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TAR DNA-binding protein 43 (TDP-43) regulates stress granule dynamics via differential regulation of G3BP and TIA-1

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TAR deoxyribonucleic acid-binding protein 43 (TDP-43) is a multifunctional protein with roles in transcription, pre-messenger ribonucleic acid (mRNA) splicing, mRNA stability and transport. TDP-43 interacts with other heterogeneous nuclear ribonucleoproteins (hnRNPs), including hnRNP A2, via its C-terminus and several hnRNP family members are involved in the cellular stress response. This relationship led us to investigate the role of TDP-43 in cellular stress. Our results demonstrate that TDP-43 and hnRNP A2 are localized to stress granules (SGs), following oxidative stress, heat shock and exposure to thapsigargin. TDP-43 contributes to both the assembly and maintenance of SGs in response to oxidative stress and differentially regulates key SGs components, including TIA-1 and G3BP. The controlled aggregation of TIA-1 is disrupted in the absence of TDP-43 resulting in slowed SG formation. In addition, TDP-43 regulates the levels of G3BP mRNA, a SG nucleating factor. The disease-associated mutation TDP-43^{R361S} is a loss-of-function mutation with regards to SG formation and confers alterations in levels of G3BP and TIA-1. In contrast, a second mutation TDP-43^{D169G} does not impact this pathway. Thus, mutations in TDP-43 are mechanistically divergent. Finally, the cellular function of TDP-43 extends beyond splicing and places TDP-43 as a participant of the central cellular response to stress and an active player in RNA storage.

INTRODUCTION

TAR deoxyribonucleic acid (DNA)-binding protein 43 (TDP-43) was first described in the transcriptional regulation of the HIV-1 genome where it was found to bind pyrimidine-rich sequence motifs in TAR DNA (1). In addition to this initially described role, TDP-43 is now known to be involved in several aspects of RNA metabolism, including transcription, alternative splicing, pre-messenger ribonucleic acid (mRNA) stability and mRNA transport (2–4). TDP-43 is composed of 414 amino acids and has all of the structural features characteristic of a heterogeneous nuclear ribonucleoprotein (hnRNP), including two highly conserved ribonucleic acid (RNA) recognition motifs (RRM1 and RRM2), a nuclear localization signal and a glycine-rich C-terminal tail (5,6). The glycine-rich C-terminal region is required for its exon skipping and inhibitory splicing activities and, as with other hnRNPs, this domain mediates protein–protein interactions (7). Indeed, a portion of this region (residues 321–366) mediates a direct interaction between TDP-43 and hnRNP A2 (2). Protein–protein interactions between hnRNPs are suspected to contribute to RNA–protein complex formation as well as direct RNA–protein interaction between hnRNPs and mRNAs (8).

In human cells, hnRNPs are concentrated in the nucleus in physiologically normal conditions. However, a subset (ex. hnRNP A1, K and Q) continuously shuttle between the nucleus and cytoplasm (9). hnRNPs are involved in the extensive processing of pre-mRNAs in the nucleus, which are subsequently transported to the cytoplasm. Several studies indicate that certain hnRNPs are directly involved in the cellular response to various stress stimuli. For example, the activation of the p38 stress-signaling pathway in mammalian

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cells results in both the phosphorylation and cytoplasmic accumulation of hnRNP A1 in stress granules (SGs) (10). Similarly, hnRNP Q redistributes to the cytoplasm and partially co-localizes to SGs and processing bodies (PBs) under specific stress conditions [e.g. thapsigargin (THAP), heat shock (HS) and arsenite] (11).

Depending on the type of cellular stress encountered, a variety of signaling pathways can be activated which ultimately modulate gene expression patterns either transcriptionally or post-transcriptionally (12). RNA-binding proteins (RBPs) play a major role in post-transcriptional regulation during stress yielding global repression of protein translation (12,13). This is facilitated by the formation of SGs which are cytoplasmic domains housing translationally arrested mRNAs (10). SGs are also now considered to be dynamic triage centers that sort mRNA for storage, decay or re-initiation during stressful conditions (14,15). The assembly of SGs can be induced by a variety of stimuli, including HS, hypoxia, osmotic and oxidative stress, and typically involves the phosphorylation of the eukaryotic initiation factor [eukaryotic translation initiation factor 2 alpha (eIF2α)]. This phosphorylation event inhibits mRNA translation through depletion of the eIF2–GTP–tRNA-met ternary complex, thus permitting the RBP TIA-1 to bind the 48S complex instead of the ternary complex. This promotes polysome disassembly and the consequent recruitment of mRNAs to SGs (10). SGs gradually disperse once the stress is removed (12). Since TDP-43 shares so many features with other hnRNPs, it is reasonable to suspect that it may also play a role in cellular stress responses. Indeed, it has recently been reported that TDP-43 is localized to SGs following oxidative stress or proteasome inhibition (16–18). However, there is currently no data to determine the functional role for TDP-43 in SGs and the cellular stress response.

We report here that TDP-43 and its binding partner hnRNP A2 are components of SGs arising from oxidative stress. Furthermore, TDP-43 down-regulation influences the stoichiometry of other SG protein components, including TIA-1 and G3BP. Moreover, TDP-43 contributes to SG formation and maintenance. In patient lymphoblastoid cells, at least one amyotrophic lateral sclerosis (ALS)-causing mutation in TDP-43 impacts SG formation and TIA-1 and G3BP levels. Our data suggest that not all TDP-43 mutations have the same mechanism and clearly define an active role for TDP-43 in the cellular response to oxidative stress.

RESULTS

Endogenous TDP-43 is localized to SGs

Various types of cellular stress insults are known to affect the expression and localization of several hnRNPs with some localizing to SGs (10,14). Since TDP-43 is a bona fide hnRNP family member, we investigated whether cellular stress could also affect endogenous TDP-43. To address this, HeLa cells were exposed to three well-established cellular stress conditions: sodium arsenite (SA), HS and THAP. SA treatment is a well-characterized model of oxidative stress, while THAP induces ER stress via calcium pump dysregulation. Endogenous TDP-43 was localized to distinct cytoplasmic puncta upon SA, HS and THAP treatment, whereas TDP-43 remained largely in the nucleus of untreated cells (Fig. 1A). Double-labeling demonstrates that these TDP-43 puncta co-localize with the well-described SG marker, TIA-1 in SA-, HS- and THAP-treated cells, consistent with published reports on TDP-43 localization to SGs with oxidative stress (16,17) (Fig. 1A). Biochemically, the formation of SGs is marked by the enhanced protein insolubility of SG proteins (and consequent depletion of the soluble pool) such as is described for TIA-1 (19). Indeed, soluble TDP-43 protein levels were decreased in HS- and SA-treated cells (37 and 56%, respectively) compared with untreated cells, and there was a reciprocal increase in TDP-43 in the insoluble fractions (Fig. 1B). However, THAP treatment did not yield similar changes in protein solubility.

