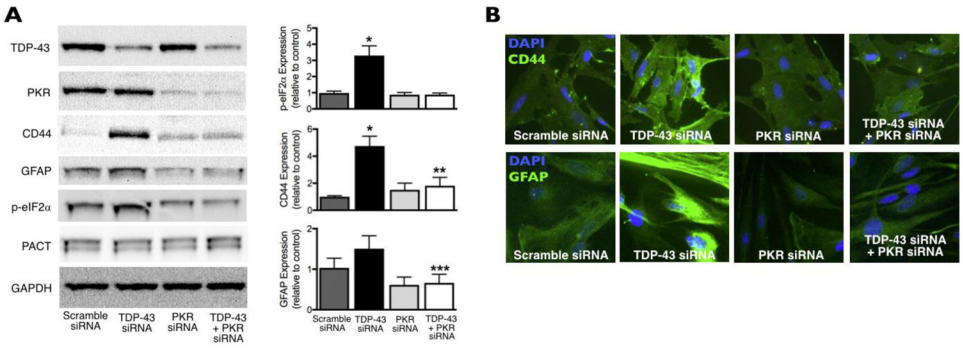

Figure 6. PKR knockdown blocks the pro-inflammatory effects of TDP-43 knockdown.

(A) Representative immunoblots showing TDP-43 knockdown and an associated increase in phosphorylation of eif2α (Ser-51), CD44 and GFAP expression, all of which are suppressed by simultaneous knockdown of PKR; no changes in PACT expression with any treatment. Quantification of p-eif2α and CD44 levels at right. (B) Representative immunofluorescence images confirming an increase in CD44 and GFAP with TDP-43 knockdown that is inhibited by simultaneous PKR knockdown. All experiments based on three or more biological replicates collected 48 h after siRNA treatments. *P<0.05 vs. scramble siRNA. **P<0.05 vs. scramble and TDP-43 siRNA. ***P<0.05 vs. TDP-43 siRNA.