Université de Montréal

TAR DNA-Binding Protein 43 (TDP-43) Regulates Stress Granule Dynamics via Differential Regulation of G3BP and TIA-1

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RÉSUMÉ

TDP-43 est une protéine multifonctionnelle possédant des rôles dans la transcription, l'épissage des pré-ARNm, la stabilité et le transport des ARNm. TDP-43 interagit avec d'autres hnRNP, incluant hnRNP A2, via son extrémité C-terminale. Plusieurs membres de la famille des hnRNP étant impliqués dans la réponse au stress cellulaire, alors nous avons émis l'hypothèse que TDP-43 pouvait y participer aussi. Nos résultats démontrent que TDP-43 et hnRNP A2 sont localisés au niveau des granules de stress, à la suite d'un stress oxydatif, d'un choc thermique, et lors de l'exposition à la thapsigargine. TDP-43 contribue à la fois à l'assemblage et au maintien des granules de stress en réponse au stress oxydatif. TDP-43 régule aussi de façon différentielle les composants clés des granules de stress, notamment TIA-1 et G3BP. L'agrégation contrôlée de TIA-1 est perturbée en l'absence de TDP-43. En outre, TDP-43 régule le niveau d`ARNm de G3BP, un facteur de granule de stress de nucléation. La mutation associée à la sclérose latérale amyotrophique, TDP-43^{R361S}, compromet la formation de granules de stress. Ainsi, la fonction cellulaire de TDP-43 s'étend au-delà de l'épissage; TDP-43 est aussi un composant de la réponse cellulaire au stress central et un acteur actif dans le stockage des ARNs.

Mots clés: TDP-43, granule de stress, stress cellulaire, TIA-1, G3BP, hnRNP A2, sclérose latérale amyotrophique

ABSTRACT

TDP-43 is a multifunctional protein with roles in transcription, pre-mRNA splicing, mRNA stability and transport. TDP-43 interacts with other 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, following oxidative stress, heat shock, and exposure to thapsigargin. TDP-43 contributes to both the assembly and maintenance of stress granules in response to oxidative stress and differentially regulates key stress granules components including TIA-1 and G3BP. The controlled aggregation of TIA-1 is disrupted in the absence of TDP-43. In addition, TDP-43 regulates G3BP mRNA levels, a stress granule nucleating factor. A mutation associated with amyotrophic lateral sclerosis, TDP-43^{R361S}, compromises stress granule formation. Thus, 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.

Key words: TDP-43, stress granule, cellular stress, TIA-1, G3BP, hnRNP A2, amyotrophic lateral sclerosis

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List of Abbreviations

ALS	Amyotrophic Lateral Sclerosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CSR	Cellular Stress Response
CTF	Carboxy-Terminal Fragments
eIF2α	Eukaryotic Initiation Factor 2 alpha
FALS	Familial Amyotrophic Lateral Sclerosis
FTLD	Frontotemporal Lobar Degeneration
FTLDU	Frontotemporal Lobar Degeneration with Ubiquitin Positive Inclusions
FUS/TLS	Fused in Sarcoma / Translocated in Liposarcoma
G3BP	Ras GTPase-Activating Protein-Binding Protein 1
GRD	Glycine Rich Domain
GW182	Glycine-Tryptophan 182
HDAC	Histone Deacetylase
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
HS	Heat Shock
HSP	Heat Shock Protein
mRNA	Messenger RNA
miRNA	MicroRNA
NLS	Nuclear Localization Signal
PB	Processing Body
PRD	Prion Related Domain
RBP	RNA Binding Protein
RRM	RNA Recognition Motif
SA	Sodium Arsenite
SALS	Sporadic Amyotrophic Lateral Sclerosis

SG	Stress Granule
SOD1	Superoxide Dismutase 1
TB	TDP Bodies
TDP-43	Transactive Response Region DNA Binding Protein 1
THAP	Thapsigargin
TIA-1	T-cell Internal Antigen-1
TIAR	TIA Related Protein
UBI	Ubiquitin Positive Inclusion

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Thank you.

A. INTRODUCTION

I. Cellular Stress

1.1. The Cellular Stress Response

Upon exposure to an environmental stress such as mechanical, chemical, heat, osmotic or oxidative, mammalian cells can activate a variety of different response mechanisms, which ultimately depend on the type of insult and severity [1]. In general, if the stress stimulus does not go beyond a certain threshold, the cell can adapt by activating protective responses. These responses are part of the Cellular Stress Response (CSR) and typically include cellular growth arrest and translational control mechanisms, the heat shock and unfolded protein responses, and the DNA damage response necessary for nucleic acid and chromatin repair [2]. However, if the insult exceeds cellular tolerance limits, cellular death pathways such as apoptosis and necrosis will be activated [2]. The CSR is an integral part of normal physiology and crucial for survival. As such, it is also highly conserved throughout evolution.

Since stressful stimuli can impinge on normal cellular functioning, multiple sensors are in place to carefully monitor the environment. Sensors can be in the form of surface/membrane-anchored receptors such as tumor necrosis factor α, cysteine-rich elements in the cytoplasm, or nuclear proteins such as ataxia-telangiectasia mutated [3]. When a threat is detected, a cascade of signals is initiated typically through the activation of secondary intracellular messengers such as stress specific kinases. These kinases phosphorylate

substrates, which in turn trigger a succession of down-stream molecules [4]. For example, heat shock activates pathways involving stress-activated protein kinase p38, and leads to the phosphorylation of the Heat Shock Protein (HSP) 27 [5, 6]. Phosphorylation of HSP27 activates a protective function either as a chaperone or as an inhibitor of apoptotic processes [7-9]. In the case of osmotic shock, activation of the stress-activated Jun NH_2-terminal kinase is triggered by the activation of the receptors for epidermal growth factor and tumor necrosis factor [10]. During oxidative stress, sensing is achieved via apoptosis signal-regulating kinase-1, a mitogen-activated protein kinase kinase kinase that can activate other mitogen-activated protein kinases such as p38 or Jun NH_2-terminal kinase [11]. These pathways exert their phenotypic influence through the modulation of transcription factors, which ultimately dictate how a cell responds to the insult by altering gene expression [1, 12].

The transcriptional regulation of gene expression can be induced by signaling pathways that implement changes in chromatin metabolism, followed by changes in the activities of transcription factors [13]. In addition, eukaryotes have developed a more complex system of gene regulation by the introduction of posttranscriptional regulation. Such regulation can occur at multiple steps during messenger RNA (mRNA) biogenesis and is particularly important during development, as there are periods when transcription is absent [14]. Posttranscriptional regulation is also now widely accepted as a potent modulator for gene expression during cellular stress, leading to a global repression of protein translation [14]. Translational repression during harmful

conditions is essential to cell survival as it permits specific stress-induced transcripts to receive highest translational priority. The silencing of non-essential stress proteins is achieved through the formation of stress granules (SG).

1.2. Stress Granules

A key element of the CSR is the re-programming of RNA metabolism in order to repair stress-induced damage [15]. During this process, the translation of "house-keeping" proteins is halted, and proteins that are essential for protection and survival become the focus of the translation machinery. Non-essential mRNAs accumulate in large, dynamic, cytoplasmic foci called SGs. Therefore, the major role of SGs is translational repression and their formation correlates with decreased global translation [16]. SGs were first identified upon heat shock in tomato cell cultures, and have since been identified in mammalian cells [17, 18]. SGs are now considered to be sites of mRNA triage where one of three mRNA fates can ensue: re-initiation, storage, or decay [18]. SGs also recruit proteins involved in metabolic signaling pathways, enabling the assembly of SGs to influence cell metabolism and survival [19, 20].

1.2.1. Induction of Stress Granules

The key event leading to the formation of SGs is the phosphorylation of the translation initiation factor, Eukaryotic Initiation Factor 2 α (eIF2α) [21]. Stress-induced phosphorylation occurs at Ser-51 of the α subunit [22-24]. There are four different protein kinases known to phosphorylate eIF2α, each of which have a unique regulatory domain in order to respond to different stress stimuli

[25]. eIF2α is a heteromeric GTP-binding protein that functions in protein translation by forming a complex with eIF2–GTP–$tRNA^{Met}$ and the 40S ribosomal subunit [25]. The phosphorylation of eIF2α during an insult reduces the availability of the eIF2-GTP-$tRNAi^{Met}$ complex, leading to stalling of the 40S ribosome on the mRNA. As a result, a pre-initiation complex is formed that cannot recruit the 60S ribosomal subunit to begin protein translation followed by polysome disassembly [26]. The stalled complexes of 40S ribosomal subunits along with initiation factors, and their associated mRNA transcripts, assemble into SGs (Fig 1.1) [25]. SG formation can also be induced by other stimuli in addition to eIF2α phosphorylation such as acute starvation or treatment with puromycin, which promote polysome disassembly [21].

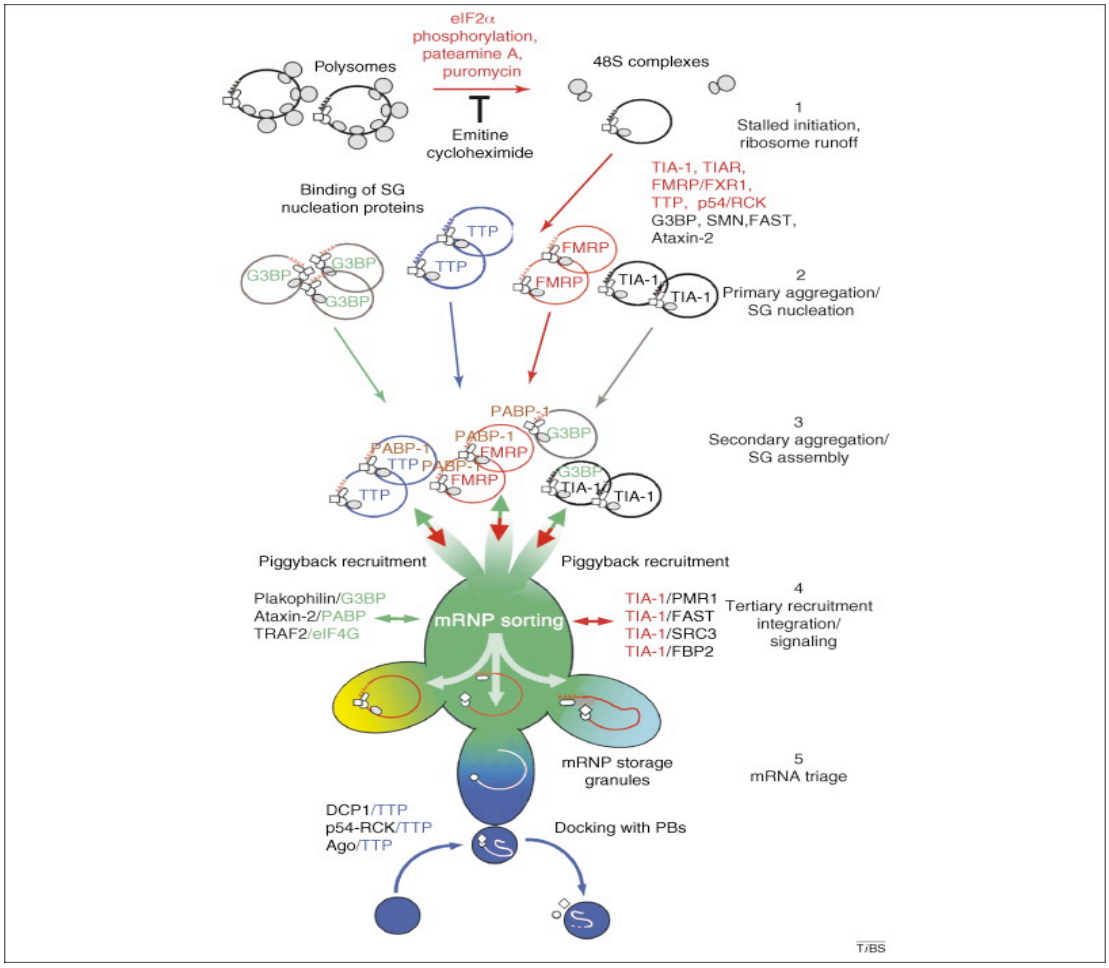

Figure 1.1. Model of stress granule assembly [18]. The process of SG assembly is initiated via the phosphorylation of eIF2α, which results in polysome disassembly. Stalled ribosomal complexes, initiation factors, and their associated mRNA transcripts, assemble into SGs. TIA-1 forms the initial SG aggregates onto which primary nucleating SG proteins bind, followed by secondary aggregation of other SG components. mRNPs sorted in SGs can either be stored, degraded, or prepared for re-initiation once the insult is removed. (Figure adapted from Anderson, P. and N. Kedersha (2008). *Trends Biochem Sci*)

1.2.2. TIA-1 and Stress Granule Assembly

Following eIF2α phosphorylation, SG formation requires T-cell Internal Antigen-1 (TIA-1) and TIA Related Protein (TIAR) [26, 27]. TIA-1 and TIAR are nucleocytoplasmic RNA Binding Proteins (RBP) that are particularly abundant in the brain, testis and spleen [26]. TIA proteins are important for proper development as mice that lack TIA-1 or TIAR exhibit high embryonic lethality [26]. They have also been shown to participate in specific splice-site selection in the nucleus however, TIA proteins are best known for their role in SGs [26]. TIA-1 is the key nucleating protein for SG assembly where over-expression of recombinant TIA-1, in the absence of an insult, can induce SGs [27]. Moreover, mouse embryonic fibroblasts lacking TIA-1 are unable to form *bonafide* SGs [28]. SGs appear to be relatively stable however, TIA-1 shuttles rapidly in and out of these structures. This indicates TIA-1 also likely plays a role in actively shuttling untranslated mRNA to SGs [26, 29]. The dynamic nature of TIA-1 may be an important aspect of gene regulation. By regulating the equilibrium between SGs and polysomes, TIA-1 can influence the frequency with which specific transcripts are sorted for translation [26]. The key role for TIA-1 in SG formation is largely attributed to its structure.