To determine whether TDP-43 is also a resident of other RNA granules, such as PBs, we double-labeled SA-stressed HeLa cells for the PB marker, GW182. TDP-43 was not robustly co-localized with GW182-labeled PBs (Fig. 1C), but rather was often located in close proximity to PBs. This is consistent with published data demonstrating the close juxtapositioning of SGs and PBs (20). Thus, TDP-43 is a resident of SGs but not PBs following acute exposure to certain stressful stimuli, in agreement with recent reports (16–18).

A cell's encounter with external stress is marked by the global repression of protein translation, and this is reflected by the phosphorylation of the eIF2α (12). Since we observed eIF2α phosphorylation most robustly in SA-treated samples, we focused on SA as a stress paradigm for subsequent experiments (Fig. 1D).

TDP-43 impacts SG assembly and disassembly

Several cellular proteins are required for SG assembly, and their reduction is associated with a muted SG response (19,21–23). SGs are dynamic entities such that oxidative stress induces TIA-1 to redistribute from the nucleus to the cytoplasm and aggregate to form SGs (12). This process occurs over ∼30 min (19). Once the stress is removed, SGs continue to aggregate and increase in size for ∼1–2 h before gradually resolving. Typically, SGs are completely resolved within 4 h of SA treatment. To determine whether TDP-43 might impact SG dynamics, we assessed the kinetics of SG formation and resolution via indirect immunofluorescent labeling for TIA-1 in cells depleted of TDP-43 via small interfering RNA (siRNA) and then exposed to SA. The number of cells exhibiting SGs was determined at 0, 15, 20 and 30 min during SA ('stress') exposure. SG-positive cells were defined as cells containing at least two TIA-1-labeled foci with a minimum area of 0.75 μm^2. Compared with control siRNA, siRNA directed to exon 1 of TDP-43 was very effective in reducing endogenous TDP-43 expression (84%, $n = 5$; Fig. 2A). We observed that the assembly of TIA-1-labeled SGs was delayed in cells depleted of TDP-43 compared with cells treated with control siRNA (Fig. 2B). In particular, we noted a modest but significant reduction in the number of cells with SG formation at 30 min following SA exposure (97 versus 81%, $P = 0.03$) as well as a marked change in SG size which will be discussed below. Similar

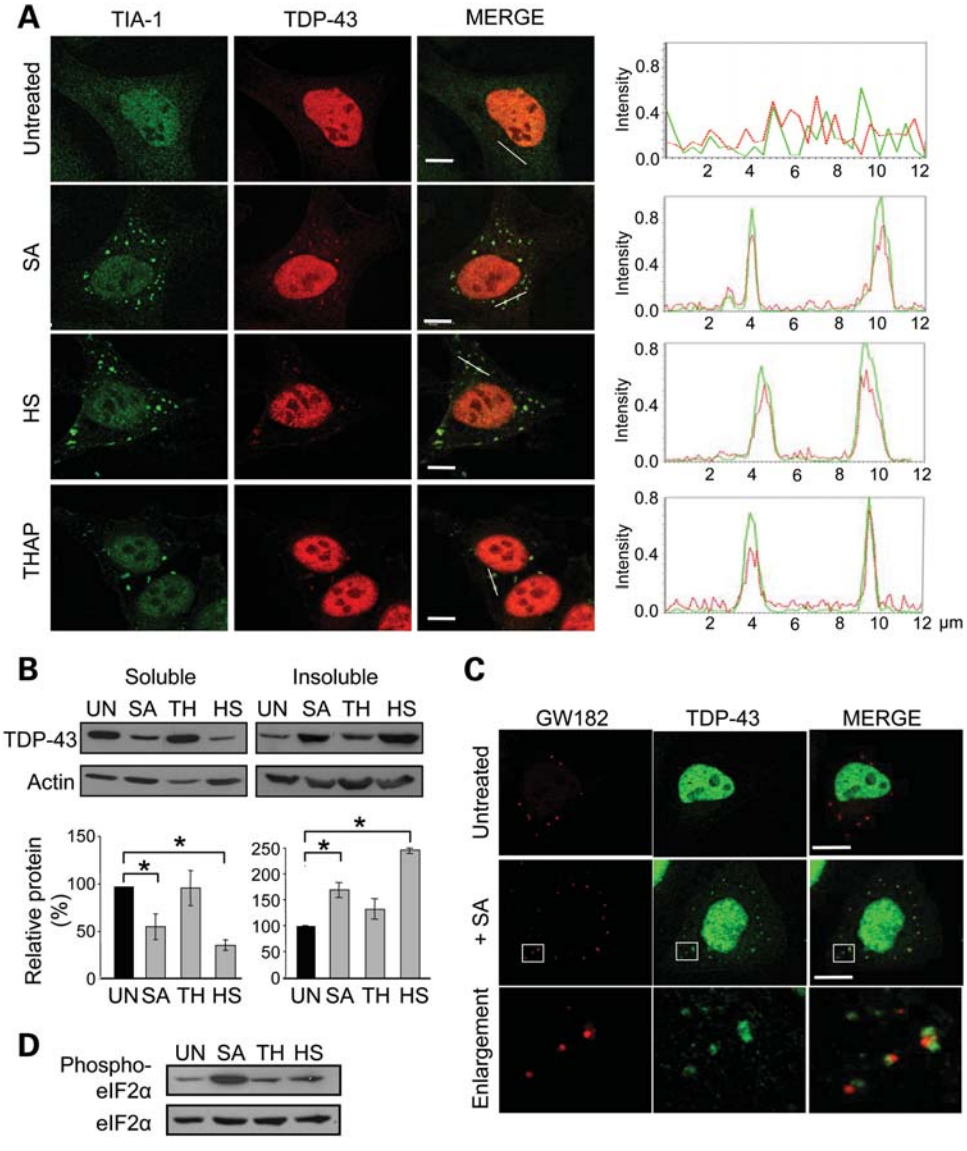

Figure 1. TDP-43 is localized to SGs. (**A**) HeLa cells cultured on cover slips were treated with 0.5 mM SA (30 min), 1 μM THAP (50 min; TH) and 43°C HS (30 min), or left as untreated (UN) and subsequently immunolabeled for TDP-43 and the SG marker TIA-1. TDP-43 localization to SGs is indicated by line scans of the merged images showing the overlap between red and green signals. Scale bar, 10 μm. (**B**) RIPA-soluble and -insoluble extracts were prepared from stressed cells and then immunoblotted for TDP-43. Actin is included as a loading control. Histograms report the mean ± SEM of three independent experiments. $^{*}P < 0.05$. (**C**) TDP-43 was not markedly co-localized with the PB marker, GW182. (**D**) Phosphorylation of eIF2α was assessed in the four stress conditions.

findings were observed in neuroblastoma cells (Supplementary Material, Fig. S1A).