TIA proteins are composed of three amino-terminal RNA Recognition Motifs (RRM) and a Carboxy (C)-terminal glutamine rich motif that is structurally related to prion protein [30]. The Prion Related Domain (PRD) can self-oligomerize forming the initial SG aggregates. TIA-1 aggregates serve as

scaffolds where the stalled pre-initiation complexes from the phosphorylation of eIF2α are routed. True SGs are then formed consisting of polyadenylated mRNAs, translation initiation factors, small ribosome subunits and RBPs [28]. Therefore, while PRD aggregation is initiated by the accumulation of stalled pre-initiation complexes, Gilks and colleagues have found that aggregation is also regulated by HSP70 [28]. By comparing the subcellular localization of exogenously expressed constructs for TIA-1 PRD and HSP70, the authors have demonstrated that in the absence of HSP70, PRD accumulates in cytoplasmic aggregates. Consistently, expression of HSP70 leads to the disappearance of the cytoplasmic aggregate and increased nuclear expression. Therefore, HSP70 is required to maintain TIA-1 in a nuclear, soluble form. Other factors that regulate TIA-1 aggregation have not yet been fully characterized; however, the identification of these factors is important since TIA-1 aggregation regulates gene expression [27]. Moreover, such factors may be important in mediating cell survival during stress since aggregation of TIA-1 is reversible in cells exposed to a sublethal stress, but irreversible in cells exposed to a lethal stress (e.g. 0.5 mM versus 2 mM arsenite for one hour, respectively) [27].

The over-expression of other SG components, such as Ras GTPase-Activating Protein-Binding Protein 1 (G3BP), is sufficient to induce SGs similar to TIA-1 [31]. Additional proteins, recruited as complexes, are also required for SG formation such as RBPs including poly(A)-binding protein 1, the mRNA stabilizing component HuR, and other eukaryotic initiation factors including eIF3, eIF4, and eIF4G [26, 29]. SGs appear 15-20 minutes following

exposure to stress, and are quite variable depending greatly on the types of Messenger Ribonucleoproteins (mRNP) present in the granules [32]. Furthermore, SG assembly is a transient event where once the stress is removed, the cell recovers with concurrent SG dispersal over 1.5-3.0 hours (provided that the damage does not exceed cellular thresholds) [26]. Some SG components, such as Staufen 1, actually promote SG disassembly [33]. SG disassembly is believed to correlate with the recovery of bulk protein synthesis such that the translation of mRNAs essential for normal cellular survival resumes [34]. SGs have been shown to interact with another type of RNA granule: Processing Bodies (PB).

1.3 Processing Bodies

PBs are cytoplasmic foci that function as small sites for mRNA degradation. There are two major mechanisms of mRNA degradation active in mammalian cells. One is initiated by deadenylation, followed by 3'-5' exonucleolytic cleavage performed in the exosome [35]. The second is the removal of the 5'cap on RNA by the decapping complex, Dcp1-Dcp2, followed by 5'-3' exonucleolytic cleavage of the transcript [36]. Components of the second pathway are found in PBs, which are essentially characterized by the presence of the RBP, glycine-tryptophan 182 (GW182), decapping enzymes, and decapping associated proteins [37]. Under physiological conditions, mRNAs destined for degradation can be targeted to PBs by microRNA (miRNA) [38]. Therefore, unlike SGs, PBs are prominent in actively

proliferating, unstressed cells, and are important in maintaining the balance between mRNA translation and decay [39]. The role of PBs in the CSR is the degradation of mRNAs that have been sorted and transported from SGs [35]. Thus, there are two subsets of PBs: one independent and motile in the cytoplasm, and the other stably attached to SGs [35]. Therefore, SGs and PBs are morphologically distinct structures and each can be assembled in the absence of the other. Moreover, they are functionally linked and represent a dynamic organization of cytoplasmic mRNPs.

In the cytoplasm of eukaryotic cells, several RBPs are frequently found in both PBs and SGs [27, 40]. In particular, several studies have indicated that one group of RBPs, the Heterogeneous Nuclear Ribonucleoproteins (hnRNP), are directly involved in cellular responses to various stresses. For example, Guil and colleagues have shown that activation of the p38 stress-signaling pathway in mammalian cells results in both the phosphorylation and cytoplasmic accumulation of hnRNP A1 in SGs [41]. Similarly, Quaresma and colleagues have demonstrated that under specific stress conditions (e.g. thapsigargin, heat shock and arsenite) nuclear hnRNP Q is redistributed to the cytoplasm and partially co-localizes to SGs and PBs [42]. hnRNP K is also a component of SGs and plays a role in the maintenance of cellular ATP levels during stress conditions [15].

II. Heterogeneous Nuclear Ribonucleoproteins

2.1. The hnRNP proteins: Overview

The hnRNP proteins are defined as RBPs that bind pre-mRNA, but are not stable components of other classes of RNP complexes [43]. mRNAs are formed from the extensive posttranscriptional regulation of primary transcripts from protein encoding genes [44, 45]. These transcripts, termed hnRNAs, are the precursors to mRNA, and hence are more commonly known as "pre-mRNA" [46]. As pre-mRNAs emerge from the transcription complex, and throughout the time in the nucleus, they are associated with proteins. These proteins include small nuclear ribonucleoproteins and splicing factors, however the majority consists of hnRNPs [43, 47]. hnRNPs associate with pre-mRNA and greatly influence their fate for example, by targeting specific processing factors to defined sites within pre-mRNA [43, 48]. These proteins, like histones, are of great interest because of their nuclear abundance [46]. Some hnRNPs are present as approximately 100 million copies per nucleus. Furthermore, in humans, at least 24 members of the hnRNP family, designated hnRNP A1 through hnRNP U, exist ranging in size from 34 to 120 kDa [46, 48, 49]. hnRNPs form complexes that are diverse, large and typically containing numerous dynamic proteins [48]. Furthermore, there is evidence that hnRNP functions, such as mRNA maturation and transport, are accompanied by changes in the protein composition of hnRNP complexes [50].

2.2. hnRNP Proteins are Multifunctional

Initially, it was hypothesized that the functions of hnRNPs were mostly structural, such as packaging nascent pre-mRNA as a means of protection and

organization [43]. It was later shown that hnRNPs have more diverse subcellular functions. For example, hnRNP K functions as a transcriptional activator by interacting with cis-acting elements and RNA polymerase II transcription machinery [51, 52]; hnRNP E2 functions as a stabilizing factor for α-globin mRNA [53]; approximately 10% of all alternative splicing events are controlled by hnRNP A1 and H [54]; hnRNP A2 binds the SET oncoprotein, a key regulator of DNA replication and chromatin remodeling [55]. The relative levels of hnRNPs are also important and can influence alternative splicing, thus affecting the expression of a wide variety of genes. Additionally, hnRNPs are responsible for the proper functioning of mRNAs by providing information that specifies their nuclear export and subcellular localization. Moreover, hnRNPs can remain bound to mRNAs up to the ribosome, indicating roles in translation and stability [48]. Therefore, these multifunctional proteins are involved in virtually every aspect of mRNA biogenesis and gene regulation.

2.3. hnRNP Structure Overview

Under physiological conditions hnRNPs are primarily concentrated in the nucleus, however a subset of the proteins shuttle constantly between the nucleus and cytoplasm [50]. The structural characterization of several hnRNPs has revealed that shuttling is accomplished via specific amino acid sequences that function as Nuclear Export Signals (NES) [56]. hnRNPs that are unable to shuttle have specific nuclear retention signals, which prevent them from exiting the nucleus. Therefore, it has been suggested that shuttling hnRNPs accompany

mRNAs during their passage through nuclear pore complexes, while non-shuttling hnRNPs are removed at the time of, or prior to, mRNA export [49, 57]. Another common feature of hnRNPs is a typical modular structure in which one or more RBDs, generally located at the N-terminus, are associated with auxiliary domains [58]. Some of the most well studied hnRNP proteins are those that belong to the hnRNP A/B family (e.g. A1, A2, B1). In fact, these proteins are the most abundant hnRNP proteins, where hnRNP A1 and A2 constitute 60% of the total protein mass of hnRNPs [59, 60]. hnRNP A/B proteins have two RRMs and a Glycine (Gly) rich auxiliary domain at the C-terminus [46]. The tandem RRM-Gly structures allow the binding of other proteins and nucleic acids, hence their pivotal roles in mRNA processing [55]. Moreover, the major hnRNP A/B proteins are among the few hnRNP proteins that assemble into splicesomes at all major splicing stages [61]. Several hnRNP genes generate multiple isoforms through alternative splicing and can undergo posttranscriptional and posttranslational modifications [62]. These include phosphorylation, methylation, and glycosylation, all of which contribute to the complexity of this family [43]. These modifications also likely modulate specific interactions with other proteins and RNA [46]. Therefore, hnRNP proteins are an inextricable part of the pathway of gene expression from the transcription complex to mRNA in the cytoplasm. The contribution of these abundant proteins varies considerably in different cellular environments [43]. A new member of the hnRNP family is Trans-Activating Response Region (TAR) DNA-Binding Protein-43 (TDP-43).

III. TAR DNA-Binding Protein-43

3.1. Protein Structure Overview

TDP-43 is a ubiquitously expressed cellular protein encoded by 414 amino acids and has a molecular weight of approximately 43kDa. Structurally, TDP-43 is similar to hnRNPs and is most closely related to the hnRNP A/B family [46, 63]. It has two RRMs, RRM1 and RRM2, and a Glycine-Rich Domain (GRD). TDP-43 is a homodimer with four RRM domains and a domain arrangement similar to hnRNP A1, where RRM1 and RRM2 are monotetramers with four copies of the RRM in each tetrameric assembly [64]. The RRMs are common RNA-binding motifs with two highly conserved hexameric and octameric segments referred to as RNP1 and RNP2 [65]. The RRMs function in RNA binding and protein-protein interactions [66, 67]. RRM1 supplies most of the requirements for specific RNA binding however, RRM2 contains elements needed for correct complex formation [67]. The RRM2 domain shares a similar fold with other RBPs proteins except it has an atypical RRM-fold with an additional β-strand involved in making domain-domain interactions [64]. The β-strand also has a role in forming a higher order thermal-stable complex [64]. The RRMs are flanked on either side with the N-terminus and the glycine-rich C-terminus.

To date, the N-terminus has not yet been fully characterized; however, there is evidence for a functional role in exon exclusion [68]. The GRD is located in the C-terminus and is involved in different aspects of splicing [66,

70]. Furthermore, the C-terminal region is responsible for protein-protein interactions including interactions with other hnRNP proteins such as hnRNP A2/B1, A1, C1/C2 and A3 [71]. The C-terminal tail of TDP-43 also contains multiple phosphorylation sites, which have a consensus sequence for casein kinase 1 [72]. There are also two possible Caspase-3 cleavage sites, which can give rise to 35 and 25kDa fragments [73]. TDP-43 contains a bipartite Nuclear Localization Signal (NLS) sequence, which is responsible for nuclear targeting as well as a leucine-rich NES [74, 75]. The structure of TDP-43 reveals a diverse protein capable of participating in many different cellular functions.

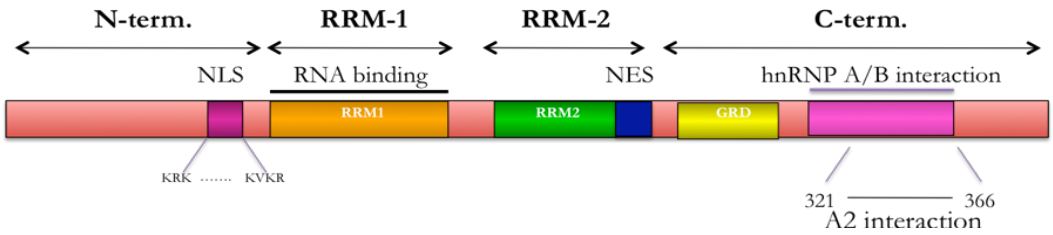

Figure 1.2. The structure of TDP-43 [53, 71, 81, 85, 105]. TDP-43 and has all of the structural features of an hnRNP protein including two highly conserved RRMs (RRM1 and RRM2), a nuclear localization signal, and a glycine-rich C-terminal. The glycine-rich C-terminal region is required for its exon skipping and inhibitory splicing activities. This domain also mediates protein-protein interactions such as a direct interaction with hnRNP A2 (residues 321-366).

3.2 The Importance of TDP-43: Functions

3.2.1 Gene Regulation and RNA Processing in the Nucleus

In the nucleus, TDP-43 is enriched in perichromatin fibrils, which are nuclear sites of transcription and co-transcriptional splicing, where TDP-43 associates with nascent transcripts [76]. The first reports of TDP-43 describe its role as a transcription factor. Transcription factors bind DNA sequences, controlling the transcription of genetic information from DNA to RNA, and can act either positively or negatively [77]. TDP-43 was described as a transcriptional repressor that bound to pyrimidine sequence motifs in TAR DNA via its RRMs [66]. Specifically, TDP-43 repressed gene expression from the human immunodeficiency virus 1 long terminal repeat. In this study, TDP-43 bound to DNA but not to RNA, despite having two RRMs. It is now established that the RRMs function in DNA and RNA binding as well as protein-protein interactions [66, 67]. RRM1 can bind to both single stranded (UG)n oligonucleotides and double-stranded pyrimidine rich DNA oligonucleotides, while RRM2 is necessary, but not sufficient for binding. TDP-43 also functions as a transcriptional repressor of the SP-10 gene, preventing its transcription in somatic tissue [78]. Reducing TDP-43 levels results in the de-repression and thus release of the SP-10-insulated transgene in a stable cell line model. Transcriptional inhibition of SP-10 by TDP-43 depends on the presence of the C-terminus and RRM1 [78].

In the nucleus, TDP-43 also plays a role in splicing regulation. Splicing

involves the removal of introns and the joining of exons in RNA, following transcription [79]. It is required for correct mRNA formation. TDP-43 binds the TG element in human Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) exon 9 pre-mRNA [67]. Over-expression of TDP-43 inhibits CFTR exon 9 splicing, while inhibition leads to an increase in exon 9 recognition. A second study has found that TDP-43 is responsible for CFTR exon 9 skipping in the presence of unfavourable $(UG)_mU_n$ sequences and that the protein plays a dominant role over other exonic or intronic regulatory elements [69]. It is the N-terminus that is required for normal TDP-43 CFTR exon exclusion such that an N-terminal deletion mutant lacks the exon skipping activity despite having intact RRM domains [68]. The GRD in the C-terminus is also important for CFTR exon-skipping activity such that transfection of a truncated form of TDP-43 lacking the GRD results in the loss of 90% of the exon skipping activity [60, 70]. Moreover, the recovery of splicing following inactivation of endogenous TDP-43 can only be restored by Wild-Type (WT) protein expression and not by mutants lacking the C-terminal tail [71]. Thus, TDP-43 plays an essential role in inhibiting splicing via its C-terminal tail. Independently TDP-43 has been identified as an exon-skipping promoter during alternative splicing of apolipoprotein A-II and cyclin-dependent kinase 6 [80, 81]. In addition to its exon exclusion activity, TDP-43 is also involved in exon inclusion. TDP-43 promotes the inclusion of exon 7 during splicing of human Survival of Motor Neuron (SMN) 2 pre- mRNA [82]. In support of its nuclear activities, TDP-43 can be found in a class of nuclear structures termed TDP Bodies (TB) [83]. TB

link different types of nuclear bodies providing a type of network for efficient trafficking and sorting of different nuclear factors. This nuclear scaffold is also where the coupled processes of transcription and splicing occur [83].

Many functions performed by TDP-43 rely on protein-protein interactions. For example, TDP-43 interacts *in vivo* with SMN, a protein involved in the assembly of spliceosomes, sites where splicing occurs [84]. Additionally, TDP-43 directly binds hnRNP A2 via residues 321-366 of its C-terminal tail [85]. This interaction provides evidence for a requirement of TDP-43 to participate in an hnRNP complex to carry out functions such as inhibiting exon splicing. TDP-43 also directly interacts with Fused in Sarcoma/Translocated in Liposarcoma (FUS/TLS) through its C-terminal domain, and together TDP-43 and FUS/TLS regulate Histone Deacetylase (HDAC) 6 mRNA [86]. By using a global proteomics approach, Friedbaum and colleagues have shown that TDP-43 interacting proteins exist in two networks: one consists of nuclear proteins that regulate RNA splicing and nuclear RNA metabolism such as hnRNPs [87]. The second is a group of cytoplasmic proteins that regulate mRNA translation such as translation and elongation factors and ribosomal subunits. Therefore, TDP-43 is implicated at multiple levels of mRNA biogenesis including those in the cytoplasm.

3.2.2. mRNA Biogenesis: An Extension to Cytoplasmic Functions

Landmark studies present TDP-43 as a primarily nuclear functioning protein. Currently, a great deal of evidence is emerging depicting a more

dynamic protein involved in other aspects of mRNA biogenesis. Similar to other hnRNPs, TDP-43 shuttles between the nucleus and the cytoplasm, suggesting a role in mRNA transport [88]. Indeed, it has been demonstrated that TDP-43 binds human Neurofilament Light (NFL) mRNA and regulates its translocation between the nucleus and cytoplasm, ultimately regulating its translation [75]. While TDP-43 motility is dependent on the C-terminus as well as the NLS and NES, shuttling is also transcription dependent. Inhibition of transcription by the RNA polymerase II inhibitor, actinomycin D, results in cytoplasmic TDP-43 accumulation [88]. Therefore, continuous mRNA synthesis is required to signal TDP-43 import via RNA or protein-protein interactions. Furthermore, disruption of RNA/DNA binding ability, particularly by RRM1, can interfere with the correct nuclear and cellular distribution of TDP-43 [88]. Not surprisingly, the expression of NLS-defective mutants results in the cytoplasmic accumulation of TDP-43, while perturbation of the NES increases nuclear TDP-43 and leads to insoluble nuclear aggregates [74]. TDP-43 also functions in mRNA transport as a component of cytoplasmic RNA granules [89]. Cytoplasmic granules include mRNA transport granules, SGs and PBs. For example, the packaging of inactive mRNAs and their transport to postsynaptic sites requires different RBPs [90]. In rodent hippocampal neuron cultures, TDP-43 resides in somatodendritic RNA granules and co-localizes with two other RBPs, fragile X mental retardation protein and Staufen 1, supporting a role for TDP-43 in RNA localization and transport [91, 92].

In addition to transport, TDP-43 is involved in mRNA stabilization,

sequestration, and storage. For instance, TDP-43 interacts with and stabilizes NFL mRNA, preventing its degradation [75]. Volkening and colleagues have shown that NFL mRNA stabilization is mediated by RRM1 such that TDP-43 binding is abolished by mutations in which the RRM1 is removed, while RRM2 is preserved [93]. Thus, supporting the essential requirement of RRM1 for RNA interactions, but not RRM2. TDP-43 also functions in the storage and stabilization of mRNA during harmful cellular conditions as a component of SGs. Both exogenous and endogenous TDP-43 localize to SGs following oxidative stress [94-96]. TDP-43 association with SGs relies on its ability to properly interact with RNA where cells expressing TDP-43 with a mutated RRM are found to contain significantly fewer SGs compared to control [89]. Moreover, TDP-43 co-localizes with both RNA transport and SG markers in motor neurons in response to sciatic axotomy [97]. This study also demonstrates TDP-43 redistribution from the nucleus to the cytosol and an increase in TDP-43 expression, indicating a more complex role for TDP-43 during cellular injury. Together, these studies indicate TDP-43 plays a role in sequestering and regulating mRNA in response to cellular damage.

TDP-43 also regulates mRNA via indirect mechanisms. In the nucleus, TDP-43 is found in both human and mouse microprocessor complexes, suggesting a role in miRNA biogenesis [15, 98]. miRNAs bind target mRNAs and regulate gene expression by regulating mRNA or translation levels. There is evidence that TDP-43 is involved in miRNA expression pathways. For example, removal of TDP-43 from the nucleus results in specific down

regulation of let-7b, a family of miRNAs important in cellular differentiation [89]. TDP-43 also associates with the Drosha complex, which is involved in primary miRNA biogenesis [98]. Therefore, the associations of TDP-43 with different complexes, and its localization to different types of RNA granules, support a role in multiple steps of RNA processing such as transport, stability and storage. With such diverse cellular functions, understanding the physiological functions of TDP-43 in different experimental models is of great interest.

3.2.3. Uncovering Physiological Functions with Mouse and Fly Models

To gain a better insight of the physiological functions of TDP-43, Wu and colleagues have inactivated the mouse *Tardbp* gene encoding TDP-43 using a gene targeting approach [99]. In this study, homozygous *Tardbp* null mice exhibit early embryonic lethality and have defective outgrowth of the inner cell mass upon *in vitro* culturing of blastocycts. Therefore, TDP-43 is an essential gene for both viability and formation of the implanting blastocysts. Sephton and colleagues have obtained similar results demonstrating that TDP-43 is necessary throughout embryonic development and is expressed predominantly in the neuroepithelium and neural progenitors during development [100]. In *Drosophila*, loss of the TDP-43 homologue, TBPD, affects locomotive skills with spastic, uncoordinated movements, incapacity to fly as well as reduced life span [101]. Interestingly, the expression of WT human TDP-43 in neurons also causes motor defects and reduces lifespan

[102]. Furthermore, homozygous null *Drosophila* larvae have altered presynaptic terminals at neuromuscular junctions. Changes include reduced number of axonal branches and synaptic boutons present inside muscles [102]. Thus, experimental models have provided insight for a role of TDP-43 in regulating synaptic terminals and locomotive behaviours as well as a role in embryonic development and life-span. Cell culture models have provided evidence for TDP-43 participating in cell cycle and survival.

3.2.4. A Role in Cell Survival

TDP-43 plays a role in cell cycle such that its depletion leads to altered levels of several cell proliferation factors, especially those associated with the retinoblastoma protein [81]. Retinoblastoma protein is essential for the control of the cell cycle, cellular differentiation, and maintenance of genome integrity, and is controlled by its phosphorylation by cyclin-dependent kinase 6. TDP-43 depletion also leads to changes in cell cycle distribution in which there is a significant decrease in the number of cells in G_0/G_1 and an increase in S and G_2/M cells [81]. In addition, the reduction of TDP-43 results in an increase in apoptotic cell death and altered nuclear morphology (e.g. membrane blebbing), highlighting a critical role for TDP-43 in cell survival. Many of the biological functions of TDP-43 are currently being unraveled. So far, researchers have uncovered a highly multifunctional protein with roles in nuclear and cytoplasmic in RNA processing, as well as an important factor in development and cell cycle (Fig 1.3). How mutations disrupt normal TDP-43 functions,

resulting in disease, is an ensuing challenge.

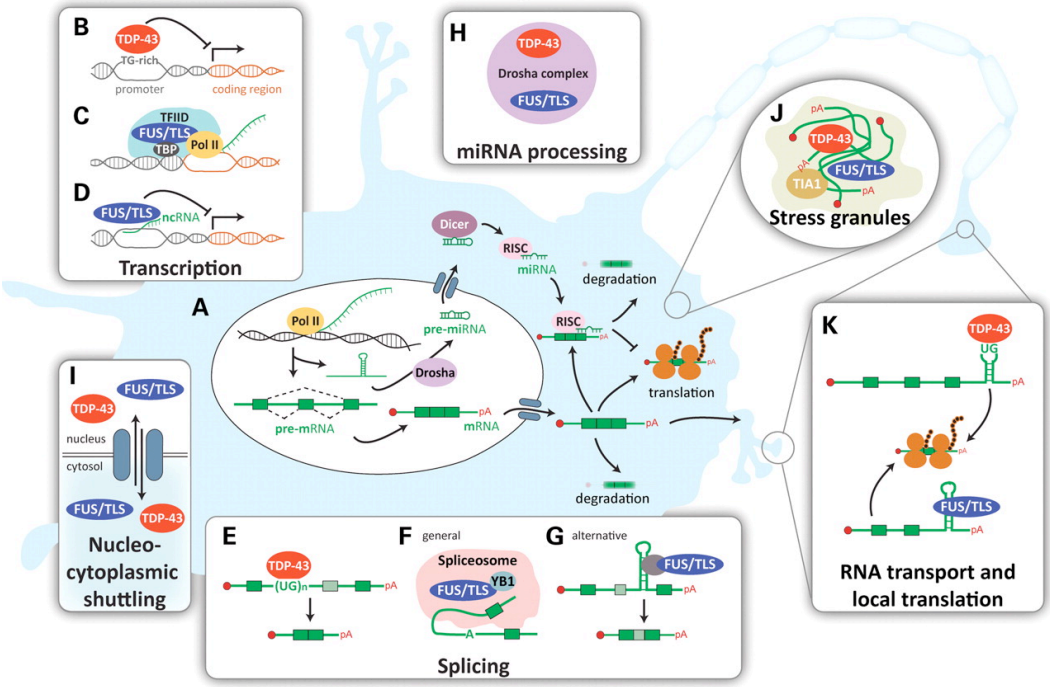

Figure 1.3. Summary of TDP-43 functions [105]. TDP-43 is involved in several aspects of RNA processing including transcription, splicing and transport. More recently TDP-43 has been described in microprocessor complexes, suggesting a role in miRNA biogenesis, as well as in SGs, suggesting roles in both mRNA silencing and storage. (Figure is adapted from Lagier-Tourenne, C., M. Polymenidou, et al. (2010). *Hum Mol Genet*)

3.3. The TARDBP Gene

3.3..1 Disease-Linked Mutations

TDP-43 is the protein product of the TARDBP gene, which consists of six exons and is located at chromosome 1p36.21 [70]. TARDBP is highly conserved from *Caenorhabditis elegans (C. elegans)* to *Drosophila* and mammals. It has a low rate of sequence divergence where human, mouse, *Drosphila*, and *C.elegans* TDP-43 are highly homologous up to the carboxyl boundary of the RNP-1 in RRM2 [70]. The similarity between *C. elegans* and the other three taxa is close to 55%, and 70% if only fly and mammal are considered. Such data suggests that TARDBP genes are likely responsible for the same fundamental roles in the various organisms.