To assess the impact of TDP-43 on SG resolution, we also assessed SG labeling at various time points following the removal of SA ('release'). After 30 min of oxidative stress, TIA-1-labeled SGs are detectable in 97% of control siRNA cells, and are small, numerous and clearly defined (Fig. 2C, left panel). Within 90 min, the number of SG-positive cells continues to be >94%; however, SGs become larger and fewer in number due to the fusion of several smaller SGs and acquire a well defined, compact shape (12). SGs then begin to gradually resolve and, by 270 min (4 h release), few if any cells contain SGs (Fig. 2C). Our observations of this process are consistent with the published kinetics of SG disassembly (12). In TDP-43-depleted cells, there is a striking difference in SG dynamics such that TDP-43-depleted cells are slower to form morphologically distinct SGs compared with control (Fig. 2C, right panel). This difference is most obvious in the first 30 min after SA treatment as the number of SG-positive cells continues to be reduced at 45 and 60 min post-stress, compared with control siRNA cells (Fig. 2B).

SGs in TDP-43-depleted cells have more diffuse TIA-1 labeling (i.e. TIA-1 is not completely localized as distinct foci) and SGs appear smaller with a less-defined and more irregular morphology. Thus, SGs in TDP-43-depleted cells are visibly different compared with the prominent SGs formed in control siRNA cells following the same stress stimulus (Fig. 2C). Quantification of SG size reveals that average SG size is reduced 43% in TDP-43-depleted cells compared with controls (0.83 ± 0.08 versus 1.46 ±

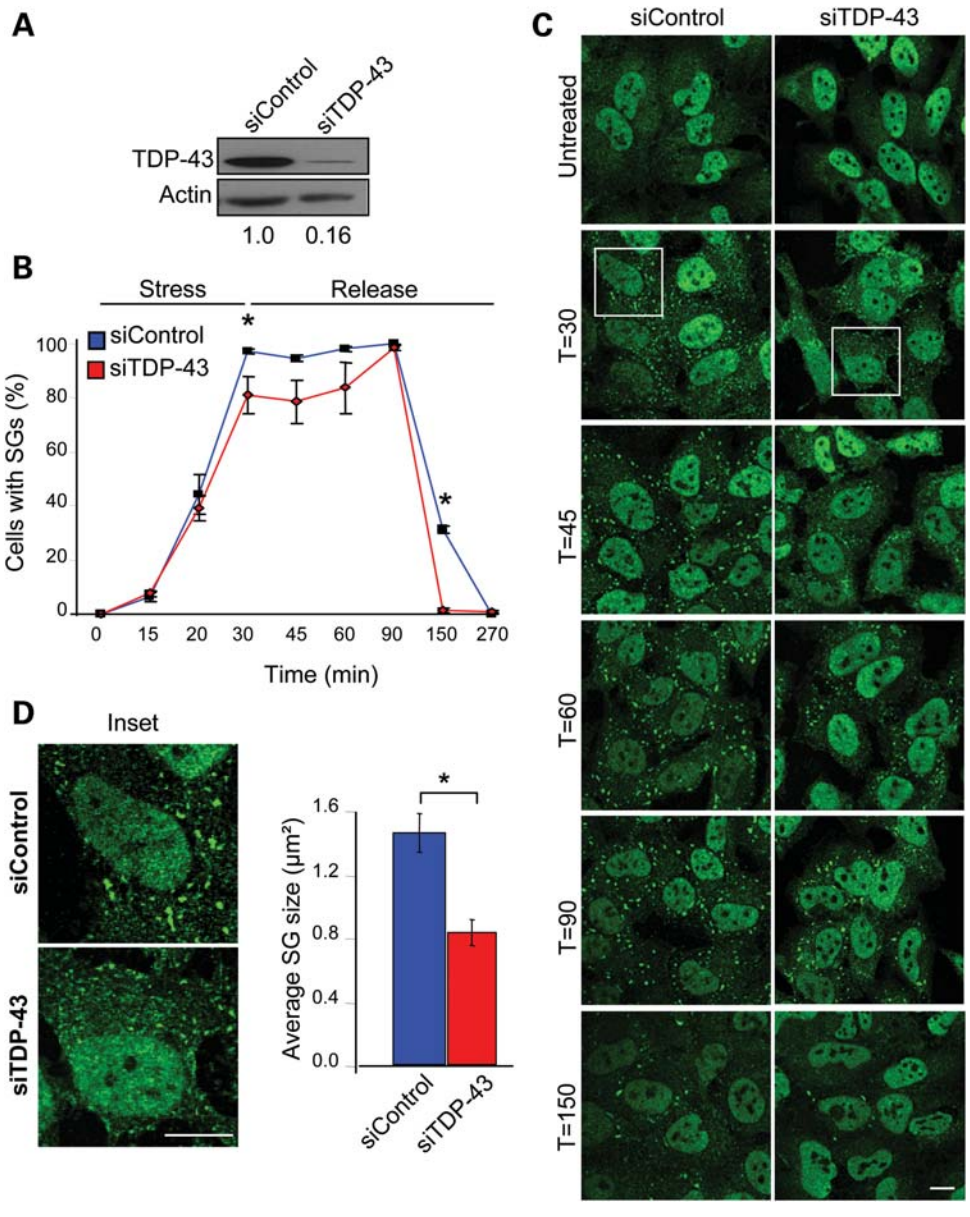

Figure 2. TDP-43 regulates SG dynamics. (**A**) TDP-43 protein levels are reduced by siRNA. Values indicate TDP-43 present as determined by densitometry and are the average of five independent experiments. Data were normalized to actin. (**B**) SG formation and resolution were assessed in HeLa cells transfected with control or TDP-43 siRNA for 72 h and subsequently treated with SA. Cover slips were collected at 0, 15, 20 and 30 min after addition of SA to assess formation ('stress'). After 30 min, the media were replaced and cover slips were collected at 45, 60, 90, 150 and 270 min after the addition of SA ('Release'). SGs were identified by TIA-1 labeling and cells were scored as positive when they had at least two foci of a minimal size of 0.75 um^2. Four fields per condition, representing at least 100 cells, were imaged and the number of cells containing SGs was counted. The means of three independent experiments ± SEM are plotted. *$P <$ 0.05. (**C**) SG morphology in siTDP-43 or siControl HeLa cells treated with SA at 'release' time points labeled with TIA-1. Scale bar, 10 μm. (**D**) SGs are smaller in cells transfected with TDP-43 siRNA. The average area ± SEM is plotted at 30 min post-stress.