Following the identification of TDP-43 in ubiquitin positive inclusions (UBI) in patients with Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration with Ubiquitin-Positive Inclusions (FTLDU), TARDBP became a strong biological candidate gene for familial forms of these diseases. This has been the case for several other neurodegenerative diseases such as amyloid precursor protein in Alzheimer's disease and α-synuclein in Parkinson's disease [103, 104]. Mutational analysis of TARDBP led to the identification of the first missense mutation in Familial ALS (FALS): Ala-315-Thr (c.1077 G>A) within exon 6 [103]. The phenotype of the affected individuals with the mutation involved a slowly progressive lower motor neuron degeneration syndrome with respiratory

involvement. Since then, a total of 38 distinct mutations have been reported as causative for ALS, all clustered in the GRD in exon 6 except D169G, which is located in RRM1 [105] (Fig 1.4). Furthermore, all of the mutations are missense mutations except for one truncation mutation, Y374X [106]. Various mutations come with different proposed mechanisms of pathology. For example, the D169G mutation in RRM1 may abrogate its RNA binding while the G348C variant may increase the propensity for aggregation by introducing a cysteine and thus forming intermolecular bridges [107]. Many mutations are also predicted to increase TDP-43 phosphorylation, particularly those substitutions which are threonine and serine residues (e.g. R361S, G287S) [107]. TARDBP mutations account for approximately 4% of FALS and less than 1% of Sporadic ALS (SALS) [108]. FALS accounts for approximately 10% of all ALS patients with mutations in Superoxide Dismutase 1 (SOD1) being the most common of all mutations [109]. Under pathological conditions, TDP-43 is commonly characterized by UBIs, protein cleavage, phosphorylation and mislocalization (Fig 1.5).

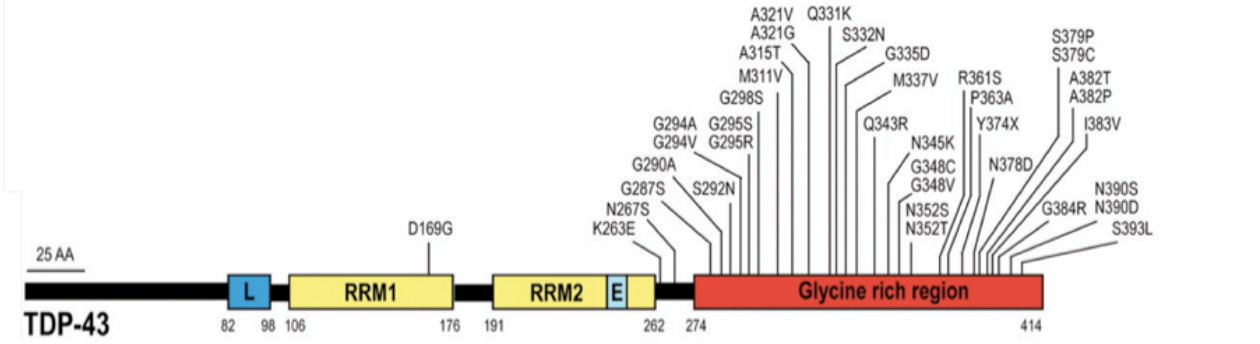

Figure 1.4. Mutations in TDP-43 [105]. A total of 38 different mutations have currently been reported as causative for ALS. All of the mutations are clustered in the GRD except D169G, which is located in RRM1. (Figure is adapted from Lagier-Tourenne, C., M. Polymenidou, et al. (2010). *Hum Mol Genet*)

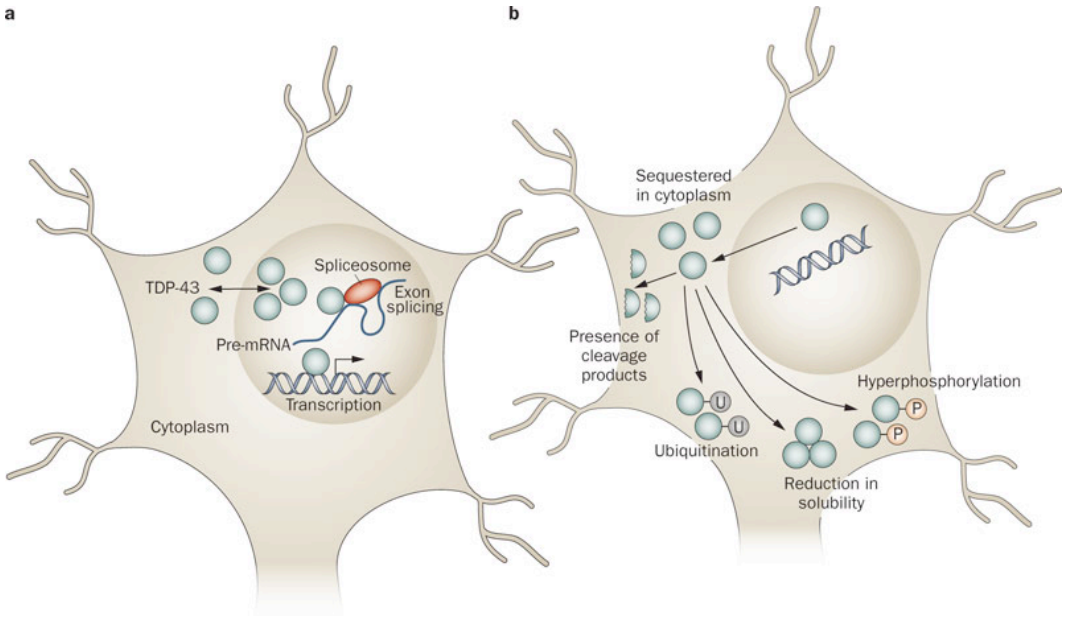

Figure 1.5. Physiological and pathological TDP4-3 [171]. Under physiological conditions, TDP-43 primarily resides in the nucleus with shuttling capabilities (a). Under pathological conditions, TDP-43 accumulates in the cytoplasm with a loss of nuclear TDP-43 (b). Pathological TDP-43 has been demonstrated to be cleaved, hyperphosphorylated and/or ubiquitinated. (Figure is adapted from Chen-Plotkin, A. S., V. M. Lee, et al. (2010). *Nat Rev Neurol*)

3.4. TDP-43 Pathology

3.4.1. Ubiquitination and Phosphorylation

TDP-43 pathology is most prominently defined by the accumulation of UBIs in the cytoplasm [110]. UBIs can appear as filamentous skeins or Lewy body-like inclusions [111]. The presence of UBIs has led some to investigate whether protein degradation systems, such as the unfolded protein pathway and autophagy-lysosomal pathway, are involved in TDP-43 pathology. Indeed, Kim and colleagues have shown that TDP-43 interacts with ubiquilin 1, which is a proteasome targeting factor [112]. Furthermore, there is evidence demonstrating that both TDP-43 and a toxic p25 fragment are degraded by the unfolded protein pathway and autophagy-lysosomal pathway [113]. For instance, TDP-43 levels are increased in cells treated with MG132, a proteasome inhibitor, and 3-methyladenine, an autophagy inhibitor. Inhibition of the autophagosome or proteasome also results in marked polyubiquitination of TDP-43, therefore implicating degradation systems [114]. However, despite such evidence, confocal analysis of patient anterior horn cells has demonstrated that cytosolic aggregates induced by the same treatment are ubiquitin negative [115]. Moreover, a separate report claims that TDP-43 is not a substrate for ubiquitination in skein-like inclusions in patients [115]. These results present the possibility that TDP-43 need not be ubiquitinated to be present in inclusions, thus presenting the uncertainty of whether defects in protein degradation systems are linked to disease.

Biochemical analysis of the detergent-insoluble UBIs extracted from brains of patients with ALS and FTLD-U has shown that TDP-43 in these inclusions is cleaved and phosphorylated [115, 116]. The involvement of caspase-cleaved C-Terminal Fragments (CTF) in inclusion formation has been investigated in mammalian cells made to express either the 35kDa or 25kDa TDP-43 fragments [68]. This study has demonstrated that the expression of the 25kDa fragment forms cytoplasmic inclusions that are ubiquitinated and phosphorylated at S409/410. An independent group has reported that the caspase-generated 35kDa CTF also becomes phosphorylated at S409/410 during conversion of the fragment into an insoluble species [118]. There are at least five sites on TDP-43 that are phosphorylated in patients with ALS and FTLD-U and immunoreactivity against pS409/410 is particularly robust [119]. Moreover, abnormal phosphorylation occurs near the C-terminal region. Immunofluorescent staining for phosphorylated TDP-43 (pTDP-43) has shown that pTDP-43-positive inclusions are often found in oligodendrocytes and in the cytoplasm of astrocytes in the frontal cortex of patients [119]. However, phosphorylation of TDP-43 is not required for inclusion formation and is considered to be a late event occurring after the deposition of the insoluble protein [68]. CTF formation is also not a prerequisite for aggregation since non-cleavable full length TDP-43 is also converted to insoluble species to the same extent as CTFs after apoptosis induction [118]. However, caspase-cleavage is thought to be a prerequisite for redistribution from the nucleus to the cytosol [73]. It has been postulated that TDP-43 mislocalization occurs due to the

aberrant cleavage of TDP-43 while it shuttles between the nucleus and cytoplasm [120]. In support of this, the appearance of C-terminal TDP-43 fragments by immunoblot often accompanies TDP-43 cytoplasmic accumulation [74].

3.4.2. Cytoplasmic localization

TDP-43 is primarily a nuclear protein. However, in the context of disease, such as ALS, it accumulates in the cytoplasm with a concurrent depletion of nuclear TDP-43 [122]. This event is also observed in response to sciatic nerve axotomy in which there is a significant reduction in nuclear TDP-43 and an increase in the cytoplasmic pool [97]. Sato and colleagues have reported similar results showing a gradual decrease of nuclear TDP-43 following ligation of the hypoglossal nerves [121]. The decrease in TDP-43 is not attributed to an inhibition of transcription since TDP-43 mRNA levels remain unchanged. In cell culture models, cytoplasmic localization of TDP-43 increases the risk of cell death two- to three-fold, regardless of whether neurons express WT or mutant TDP-43 [122]. Moreover, the amount of cytoplasmic TDP-43 in a cell is a strong predictor of cell death. However, cytoplasmic mislocalization is significantly more frequent in neurons expressing mutant TDP-43 then WT [122]. Interestingly, the A315T mutant is able to specifically induce mislocalization of TDP-43 from the nucleus to the cytoplasm [122]. In a model of TDP-43 proteinopathy in rat primary cortical neurons (expressing WT or TDP-43 bearing mutations) the authors provide evidence that mutant TDP-

43 forms ubiquitinated and detergent-resistant cytoplasmic inclusions [122]. Furthermore, Winton and colleagues have shown that disturbance of endogenous TDP-43, by mutations in the NLS or NES, can also result in the formation of aggregates in the cytoplasm and nucleus [74]. Such evidence suggests that the trafficking of TDP-43 is delicately balanced and perturbations of this can be a trigger for aggregation.

To assess the factors that initiate the sequestration of TDP-43 to the cytoplasm, Johnson and colleagues have developed a TDP-43 proteinopathy model in yeast [123]. In this model, over-expression of human TDP-43 has led to cytoplasmic aggregation and cellular toxicity. Moreover, an experiment using truncated TDP-43 mutants has demonstrated that sequences within the N-terminus are necessary to direct nuclear localization, while the C-terminus with an intact RRM region is required for cytoplasmic aggregation [123]. The C-terminal region with the entire RRM2 domain is necessary for toxicity [123]. In a similar yeast model, several TDP-43 mutants have an increased number of cytoplasmic aggregates, accelerated aggregation (as determined by the extent of turbidity or sedimentation over time) and enhanced toxicity compared to WT TDP-43 [124].

There is some debate regarding the toxicity of cytoplasmic aggregates. For instance, inclusion formation has been reported to enhance cellular toxicity [74, 123]. However, cytoplasmic aggregates have been reported in small atrophic neurons as well as large, otherwise healthy, neurons in humans [74,

123, 125]. Furthermore, inclusion formation is not required for mutant TDP-43 toxicity as soluble forms of the protein have a neurotoxic effect that is independent of inclusion formation [122]. Similarly, in mice expressing a mutant form of TDP-43, cytoplasmic aggregates are not seen suggesting they are not necessary for TDP-43 induced neurodegeneration [126]. However, despite evidence opposing the cytotoxicity of TDP-43 aggregates, it is possible that their formation simply enhances TDP-43 mediated neurodegeneration. Therefore, the mechanism(s) of TDP-43 toxicity continues to be a matter of intense investigation. Insight into the role of TDP-43 under pathological conditions is required in order to understand neurodegenerative proteinopathies.

3.4.3. TDP-43 Pathology in FTLDU and ALS

TDP-43 pathology underlies major forms of sporadic and familial FTLD and ALS, and together the diseases are now referred to as TDP-43 proteinopathies [111, 127]. Linking these two disorders is based on their shared accumulation of ubiquitin-positive cellular inclusions and the overlap in symptoms. FTLD is the second most common form of dementia after Alzheimer's disease, with a prevalence ranging from 3.5 to 25.4 per 100 000 [128, 129]. Clinically it is defined by behavioural and/or language dysfunction, and reflects the prominent frontal and temporal atrophy associated with neuronal loss and gliosis [111, 120]. TDP-43 pathology is found throughout the central nervous system however, not all FTLD-U cases show TDP-43 pathology [120, 127]. In ALS, TDP-43 inclusions are observed in the majority

of cases, except in those cases resulting from mutations in the SOD1 gene [131, 132]. Thus, TDP-43 pathology can be seen in SALS and non-*SOD1*-FALS, but not *SOD1*-FALS [133]. TDP-43 mutations in the TARDBP gene in ALS patients have provided a direct-link between TDP-43 abnormalities and neurodegeneration.