0.12 μm^2; $P = 0.0002$) (Fig. 2D). A similar reduction in SG size was also observed in human neuroblastoma cells (SK-N-SH) depleted of TDP-43 (Supplementary Material, Fig. S1B).

Our data indicate that the lag in SG assembly in siTDP-43 cells is overcome by 90 min, such that the number of cells with SGs is equivalent in both TDP-43 and control siRNA cells, and SG size and morphology are comparable. However, at 150 min, only 1% of TDP-43 siRNA cells have obvious SGs, while SGs persist in 31% of control siRNA cells ($P = 0.04$) (Fig. 2C). Therefore, depletion of TDP-43 results in a lag in SG assembly such that SG nucleation and secondary aggregation seem to be delayed. Furthermore, SGs in TDP-43-depleted cells are smaller and only later attain normal-appearing SGs, suggesting a role for TDP-43 in TIA-1 aggregation and SG coalescence. Lastly, these SGs are not sustained and quickly resolve. Thus, endogenous TDP-43 contributes to the establishment of SGs and is required to maintain SGs.

To determine whether TDP-43 depletion confers increased vulnerability to exogenous stress, we evaluated cell death via Annexin V labeling after SA exposure (Supplementary

Material, Fig. S2). Twenty-four hours after the removal of SA, TDP-43-depleted cells were found to be more sensitive than control siRNA cells following acute exposure to oxidative stress (65 ± 3.5 versus $49 \pm 6\%$; $P = 0.05$). Thus, TDP-43 positively contributes to cellular recovery following an acute stress insult.

It has been reported that exogenous expression of some SG components is sufficient to nucleate SGs (19,22). Since our earlier data suggest a role for TDP-43 in SG nucleation, we assessed SG formation in cells transiently transfected with green fluorescent protein-tagged full-length TDP-43. In our hands, overexpression of TDP-43 was not sufficient to nucleate SGs but does itself correctly localize to SGs following SA treatment (data not shown) (16,18).

TDP-43 differentially regulates SG nucleating proteins

TIA-1 and G3BP are both considered primary nucleators of SGs (24). We hypothesized that TDP-43 may regulate the levels of these proteins, either at the protein or mRNA level. Immunoblot analysis of steady-state levels of key SG proteins, including TIA-1, TIAR and G3BP, revealed that TIA-1 was up-regulated 130% ($P = 0.03$) in TDP-43 siRNA cells, while G3BP was down-regulated 79% ($P = 0.05$; Fig. 3A). At the transcriptional level, qPCR with gene-specific primers revealed marked alterations in steady-state mRNA levels. Specifically, we noted a 2.6-fold increase ($P = 0.01$) in TIA-1, while G3BP mRNA was reduced 3-fold ($P = 0.007$; Fig. 3B). A trend towards upregulation of TIAR was also noted at both the protein and mRNA level, but it did not reach statistical significance ($P = 0.059$). Moreover, the effect of TDP-43 depletion was selective for TIA-1 and G3BP since another SG component HuR remained unchanged (Fig. 3B). Thus, TDP-43 differentially modulates the mRNA levels of SG-nucleating proteins.

The phosphorylation of the eukaryotic initiation factor eIF2α at serine 51 is an important early initiating step in SG assembly in response to oxidative stress (10). To determine whether eIF2α signaling was intact in TDP-43-depleted cells, we immunoblotted control and TDP-43 siRNA cell lysates in the presence and absence of SA with an antibody specific for serine-51 phospho-eIF2α. Following SA treatment, we did not observe a remarkable change in phospho-eIF2α levels in the presence or absence of TDP-43, placing TDP-43 downstream of this step of the stress response (Fig. 3C). Moreover, in untreated conditions, the depletion of TDP-43 itself did not induce phosphorylation of eIF2α suggesting that the removal of TDP-43 does not outright trigger an intracellular stress response.

TDP-43 impacts G3BP SGs

Given the significant down-regulation of G3BP in TDP-43 siRNA cells, we investigated the ability of cells to form G3BP-labeled SGs immediately following SA treatment. Control siRNA cells treated with SA contain numerous large SGs labeled with both G3BP and TIA-1. In contrast, when TDP-43 is reduced, there is a generally more diffuse labeling of G3BP and the number of cells with SGs is reduced (Fig. 4). In cells which do form SGs, it appears that the number of G3BP-labeled SGs is markedly reduced and these SGs are much reduced in size compared with their control counterparts. TIA-1 and G3BP co-localization appears to be maintained (Fig. 4).

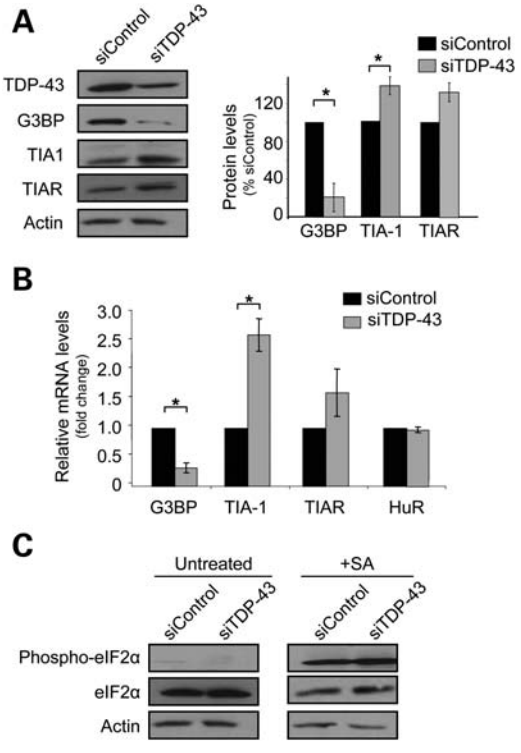

Figure 3. TDP-43 regulates G3BP and TIA-1. (**A**) Western blot analysis of soluble fractions of control and TDP-43 siRNA cells indicates decreased levels of G3BP and increased accumulation of TIA-1 and to a lesser extent TIAR. Data were normalized to actin via densitometry. Data from two to three independent experiments are expressed as the mean fold change ± SEM relative to siControl cells, $^*P < 0.05$. (**B**) qPCR analysis of G3BP, TIA-1 and TIAR. HuR remained unchanged. Data normalized to β-actin and fold change are plotted. $^*P < 0.01$. (**C**) TDP-43 siRNA is not sufficient to induce eIF2α phosphorylation, and this event is not disrupted by TDP-43 depletion.