3.4.4. ALS

ALS is a late-onset and typically fatal neurodegenerative disease [134]. Approximately 90% of ALS cases are sporadic and the remaining 10% familial with a clear genetic link. The disease is characterized by the premature loss of upper and lower motor neurons resulting in progressive weakness, muscular wasting and spasticity leading to paralysis and death within three to five years [135]. The etiology is unknown and there are currently no effective treatments. It is widely accepted that multiple aberrant biological processes, such as enhanced oxidative injury, mitochondrial dysfunction, excitoxicity, and proteasome inhibition, result in motor neuron death in ALS [136-149]. Non-neuronal cells such as microglia and astrocytes are also involved in the disease process [150, 151] (Fig 1.6). A new emerging theme in ALS pathology is aberrant RNA processing. In support of this, five RNA processing genes have now been linked to ALS in addition to TDP-43 including angiogenin, Elongatoprotein 3, senataxin, FUS/TLS and SMN [152]. Therefore, dysfunction in RNA processing may be a central feature in the pathology and development of ALS.

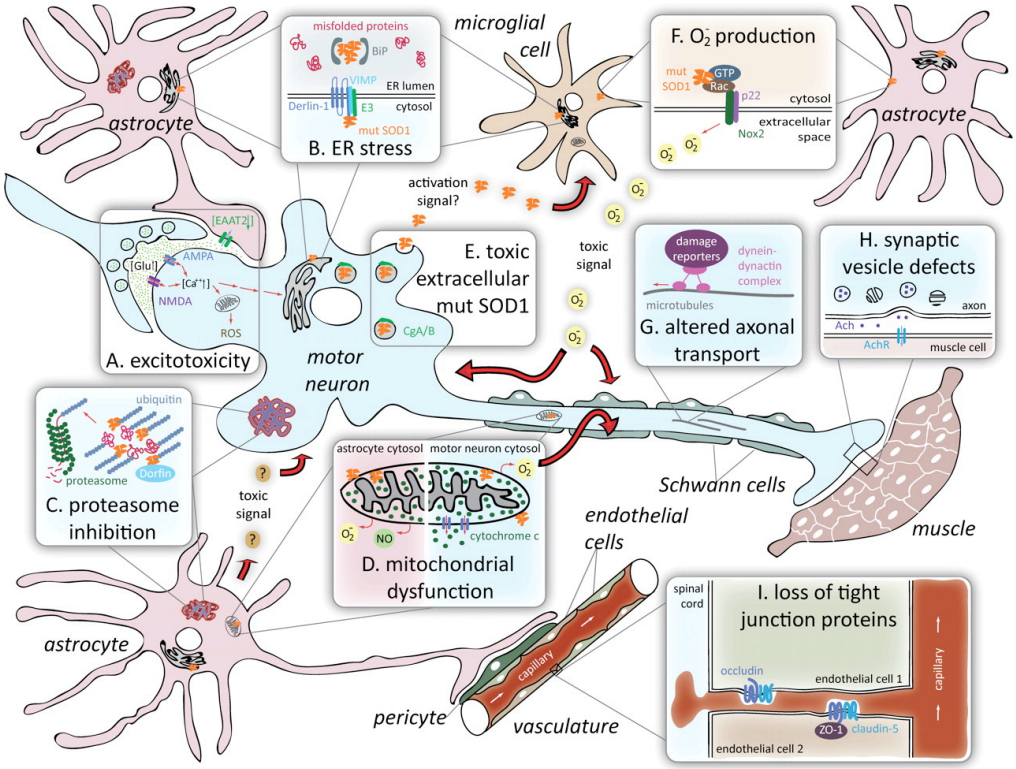

Figure 1.6. Proposed disease mechanisms in ALS [140]. Motor neuron death in ALS is believed to be the result of a number of aberrant processes, some of which include: excitotoxicity which results primarily from a failure to remove glutamate from synapses; proteasome inhibition due to the over accumulation of ubiquitinated misfolded protein aggregates; mitochondrial dysfunction which is mediated by mutant SOD1 deposition on the mitochondrial membrane; and oxidative damage due to excessive superoxide production from microglia or astrocytes. (Figure adapted from Ilieva, H., M. Polymenidou, et al. (2009). *J Cell Biol*)

IV. Aim of Study

RNA processing is a complex and tightly regulated pathway. Disturbances in TDP-43, a protein involved in many different aspects of RNA metabolism, can lead to a number of disrupted processes such as transcriptional activation, pre-mRNA splicing, mRNA stabilization, and mRNA transport, all of which are coupled to the CSR. Therefore, since RNA regulation is critical for survival during cellular stress, a great deal of research is currently being conducted on impaired CSRs in neurodegenerative diseases. In order to fully elucidate the role of TDP-43 in disease, one must have a clear understanding of its normal biological functions, which remain largely unknown. It is well established that TDP-43 localizes to SGs with oxidative stress, however its role in SGs remains unknown. We hypothesize that TDP-43 participates in the CSR. Therefore, our study aims to define the role of TDP-43 in the CSR in relation to SGs and whether ALS-linked mutations alter this role.

B. METHODS

1. Cell Culture

HeLa and patient lymphoblast cell lines were grown in DMEM and IMDM, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin/glutamate.

2. Stress Conditions

Cells were treated with various stresses including 0.5 mM sodium arsenite (30 min, 37ºC; Sigma), heat shock (30 minutes, 43ºC), and 1μM thapsigargin (50 min, 37ºC; Sigma).

3. siRNA Transfection

Cells were grown in media (without antibiotics) for 24 hr to a confluency of approximately 30-50% on the day of transfection. For a 6-well plate (growth area per well: 9,6 cm^2), 5 μl of Lipofectamine 2000 (Invitrogen) was diluted in 250 μl of Opti-MEM1 media (Invitrogen) and incubated for 5 min at Room Temperature (RT). Separately, 125 pmol of either Control or TDP-43 siRNA (Invitrogen) was diluted in 250 μl of Opti-MEM1. The two dilutions were combined, mixed gently, and incubated for 20 min at RT. The growth media on the cells was replaced with 2 ml of Opti-MEM and 500 μl of the transfection mix was added. The transfection mix was incubated with cells for 5 hr at 37°C, and subsequently replaced by fresh growth media. Cells were incubated for 72 hours at 37°C before harvesting.

Control siRNA sequence: 5'-AAUUCCAGUUAUUGUAUAUAUUCAG-3'

TDP-43 siRNA sequence: 5'-AAGCAAAGCCAAGAUGAGCCUUUGA-3'

4. Cell Harvesting and Immunoblot

The growth media was collected in a 15 ml conical. 1 ml of cold PBS was added to cells which were then scraped with cell scrapers. The cells were transferred to the 15 ml conical containing the media, followed by centrifugation at 16800 g, 5 min, RT. The supernatant was aspirated. The pellet was resuspended in 1 ml of cold PBS, transferred to 1.5 ml tube and centrifuged 900 g, 5 min, 4˚C. The supernatant was aspirated and the pellet was resuspended in x µl (volume varied between 30 µl -60 µl depending on the size of pellet) of RIPA lysis buffer (150mM NaCl, 50mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate). The tube was centrifuged 16200 g, 5 min, 4˚C. The supernatant was separated from the pellet and stored at -20˚C. For fractionation of soluble and insoluble components, cells lysed in RIPA buffer were passed through a 25G syringe 6 times and centrifuged at maximum speed. Supernatants (soluble) were recovered and pellets (insoluble) were resuspended directly in 1x 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.

5. Protein Concentration: BCA Assay

A standard curve was prepared in cuvettes as follows:

Standards (µg/ml)	0	1	2	4	6	8	10	12
BSA (2µg/µl)	0	0.5	1	2	3	4	5	6
ddH2O	50	49.5	49	48	47	46	45	44

Table 1.1. Standard Curve

1 µl of protein was added to the cuvette containing 49 µl of ddH2O in duplicates. BCA Reagent A was mixed with Reagent B (Thermo Scientific) in a ratio of 50:1. 1 ml of Reagent A/B was added mix to each cuvette with the protein. The mixes were incubated at 37˚C for 30 min. Protein concentrations were determined via spectrophotometry at 562 nm.

6. Immunofluorescence and Antibodies

Cells grown on coverslips were fixed in 1% formaldehyde (FA) in PBS for 20 min, washed with PBS and subsequently permeabilized with 0.1% Triton-X100 in PBS for 15 min. Coverslips were blocked with 0.1% BSA in PBS for 30 min and incubated with primary antibodies diluted in blocking buffer for 1 hr at RT. Coverslips were subsequently washed once with 0.1% Triton-X100 in PBS for 5 min and twice with 0.1% BSA in PBS for 5 min. Labeling was visualized with the fluorescently conjugated secondary antibodies

diluted in blocking buffer. Coverslips were them washed as before, and mounted with ProLong Antifade reagent (Invitrogen). Images were collected on a Leica SP5 confocal microscope. Primary antibodies consisted of 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). Secondary antibodies consisted of donkey anti-rabbit Texas Red (Jackson Immunochemicals), donkey anti-goat Alexa 488 (Invitrogen), and donkey anti-goat 633 (Invitrogen).

7. Lymphoblastoid Immunofluroescence

100 µl of cells (in suspension, grown in 25 ml flasks) were transferred into cytospin chambers which were mounted on Superfrost charged slides (Thermo Scientific). Chambers were centrifuged at 7000 RPM, 4 min, RT. The excess media was aspirated and the apparatus dissembled. Slides were left to dry and outlined with a hydrophobic pen. The cells were fixed with 1% FA for 20 min, RT. Cells were then washed with 50 mM NH_4Cl for 15 min and permeabilized with PBS/0.2%tx100 for 10 min. Following one wash with PBS, cells were blocked with PBS/0.1%tx100/5%BSA for 1hr, RT. The primary antibody, diluted in PBS/0.1%tx100, was then added and incubated for 1hr, RT. The slides were washed 3 times with PBS/0.1%tx100 and the secondary antibody, diluted in PBS/0.1%tx100, was then added and incubated for 1 hr, at RT. Slides were washed 3 times PBS/0.1%tx for 10 min and mounted with Pro Long Antifade Reagent (Invitrogen).

8. Stress Granule Formation and Recovery

Cells were transfected with either Control or TDP-43 siRNA as described in Methods 3. 72 hr post-transfection, 0.5 mM SA was added to cells and coverslips were collected at the following time points after the addition of SA for "Stress Granule Formation": 0, 10, 15, 20, 30 min.

For "Stress Recovery", 0.5 mM SA was added to cells for 30 min at 37˚C. The media was then replaced with fresh growth media and coverslips were collected at the following time points: untreated, 0 min, 15 min, 30 min, 1hr, 2 hr, 4 hr.

Coverslips were fixed and stained as described in Methods 5.

Slides were imaged at 63x via confocal microscopy. Four fields were imaged for a minimum of 100 cells per condition. Imaging settings were kept constant between each condition. SG-positive cells were defined as cells with at least 2 SGs. The total number of SG-positive cells was compared for siTDP-43 and siControl.

9. RNA Extraction

RNA was extracted with RNAeasy kit (Qiagen). Specifically, cells were collected in 1 ml of trypsin (Invitrogen) and centrifuged at 9600 g, 5 min, RT. The media was aspirated and the pellet was resuspended in 350 µl RLT and transferred to 1.5 ml tube. Cells were then homogenized with 1 cc syringe equipped with a 25G needle and 1 volume of 70% ethanol was added. 700 µl of was added to RNeasy spin column and centrifuged 9600 g, 15 sec, RT. The flow through was discarded and 350 µl RW1 was added and centrifuged 9600 g, 15 sec, RT. The flow through was discarded and 80 µl of DNase I mix was

added to the column (consisting of 10 µl DNase I in 70 µl RDD). The mix was centrifuged 9600 g, 15 sec, RT and 350 µl RW1 was added. The tubes were centrifuged 9600 g, 15 sec, RT, and the flow through was discarded. Twice, 500 µl of RPE was added to the column and centrifuged 9600 g, 15 sec, RT. The flow through was discarded and using a new collection tube, the columns were centrifuged 9600 g, 1 min, RT. 30 µl of RNase free water was then added and centrifuged 9600 g, 1 min, RT. The eluate was added to column again and centrifuged 9600 g, 1min, RT. The resulting eluate was RNA. RNA was stored at -80˚C and concentrations were determined via spectrophotometry at 260/280 nm.

10.Reverse Transcription and cDNA Synthesis

Reverse Transcription was performed with QuantiTect (Qiagen).

RNA was thawed on ice. The following mix was prepared for each sample on ice: 2 µl gDNA wipeout buffer, x µl RNA (500 ng), x µl ddH2O (total volume=14 µl). Tubes were heated at 42ºC for 2 min, then iced immediately. The following mix was added directly to the tubes:1µl reverse transcriptase (RT), 4 µl 5x RT buffer, 1 µl RT primer mix (total volume=20 µl). Tubes were heated at 42ºC, 30 min followed by 95ºC, 3mins, then placed on ice. cDNA was stored at -20ºC. cDNA was processed for qPCR with SybrGreen (Biorad).

The following primer sets were used: 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'-CGCAGAGATTCAGGTTCTCC-3'; and HuR exon 5 R: 5'-CCAAACCCTTTGCACTTGTT-3'.

RT-PCR conditions:

Reagent	**Volume (µl)**
DNA	1
Forward Primer (10 uM)	1.25
Reverse Primer (10 uM)	1.25
dNTPs (10 uM)	0.5
10x PCR buffer *	2.5
TAQ *	0.5
ddH2O	18

*Purchased from New England Biolabs

Table 1.2. RT-PCR Conditions

12. Quantification of SG Size

The area of 10 SGs randomly selected in at least ten cells per condition was manually measured with ImageJ. The average SG size of at least 100 SGs in ten

cells is presented.. SGs measured ranged in size from 0.75um^2-5 um^2.