Endogenous hnRNP A2 is a resident of SGs

hnRNP A2 directly interacts with TDP-43, albeit the functional significance of this interaction remains to be defined (2). Therefore, we hypothesized that hnRNP A2 may also be a component of SGs and/or influenced by TDP-43. Following SA exposure, endogenous hnRNP A2 redistributed so that it was co-localized with TIA-1 (Fig. 5A). Moreover, triple labeling revealed that hnRNP A2 co-localized with TDP-43 in SGs (Fig. 5B) indicating that endogenous TDP-43 and hnRNP A2 are residents of the same SGs in conditions of oxidative stress. We also examined whether TDP-43 is required for hnRNP A2 localization to SGs. Thus, we examined the localization of hnRNP A2 to SGs in cells treated with TDP-43 siRNA and subjected to SA treatment. As before, fewer cells with SGs were observed and SG size was decreased following TDP-43 depletion (Fig. 5C). hnRNP A2 labeling in the absence of TDP-43 showed few distinct puncta which did colocalize to TIA-1-marked SGs. However, the cells appeared to have an

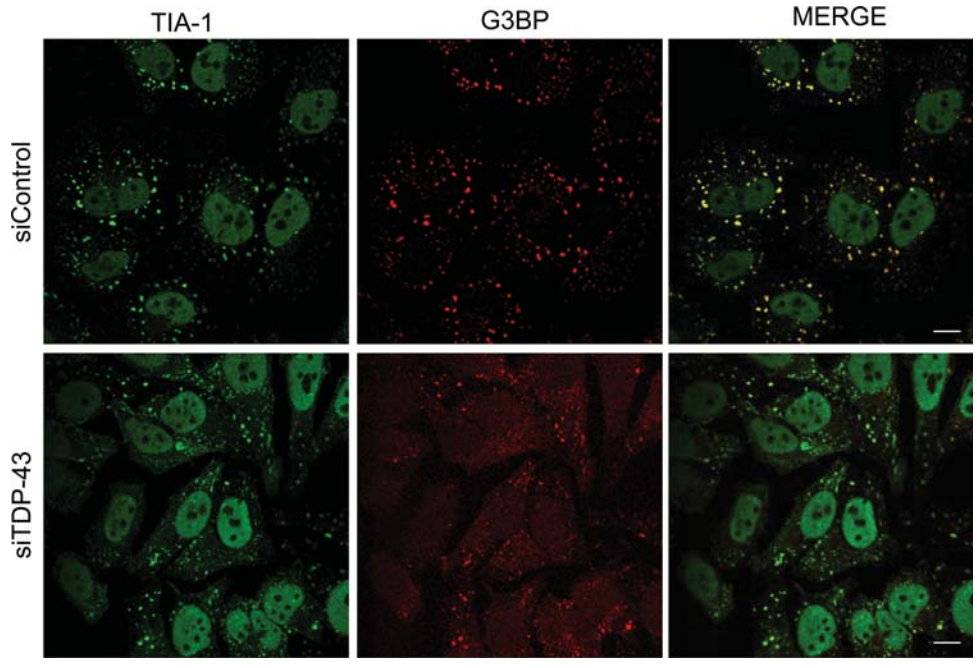

Figure 4. G3BP SG formation is impaired by TDP-43 siRNA. Formation of G3BP SGs was assessed in HeLa cells transfected with control or TDP-43 siRNA for 72 h and subsequently treated with SA. Cover slips were collected immediately and labeled for TIA-1 and G3BP. Data are representative of two independent experiments.

increased patchy distribution within the cytosol compared with control siRNA cells. Thus, TDP-43 influences hnRNP A2 localization to SGs (Fig. 5C).

SG assembly is disrupted by TDP-43^{R361S} mutation

In a cellular overexpression model, mutant fused in sarcoma/translocated in liposarcoma (FUS/TLS) has recently been described to be sufficient to induce SGs (25–27). Thus, we hypothesized that mutations in TDP-43 may alter SG formation in response to oxidative stress. In order to avoid artifacts potentially introduced by transient overexpression of TDP-43, we took advantage of patient lymphoblasts expressing physiological levels of wild-type and two different TDP-43 mutations, TDP-43^{D169G} (located in RRM1) and TDP-43^{R361S} (located in C-terminus). We verified that steady-state levels of the TDP-43 protein were not reduced in the different cells (Fig. 6A). In fact, a slight increase in mutant TDP-43 protein levels was observed such that TDP-43^{D169G} and TDP-43^{R361S} proteins accumulated to 1.2-fold ($P = 0.04$) and 1.3-fold ($P = 0.01$) relative to controls cells expressing wild-type TDP-43. Lymphoblasts were treated with SA, and SG formation was scored as described earlier using TIA-1 as a marker. In control cells expressing a non-pathogenic silent polymorphism (A66A), distinct SGs are robustly present in 10% of the population following oxidative stress (Fig. 6B). In contrast, there was a 2-fold reduction in the number of cells forming SGs in cells expressing TDP-43^{R361S} mutation ($P < 0.03$; Fig. 6C). Interestingly, SG formation in cells expressing TDP-43^{D169G} was comparable with control.

To determine whether mutations in TDP-43 conferred an effect on the accumulated levels of SG components, we examined G3BP and TIA-1 protein levels by immunoblot. While no significant differences in these proteins were detected in cells expressing TDP-43^{D169G}, the G3BP protein was decreased 26% ($P = 0.04$) and TIA-1 was increased 20% ($P = 0.02$) in TDP-43^{R361S} cells (Fig. 6D). These data are similar to what is observed with siTDP-43 and thus suggest that TDP-43^{R361S} is a loss-of-function mutation with respect to SG dynamics.

DISCUSSION

Our studies indicate that TDP-43 contributes to the cellular response to acute stress. Specifically, endogenous TDP-43 is recruited into SGs which are considered to be key elements in the protective response to cellular stress. Moreover, our data demonstrate that TDP-43 participates in regulating SGs such that depletion of TDP-43 delays SG nucleation and secondary aggregation via the differential deregulation of key nucleating factors TIA-1 and G3BP at the mRNA level. Furthermore, the number and size of TIA-1- and G3BP-positive SGs are reduced in cells depleted of TDP-43 and subsequently treated with oxidative stress. TDP-43 therefore contributes positively to SG assembly and their maintenance, as well as cellular survival following acute oxidative stress. Our use of TDP-43 siRNA demonstrates that formed SGs are unable to persist and that they resolve quickly in cells depleted of TDP-43. It is well accepted that following the removal of a stress, SGs disassemble, and the majority of released mRNAs are recruited back to the translation machinery (28). Thus, the contribution of TDP-43 to the assembly and disassembly of SGs offers an important mechanism by which TDP-43 may regulate gene expression in response to stress as well as cellular recovery/survival.

Interestingly, TDP-43 has been reported to interact with TIA-1 and TIAR, both core nucleating components of SGs

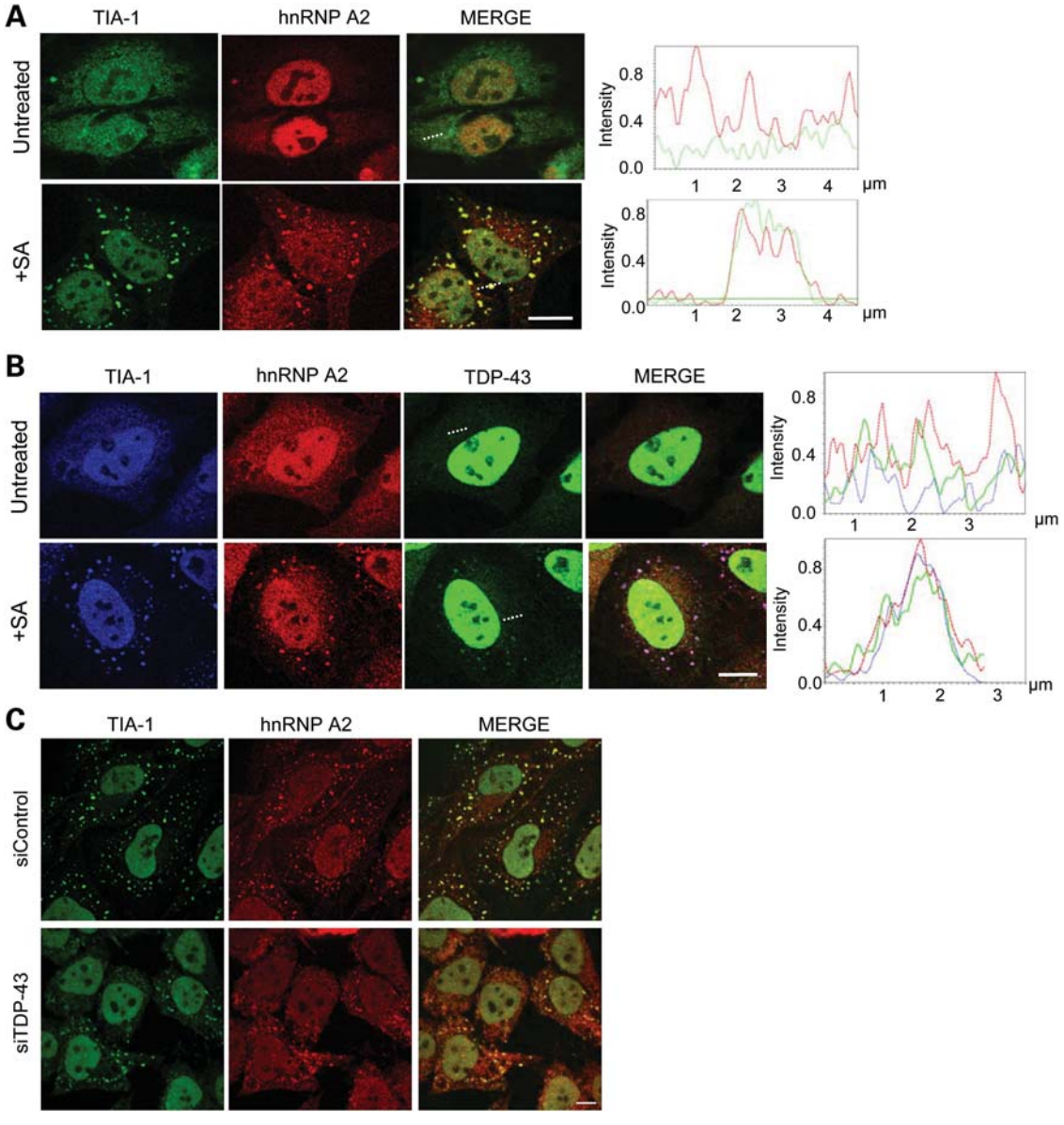

Figure 5. Endogenous hnRNP A2 is localized to SGs. HeLa cells cultured on cover slips were treated with SA or left as untreated (UN) and subsequently immunolabeled for (**A**) hnRNP A2 and TIA-1 or (**B**) hnRNP A2, TDP-43 and TIA-1. Scale bar, 10 μm. hnRNP A2 co-localization to SGs is quantified by line scans of the merged images showing the overlap between red and green (and blue) signals. Scale bar, 10 μm. (**C**) Localization of hnRNP A2 to SGs (marked with TIA-1) was assessed in HeLa cells transfected with control or TDP-43 siRNA for 72 h and subsequently treated with SA. Cover slips were collected immediately and labeled for TIA-1 and hnRNP A2. Scale bar, 10 μm. Data are representative of three independent experiments.

(16,17). Our study shows that TDP-43 directly modulates the expression of TIA-1 and G3BP (and less so TIAR), providing a potential mechanism for the impact of TDP-43 on SG dynamics. Our immunofluorescence data demonstrate that TDP-43 can modulate TIA-1 aggregation, thus we speculate that the association between TDP-43 and TIA-1 facilitates the homotypic interactions of TIA-1 which are required for SG assembly and maintenance. The down-regulation of G3BP mRNA levels by TDP-43 also yields fewer SGs. Thus, we propose that TDP-43 regulates SG formation via two independent mechanisms. However, we cannot rule out that the impact of TDP-43 on G3BP is the mechanism which affects the controlled aggregation of TIA-1.

hnRNP A2 interacts with TDP-43 (2). In the presence of acute oxidative stress, endogenous hnRNP A2 was also recruited to SGs. This is the first description of hnRNP A2 localization to SGs and our data indicate that it may be partially dependent on TDP-43. Specifically, the down-regulation of TDP-43 yielded fewer cells with SGs and smaller hnRNP A2-labeled SGs. Whether this is due to the direct action of TDP-43 on hnRNP A2 or a consequence of slowed SG assembly due to TDP-43 regulation of G3BP transcripts and/or TIA-1 aggregation remains to be clarified.