13. Statistical Analysis

Data was analyzed by Student t-test or one-way ANOVA, where appropriate. Significance was achieved with a P-value of <0.05. Error bars represent standard error of the mean (SEM).

C. MANUSCRIPT

TAR DNA-Binding Protein 43 (TDP-43) Regulates Stress Granule Dynamics via Differential Regulation of G3BP and TIA-1

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ABSTRACT

TDP-43 is a multifunctional protein with roles in transcription, pre-mRNA splicing, mRNA stability and transport. TDP-43 interacts with other 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, following oxidative stress, heat shock, and exposure to thapsigargin. TDP-43 contributes to both the assembly and maintenance of stress granules in response to oxidative stress and differentially regulates key stress granules components including TIA-1 and G3BP. The controlled aggregation of TIA-1 is disrupted in the absence of TDP-43. In addition, TDP-43 regulates G3BP mRNA levels, a stress granule nucleating factor. The disease-associated mutation TDP-43^{R361S} compromises stress granule formation. Thus, the cellular function of TDP-43 extends beyond splicing and places TDP-43 as a participant of in the central cellular response to stress and an active player in RNA storage.

INTRODUCTION

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-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 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 have hnRNP, are directly involved in cellular responses to various stresses. For example, Guil and colleagues (2006) have shown that activation of the p38 stress-signaling

pathway in mammalian cells results in both the phosphorylation and cytoplasmic accumulation of hnRNP A1 in SGs (10). Similarly, Quaresma and colleagues (2009) have demonstrated that under specific stress conditions (e.g. thapsigargin, heat shock and arsenite) nuclear hnRNP Q is redistributed to the cytoplasm and partially co-localizes to SGs and PBs (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 (RBP) 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 heat shock, hypoxia, osmotic and oxidative stress and typically involves the phosphorylation of the eukaryotic initiation factor eIF2α. This phosphorylation event inhibits mRNA translation through depletion of the eIF2-GTP-tRNA-met ternary complex thus permitting the RNA-binding protein 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 it may also play a role in cellular stress responses.

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, TIAR, and G3BP. Moreover, TDP-43 contributes to SG formation and maintenance. In patient lymphoblastoid cells, at least one ALS-causing mutation in TDP-43 impacts SG formation. Our data suggests 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 stress granules. Various types of 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), heat shock (HS), and thapsigargin (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 (**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 (17,18). Indeed, soluble TDP-43 protein levels were decreased in HS and SA treated cells (37% and 56%, respectively) compared to untreated cells and there was a significant reciprocal increase in TDP-43 in the insoluble fractions (**Fig. 1B**). However, THAP treatment does not yield similar changes in protein solubility.

To determine if TDP-43 is also a resident of other RNA granules, such as Processing Bodies (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 (19). Thus, TDP-43 is a resident of SGs but not PBs following acute exposure to certain stressful stimuli, in agreement with recent reports (20,21).

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 eukaryotic translation initiation factor 2 alpha (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 (17,22-24). 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 approximately 30 minutes (17). Once the stress is removed, SGs continue to aggregate and increase in size for approximately 1-2 hours before gradually resolving. Typically, SGs are completely resolved within 4 hours of SA treatment. To determine if 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 siRNA and then exposed to SA. The number of cells exhibiting SGs was determined at 0, 15, 20 and 30 minutes during SA ("stress") exposure. Compared to 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 to cells treated with control siRNA (**Fig. 2B**). In particular, we noted a modest but significant reduction in the number of cells with successful SG formation at 30 minutes following SA exposure (97% vs. 81%, p=0.03) as well as a marked change in SG size which will be discussed below.

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 minutes 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 minutes, 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 minutes (4 hours release) will few if any cells still containing 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 to control (**Fig. 2C**, right panel). This difference is most obvious in the first 30 minutes after SA treatment as the number of SG-positive cells continues to be reduced at 45 and 60 minutes post stress, compared to control siRNA cells (**Fig. 2B**). Furthermore, SGs in TDP-43 depleted cells have more diffuse TIA-1 labeling (ie. 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 to 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 to controls (0.83 ± 0.08 vs. 1.46 ± 0.12 μm^2; p=0.0002) (**Fig. 2D**).

Our data indicates that the lag in SG assembly in siTDP-43 cells is overcome by 90 minutes, 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 minutes, 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. However, these SGs are not sustained and quickly resolve. Thus, endogenous TDP-43 contributes to both the establishment of SGs and is required to maintain SGs.

It has been reported that exogenous expression of some SG components is sufficient to nucleate SGs (17,23). Since our earlier data suggests a role for TDP-43 in SG nucleation, we assessed SG formation in cells transiently transfected with GFP-tagged full-length TDP-43. Overexpression of TDP-43 was not sufficient to nucleate SGs but does itself correctly localize to SGs following SA treatment (data not shown).

TDP-43 differentially regulates SG nucleating proteins. TIA-1 and G3BP are both considered as primary nucleators of SGs (25). We hypothesized that TDP-43 may regulate the levels of these proteins, either at the transcriptional or post-transcriptional level. Immunblot 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 even more marked alterations were seen in steady state mRNA levels. Specifically, we noted a 2.6-fold increase (p=0.01) for 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 regulates the transcription 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 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 facilitates TIA-1 redistribution. Over-expression of TIA-1 is sufficient to induce SG formation (17) and TDP-43 interacts with TIA-1 (20). However, our data indicates diffuse TIA-1 labeling in TDP-43 depleted cells and slowed SG assembly but paradoxically higher TIA-1 steady state levels. However, it is well described that the recruitment of TIA-1 into SGs is marked by the redistribution of TIA-1 from soluble to insoluble compartments (17,18). Thus, we speculated that TDP-43 might influence TIA-1 aggregation. To this end, we examined TIA-1 distribution in soluble and insoluble fractions of control and TDP-43 depleted cells. In contrast to control siRNA cells, we observed a 39% increase in soluble TIA-1 when TDP-43 is reduced compared to cells treated with control siRNA ($p=0.03$; **Fig. 4A**) with a concomitant decrease in the insoluble pool. Therefore, our data suggest that TDP-43 impacts the compartmentalization of TIA-1.

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. 4B**). 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 to their control counterparts. TIA-1 and G3BP co-localization appears to be maintained (**Fig. 4B**).

Endogenous hnRNP A2 is a resident of SGs. TDP-43 interacts with hnRNP A2 (2). Thus, we investigated whether hnRNP A2 could also be a component of SGs. 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 hypothesized that TDP-43 may also influence hnRNP A2 localization. Thus, we examined the localization of hnRNP A2 to SGs in cells treated with TDP-43 siRNA and subjected to SA treatment. As before, siTDP-43 cells had fewer SGs per cell and SG size was decreased (**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 increased patchy distribution within the cytosol compared to 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 FUS/TLS has recently been described to be sufficient to induce SGs (26,27). 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} and TDP-43^{R361S}. We verified that steady-state levels of TDP-43 protein were not reduced in the different cells (**Fig. 6A**). In fact, we noted a slight increase in mutant TDP-43 protein levels such that TDP-43^{D169G} and TDP-43^{R361S} proteins accumulated to 1.2x ($p=0.04$) and 1.3x ($p=0.01$) relative to controls cells expressing TDP-43^{WT}. Lymphoblasts were treated with SA and SG formation was scored 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, we observed a 2-fold reduction in the number of cells forming SGs in cells expressing TDP-43^{R361S} mutation ($p=0.01$; **Fig. 6B**). Interesting, SG formation in cells expressing TDP-43^{D169G} was comparable to control.

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 a key element to the protective response to cellular stress. Moreover, our data demonstrates 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 transcriptional 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 both SG assembly and their maintenance. 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.

Interestingly, TDP-43 has been reported to interact with TIA-1 and TIAR, both core nucleating components of SGs (20). 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. It has been previously shown that the levels of SG-associated proteins significantly increase in the insoluble fraction during biochemical fractionation

of cells treated with various stresses (17,18). The efficiency with which TIA-1 can transition between its soluble and insoluble forms is essential for SG assembly and disassembly. Our data demonstrate that TDP-43 can modulate TIA-1 redistribution, thus we speculate that the association between TDP-43 and TIA-1 facilitates the homotypic interactions of TIA-1 (and thus modifies its solubilization) which are required for SG assembly and maintenance. The transcriptional down-regulation of G3BP by TDP-43 also yields fewer SGs. Thus, TDP-43 regulates SG formation via two independent mechanisms.

TDP-43 interacts with hnRNP A2 (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 indicates that it may be partially dependent on TDP-43. Specifically, the down-regulation of TDP-43 yielded fewer 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 transcription and/or TIA-1 redistribution 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 suggests a novel function for TDP-43 in acute stress. It will be interesting to evaluate the role of TDP-43 in chronic stress. Our observation that the TDP-43^{R361S} mutation is hampered in its ability to form SGs is interesting and suggests a potentially disease-relevant mechanism. (Note, continuous life-time expression of a mutant protein may itself be a model of chronic stress.) 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 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 as has been proposed in Parkinson's disease (33). This aspect remains to be tested in ALS. Importantly, we report here SG formation is not disturbed by the TDP-43^{D169G} mutation suggesting that it may be mechanistically independent of the reported C-terminal mutants. The nature of this mutation, predicted to be mRNA binding, remains to be demonstrated.

We describe a mechanistic role for endogenous TDP-43 in the cellular stress response. Our data suggests that TDP-43 regulation of stress granule

pathways 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.

MATERIALS AND METHODS

Plasmids, Cell Culture and Transfection- HeLa patient lymphoblast cell lines were grown in DMEM and IMDM, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin/glutamate. For transfection of small interfering RNA (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 was replaced with regular culture media without antibiotics after five hours. Cells were collected after 72 hours of siRNA treatment.

Cells were treated with various stresses including 0.5mM sodium arsenite (30 minutes, 37ºC; Sigma), heat shock (30 minutes, 43ºC), and 1μM thapsigargin (50 minutes, 37ºC; Sigma). For stress recovery experiments, cells were stressed and then media was replaced, and cells were permitted to recover for various times prior to collection and fixation.

Immunofluorescence and antibodies- Cells grown on coverslips were fixed in 1% formaldehyde (FA) in PBS and subsequently permeabilized with 0.1% Triton-X100 in PBS for 15 minutes. Coverslips were blocked with 0.1% BSA in PBS for 15 minutes 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 hour at room temperature. Coverslips were subsequently washed once with 0.1% Triton-X100 in PBS and twice with 0.1% BSA in PBS. Labeling was visualized with the fluorescently conjugated secondary antibodies donkey anti-rabbit Texas Red (Jackson Immunochemicals) and donkey anti-goat Alexa 488 (Invitrogen). Coverslips 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 (16). Images were collected on a Leica SP5 confocal microscope.

Quantification of SG size- The area of ten SGs (ranging from 0.75um^2-5 um^2), randomly selected in at least ten cells per condition was manually measured with ImageJ. The average SG size of at least 100 SGs in ten cells is presented.

Cell lysates and immunoblot analysis- Cells were collected in ice cold PBS, lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate), and centrifuged at 16 000x*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 25G syringe 6 times and centrifuged at maximum speed. Supernatants (soluble) were recovered and pellets (insoluble) were resuspended directly in 1x 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'-CGCAGAGATTCAGGTTCTCC-3'; and HuR exon 5 R: 5'-CCAAACCCTTTGCACTTGTT-3'.

Statistical analysis- Data was analyzed by Student t-test or one-way ANOVA, where appropriate. Error bars represent standard error of the mean (SEM).

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CONFLICT OF INTEREST STATEMENT

None declared.

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FIGURE LEGENDS

Figure. 1: TDP-43 is localized to stress granules. (A) HeLa cells cultured on coverslips were treated with 0.5mM sodium arsenite (30 mins; SA), 1 μM thapsigargin (50 mins; TH), and 43°C heat shock (30 mins; HS), or left as untreated (UN) and subsequently immunolabled 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. Data are representative of three independent experiments. (C) TDP-43 was not markedly co-localized with the PB marker, GW182. (D) Phosphorylation of eIF2α was assessed in the four stress conditions.

Figure 2: TDP-43 regulates stress granule 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 hours and subsequently treated with SA. Coverslips were collected at 0, 15, 20, and 30 minutes after the addition of SA to assess formation ("Stress"). After 30 minutes the media was replaced and coverslips were collected at 45, 60, 90, 150 and 270 minutes after the addition of SA ("Release"). SGs were scored via TIA-1 labeling. 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, 10um. (D) SGs are smaller in cells transfected with TDP-43 siRNA. The average area +/- SEM is plotted at 30 minutes post-stress.

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 was 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 is 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.

Figure 4: TDP-43 alters TIA-1 solubility and reduces G3BP SG formation. (A) RIPA soluble and insoluble fractions from control siRNA and TDP-43 depleted cells were analyzed via western blot for TDP-43 and TIA-1. Data is representative of three independent experiments. (B) Formation of G3BP SGs was assessed in HeLa cells transfected with control or TDP-43 siRNA for 72 hours and subsequently treated with SA. Coverslips were collected immediately and labeled for TIA-1 and G3BP.

Figure 5: Endogenous hnRNP A2 is localized to stress granules. HeLa cells cultured on coverslips were treated with 0.5mM sodium arsenite (30 mins; 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 hours and subsequently treated with SA. Coverslips were collected immediately and labeled for TIA-1 and hnRNP A2. Scale bar, 10 μm.

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 is representative of four experiments. Histogram indicates quantification via densitometry. *, $p<0.05$. (B) Control and mutant patient cells were treated with SA or left untreated (UN). Cells were labeled with TIA-1 and 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. *, $p<0.05$.

Figure 7: Model of TDP-43 in the regulation of SGs. Reduced TDP-43

protein levels or TDP-43 mutations yield a reduction in G3BP 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.

ABBREVIATIONS

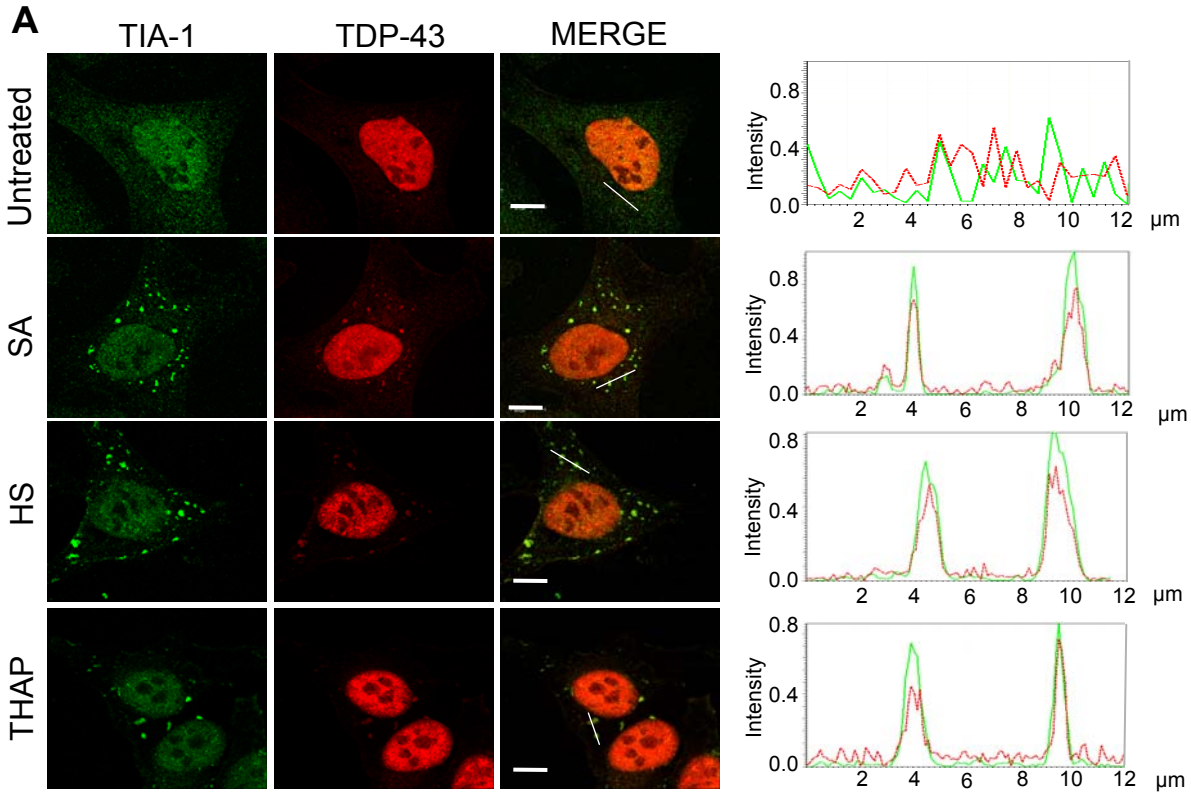
SG, stress granule

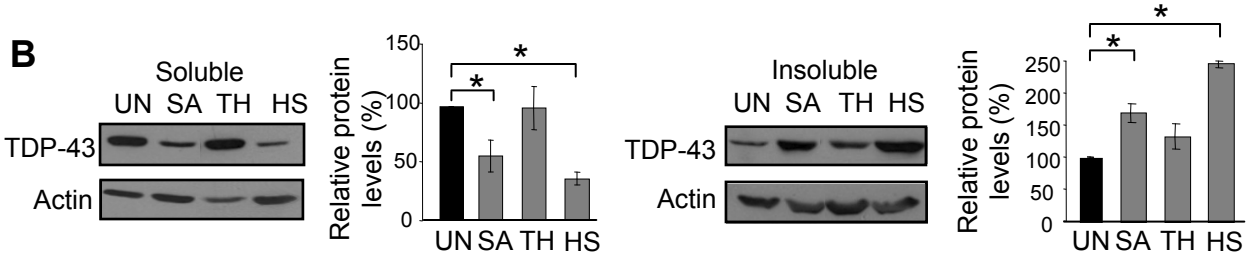
PB, processing body

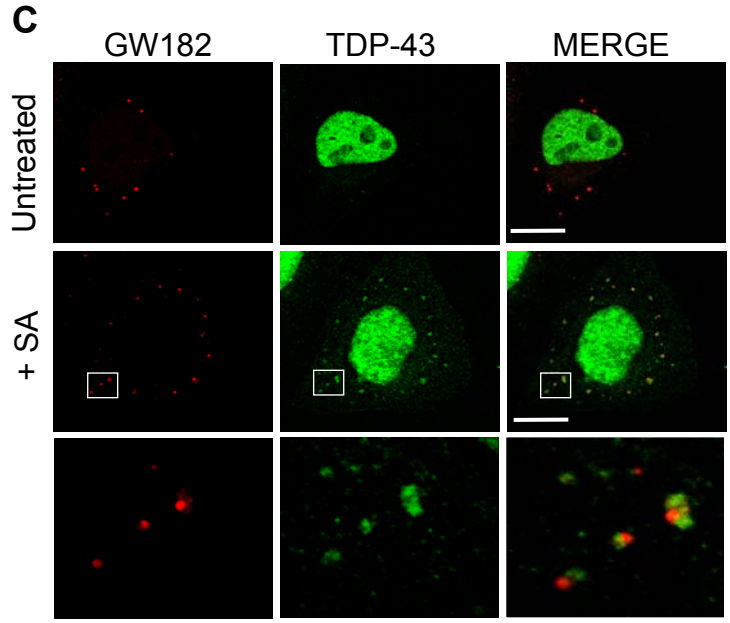
ALS, amyotrophic lateral sclerosis

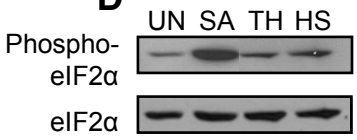
FTD, frontotemporal dementia

hnRNP, heterogeneous nuclear ribonucleoprotein

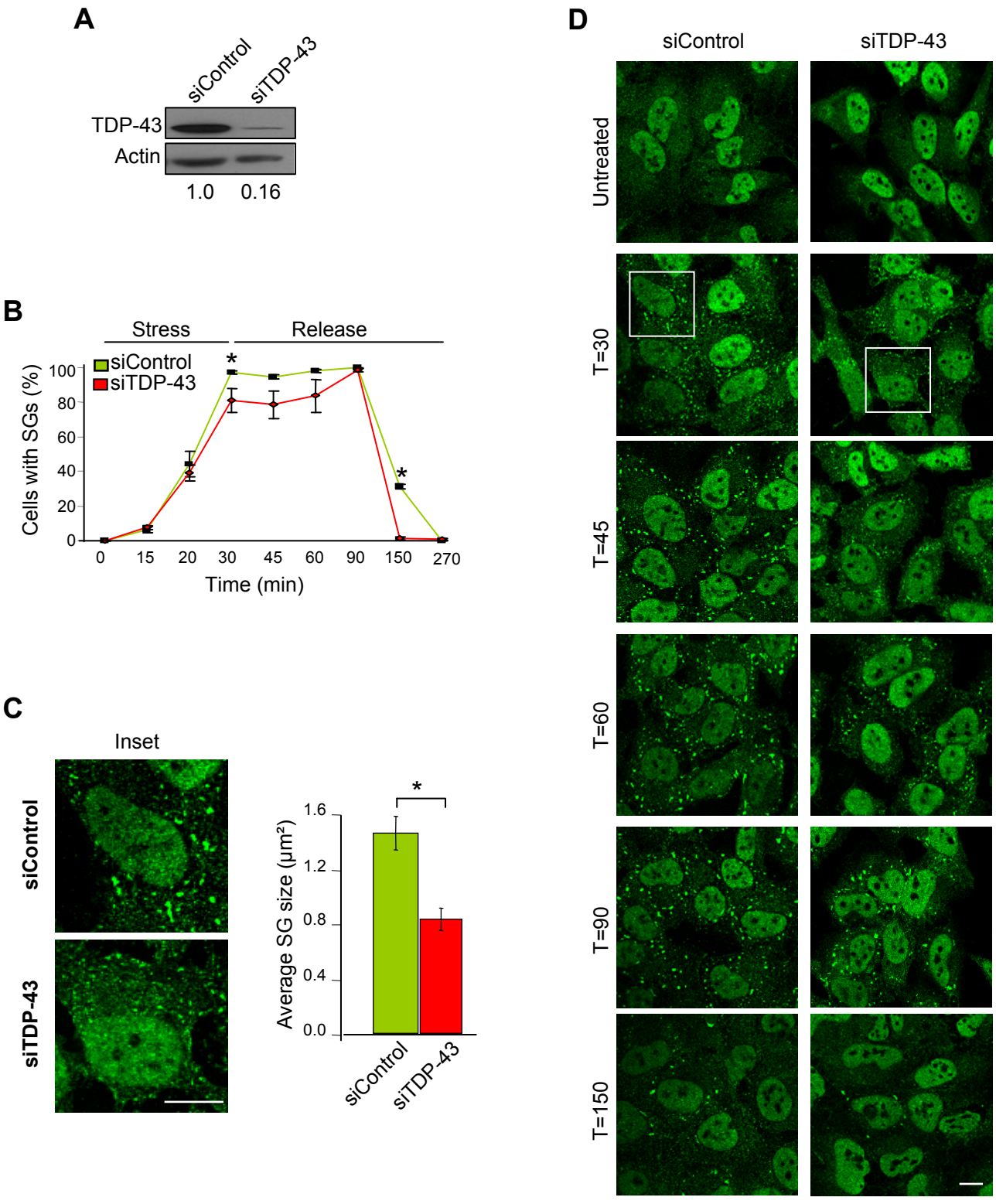








McDonald et al. Figure 1



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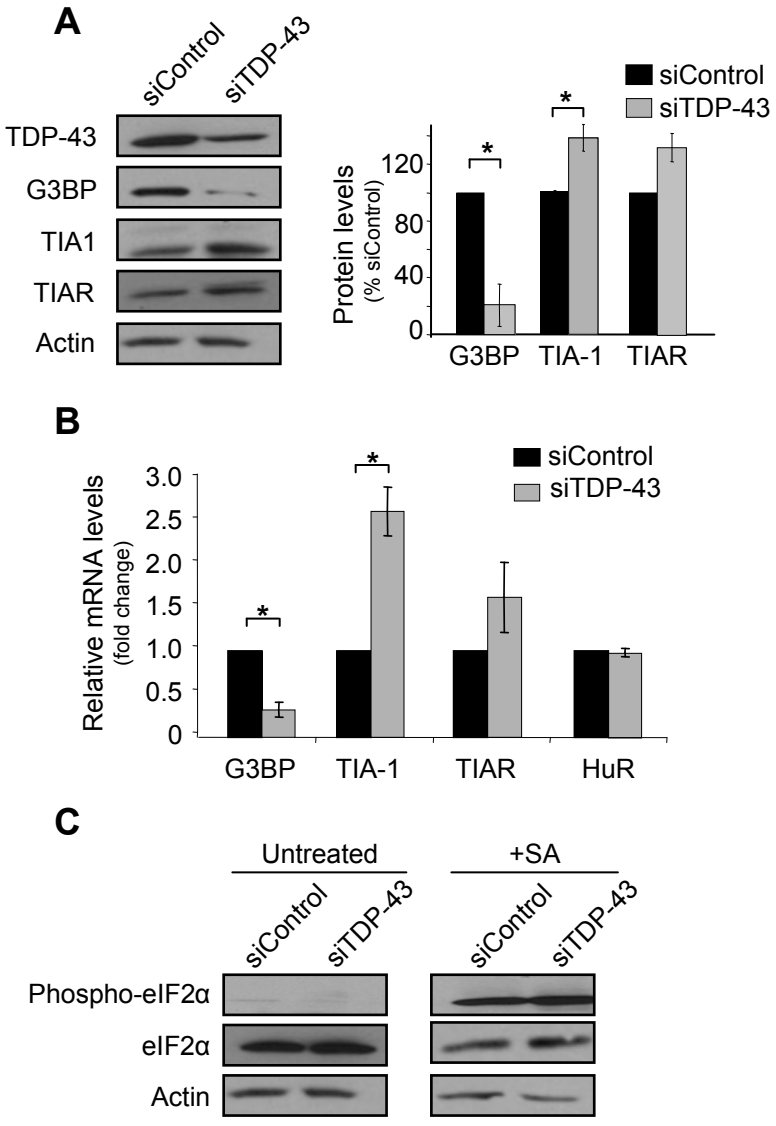