The localization of TDP-43 to SGs, the regulation of SG proteins by TDP-43 and the delay in SG assembly and maintenance in the absence of TDP-43 suggest a novel function for

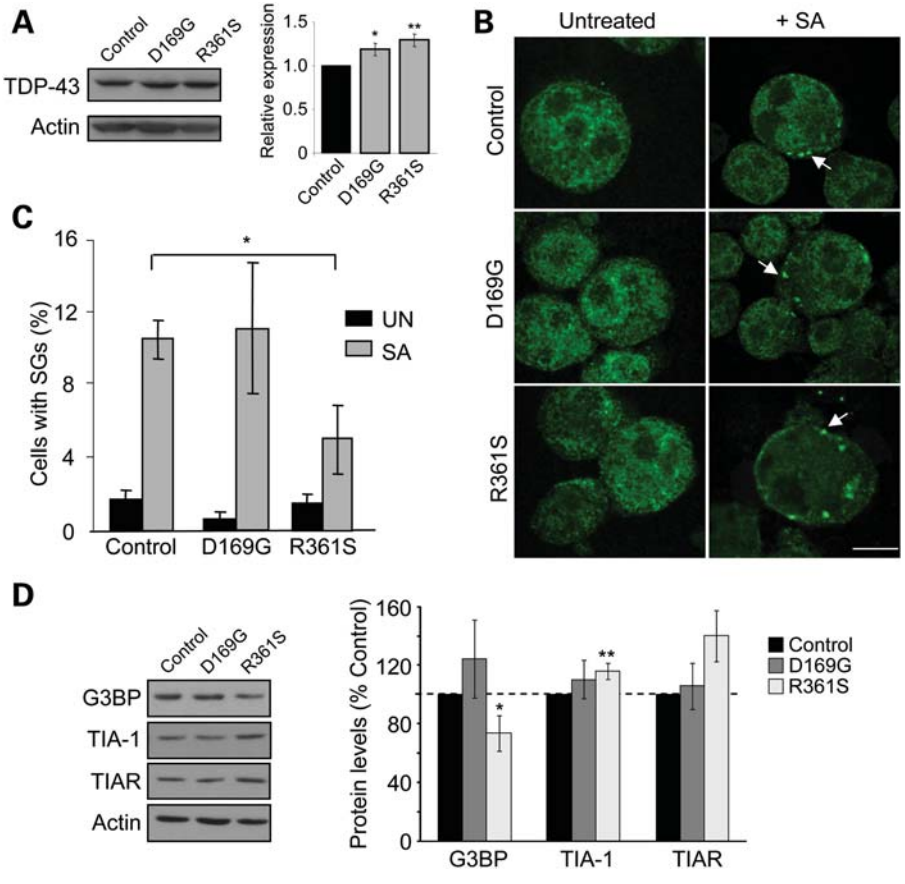

Figure 6. The disease-causing mutation TDP-43^{R361S} impacts SG formation. (**A**) Immunoblot of steady-state TDP-43 protein levels in RIPA cell lysates from control human lymphoblasts or patients expressing the disease-causing mutations TDP-43^{D169G} and TDP-43^{R361S}. Data are representative of four experiments. Histogram indicates quantification via densitometry. $^{*}P = 0.04$; $^{**}P = 0.01$. (**B**) Control and mutant patient cells were treated with SA or left untreated (UN) and labeled with TIA-1. SGs are indicated with arrows. (**C**) The number of SG-positive cells was counted from a minimum of three fields representing at least 100 cells. The mean ± SEM of four independent experiments is plotted. $^{*}P < 0.03$. Scale bar, 10 μm. (D) G3BP, TIA-1 and TIAR protein levels in control and patient cell lysates. Actin is a loading control. Data are representative of three to six independent experiments. Histogram indicates quantification via densitometry. $^{*}P = 0.04$; $^{**}P = 0.02$.

TDP-43 in acute stress. It will be interesting to evaluate the role of TDP-43 in models of chronic stress. Our observation that cells expressing the mutation TDP-43^{R361S} have deregulated G3BP and TIA-1 levels and are hampered in their ability to form SGs is interesting and suggests a potentially disease-relevant mechanism. The observations in these cells are reminiscent of that observed when TDP-43 is depleted, thus suggesting that TDP-43^{R361S} is a loss-of-function mutation with regard to SG dynamics. In a transient overexpression culture model, it has previously been reported that TDP-43^{R361S} reduces mRNA expression of the histone deacetylase HDAC6 (29). Separately, the down-regulation of TDP-43 results in reduced transcription of HDAC6 (29) (data not shown). HDAC6 has been published as an important determinant in SG assembly (30), and TDP-43 and FUS/TLS have recently been reported to cooperatively regulate HDAC6 mRNA (31). In addition to its role in SG dynamics, HDAC6 is also involved in the removal of misfolded proteins and aggresome formation (32). Large cytoplasmic aggregates are a feature of ALS, thus deregulation of HDAC6 by TDP-43 mutations is a very interesting target worthy of future investigation.

In the context of ALS, mutations in TDP-43 could compromise the cellular stress response such that one could envision successive cycles of a weakened stress response leading to a maladaptive state. Successive encounters with oxidative stress would eventually overcome the cell's ability to manage the stress, and ultimately result in cellular demise (Fig. 7). Furthermore, it remains possible that large pathological aggregates arise due to disrupted SG dynamics (and/or prolonged exposure to stress) as has been proposed in Parkinson's disease (33). This hypothesis has gained support from the recently described colocalization between SG makers and neuronal cytoplasmic inclusions in cases of familial ALS bearing a mutation in the RBP FUS/TLS (27). In addition, the multiple reports of TDP-43 and FUS/TLS localization to SGs in response to acute exogenous stress (16–18,25–27) emphasize that the links between disturbed cellular SG dynamics and neurodegenerative disease may be relevant to pathogenesis. Importantly, we report here SG formation is not disturbed by the TDP-43^{D169G} mutation, suggesting that it is mechanistically independent of the reported C-terminal mutants. The nature of this mutation, predicted to be mRNA binding, remains to be demonstrated.

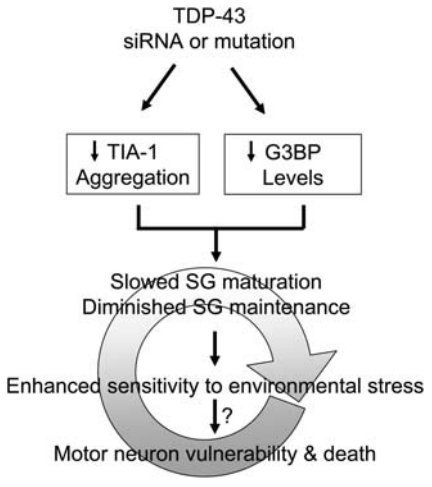

Figure 7. Model of TDP-43 in regulation of SGs. Reduced TDP-43 protein levels or TDP-43 mutations yield a reduction in G3BP levels and disrupt TIA-1 aggregation. These events yield slowed and diminished SG formation and poor maintenance. This may increase cellular susceptibility to acute stress stimuli and contribute to cellular death. This could set up a feed-forward amplification loop resulting in a maladaptive state in motor neurons, thereby contributing to an increased vulnerability over time.

We describe a mechanistic role for endogenous TDP-43 in the cellular stress response which may be a disease relevant mechanism. Defining the relevance of this aspect of TDP-43 biology in motor neurons and ALS is now an ensuing challenge. Finally, our data emphasize that the pathogenic affects of TDP-43 mutations may be mechanistically divergent.

MATERIALS AND METHODS

Plasmids, cell culture and transfection

HeLa and patient lymphoblast cell lines were grown in Dulbecco's modified Eagle medium and Iscove's modified Dulbecco's medium, respectively, supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin/glutamate. For transfection of siRNA, 125 pmol of custom siRNAs were transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Control and TDP-43 siRNA sequences were 5′-AAUUCCAGUUAUUGUAUAUAUUCAG-3′ and 5′-AAGCAAAGCCAAGAUGAGCCUUUGA-3′, respectively (Invitrogen). Cells were transfected at 30–50% confluency. Transfection media were replaced with regular culture media without antibiotics after 5 h. Cells were collected after 72 h of siRNA treatment.

Cells were treated with various stresses, including 0.5 mM SA (30 min, 37°C; Sigma), HS (30 min, 43°C) and 1 μM THAP (50 min, 37°C; Sigma). For stress recovery experiments, cells were stressed and then media were replaced, and cells were permitted to recover for various times prior to collection and fixation. Cell death was measured using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's instructions. Total Annexin V-positive cells are plotted.

Immunofluorescence and antibodies

Cells grown on cover slips were fixed in 1% formaldehyde in phosphate buffered saline (PBS) and subsequently permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cover slips were blocked with 0.1% bovine serum albumin (BSA) in PBS for 15 min and incubated with antibodies to TDP-43 (1:300; Proteintech), TIA-1 (1:100; Santa Cruz), hnRNP A2 (1:100; Abnova), G3BP (1:400; BD Biosciences) and GW182 (1:50; M. Fritzler) diluted in blocking buffer for 1 h at room temperature. Cover slips were subsequently washed once with 0.1% Triton X-100 in PBS and twice with 0.1% BSA in PBS. Labeling was visualized with the fluorescently conjugated secondary antibodies donkey anti-mouse Texas Red (Jackson Immunochemicals), donkey anti-rabbit FITC (Jackson Immunochemicals), goat anti-human Alexa 555 (Invitrogen) and donkey anti-goat Alexa 633 (Invitrogen). Cover slips were washed as before, and mounted with ProLong Antifade reagent (Invitrogen). Lymphoblasts were affixed to Superfrost charged slides via cytospin and then subsequently fixed and labeled, as previously described (34). Images were collected on a Leica SP5 confocal microscope.

Quantification of SG size

SGs were identified by TIA-1 staining and cells were scored as positive when they had at least two foci of a minimal size of 0.75 μm^2. The area of 10 SGs (ranging from 0.75 to 5 μm^2), randomly selected in at least 10 cells per condition, was manually measured with ImageJ. The average SG size of at least 100 SGs in 10 cells is presented.

Cell lysates and immunoblot analysis

Cells were collected in ice-cold PBS, lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) and centrifuged at 16 000*g*. Supernatants were collected and quantified with the BCA Protein Assay Kit (Thermo Scientific). For fractionation of soluble and insoluble components, cells lysed in RIPA buffer were passed through a 25 G syringe six times and centrifuged at maximum speed. Supernatants (soluble) were recovered and pellets (insoluble) were resuspended directly in 1× Laemmli sample buffer. Equal volumes of each fraction were separated by SDS–PAGE. The following antibodies were used in immunoblotting: rabbit anti-TDP-43 (1:5000; Proteintech), goat anti-TIA-1 (1:500; Santa Cruz), goat anti-TIAR (1:200; Santa Cruz), mouse anti-G3BP (1:600; BD Biosciences), rabbit anti-eIF2α (1:1000; Cell Signaling), rabbit anti-Phospho eIF2α (1:1000; Cell Signaling) and mouse anti-actin (1:400 000; MP Biomedicals). Blots were visualized with peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific). Densitometry was performed with ImageJ.

qPCR

RNA was extracted with RNAeasy kit (Qiagen) and reverse transcribed with QuantiTect (Qiagen). Resulting cDNA was processed for qPCR with SybrGreen (Biorad) according to the manufacturer's instructions using the following primer sets: β-actin exon 5 F: 5′-CGTTGGCATCCACGAAACTA-3′; β-actin exon 6 R: 5′-AGTACTTGCGCTCAGGAGGA-3′; TIA-1 exon 12 F: 5′-CATGGAACCAGCAAGGATTT-3′;

TIA-1 exon 13 R: 5′-CACTCCCTGTAGCCTCAAGC-3′; TIAR exon 11 F: 5′-GCCAATGGAGCCAAGTGTAT-3′; TIAR intron 12 R: 5′-CATATGCGGCTTGGTTAGGA-3′; G3BP exon 11 F: 5′-TAATCGCCTTCGGGGACCTG-3′; G3BP exon 11 R: 5′-AAGCCCCCTTCCCACTCCAA-3′; HuR exon 4/5 junction: 5′-CGCAGAGATTCAGGTTC TCC-3′; and HuR exon 5 R: 5′-CCAAACCCTTTGCACT TGTT-3′.

Statistical analysis

Data were analyzed by Student's *t*-test or one-way ANOVA, where appropriate. Error bars represent standard error of the mean (SEM).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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