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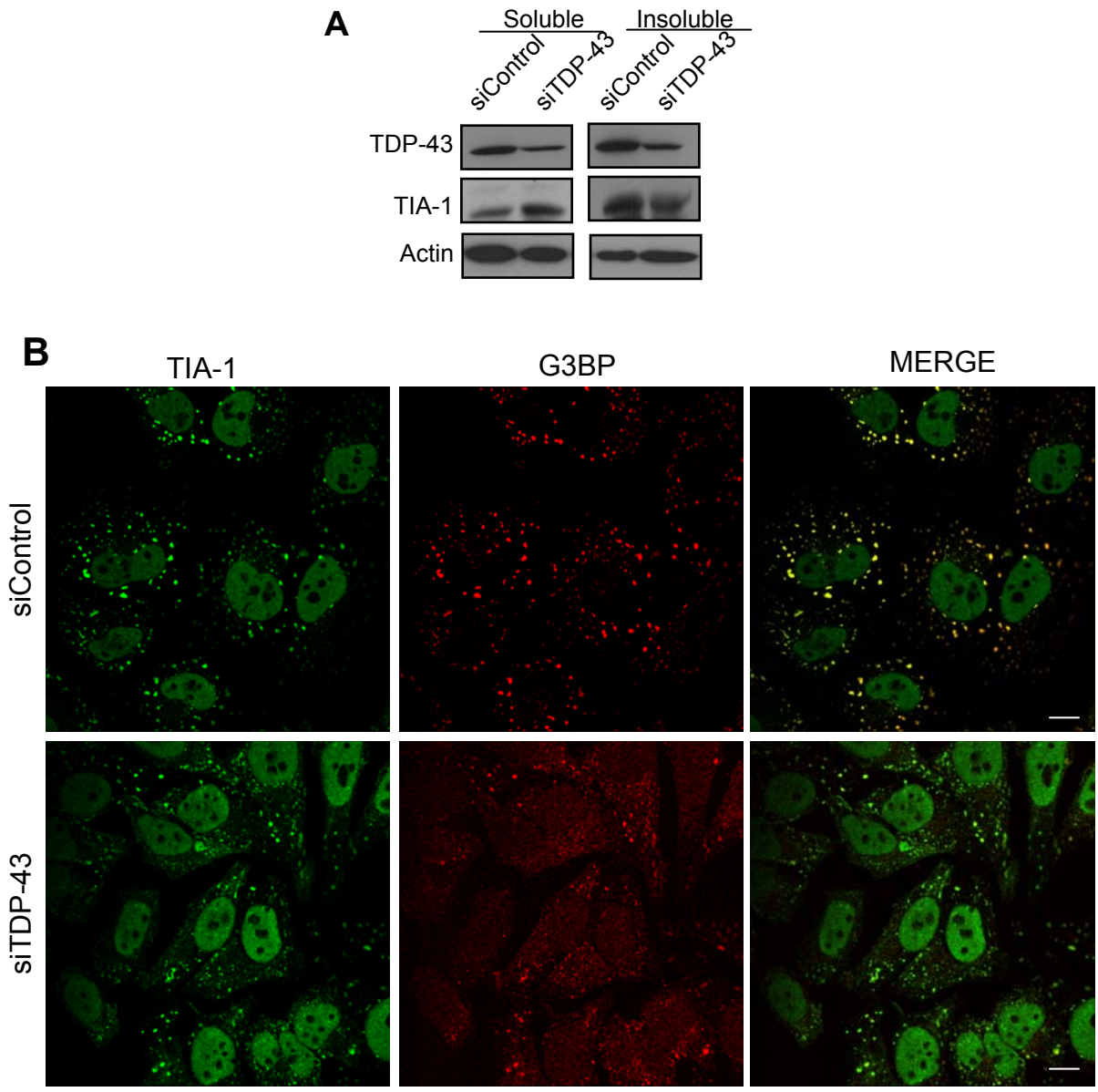

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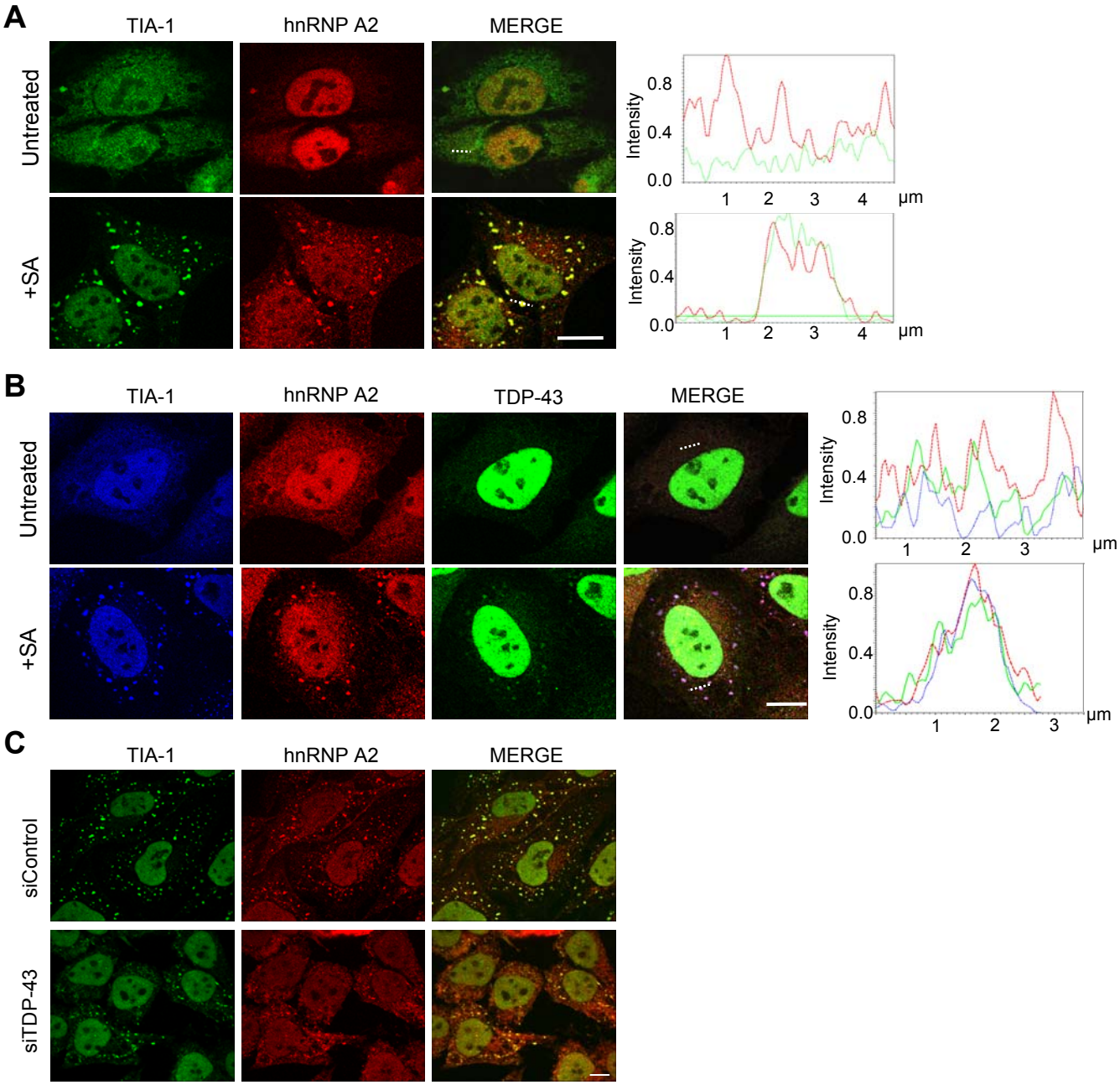

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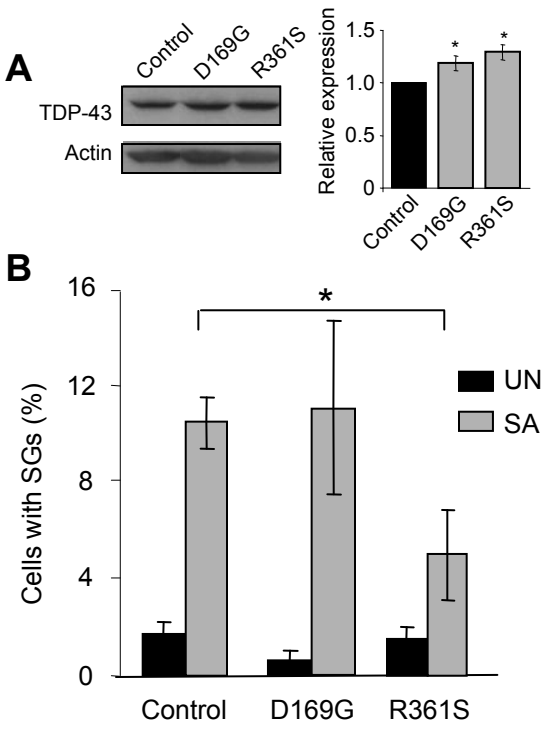

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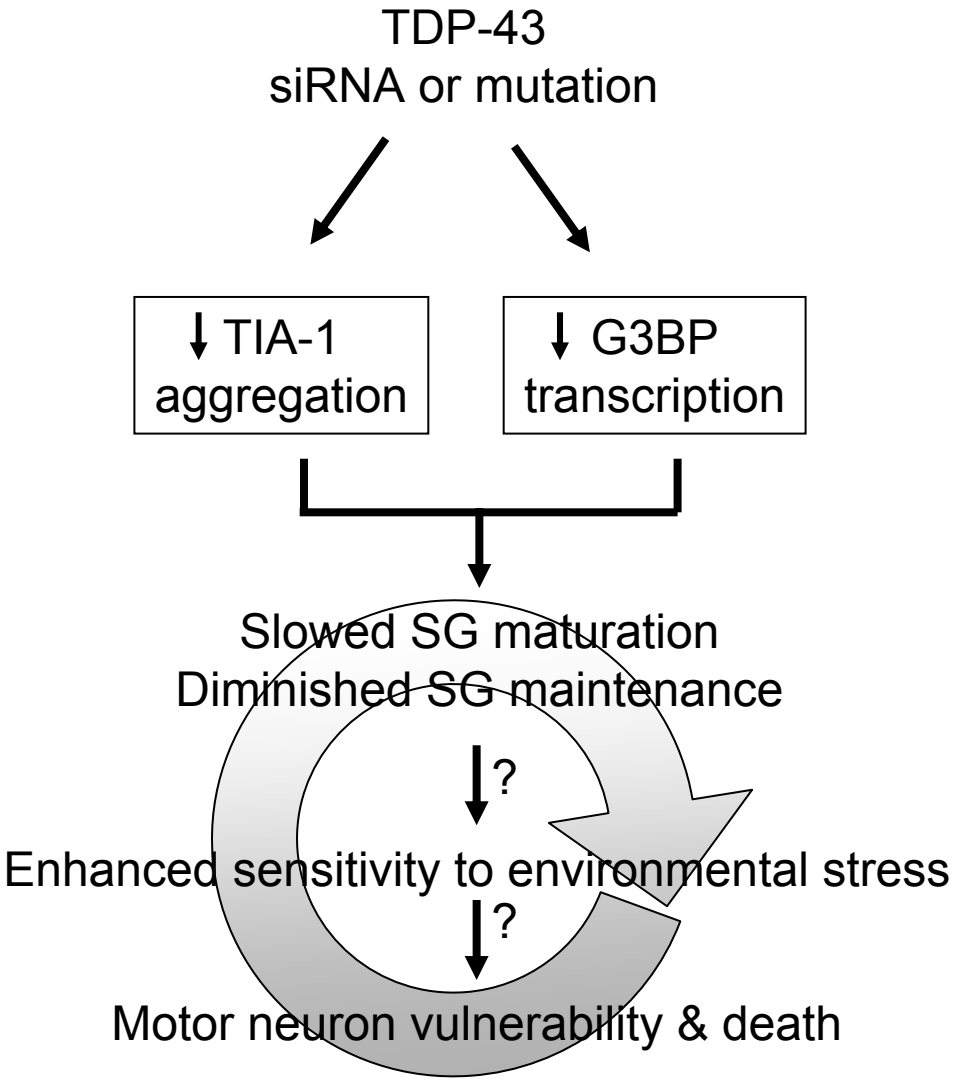

McDonald et al. Figure 7

D. DISCUSSION

TDP-43 redistribution

In this study we define a pivotal role for TDP-43 in the CSR. The CSR represents an evolutionary evolved mechanism by which cells respond and defend against changes in their environment [154]. One of the most immediate responses to cellular stress is a very rapid reversible suppression of mRNA translation, triggered by the phosphorylation of the translation initiation factor eIF2α. Translationally stalled mRNAs are recruited to discrete cytoplasmic foci called SGs. Our results demonstrate that endogenous TDP-43 forms cytoplasmic puncta following exposure to oxidative stress which colocalize with SGs, consistent with recently published reports [94, 95]. TDP-43 also localizes to SGs with HS and THAP treatment. Furthermore, we show TDP-43 redistribution with both SA and HS however, SA induces a much more robust stress response in our experimental model. Arsenite is a potent environmental toxin that contributes to human pathogenesis resulting in skin disease, cancer, and cardiovascular disease [25]. Arsenite also represents an oxidative stress, which is of particular interest in the case of TDP-43 since one of the major commonalities of neurodegenerative diseases is the progressive accumulation of damage resulting from oxidative stress in the CNS [155]. There is evidence of increased oxidative stress in Alzheimer's disease, Parkinson's disease, ALS and FTLD. In our study, TDP-43 protein expression is decreased in RIPA-soluble fractions and increased in RIPA-insoluble fractions. RIPA insoluble cellular fractions typically consist of chromatin and/or nuclear matrix components of structure bound portions of the nucleus [88]. TDP-43 has

previously been described in the cellular nuclear fraction as a transcriptional regulator of the spermatid-specifc SP-10 gene [78]. Therefore, TDP-43 redistribution in our study may represent an important mechanism of increased transcriptional regulation by TDP-43 during stress.

TDP-43 is also an important splicing regulator. Alternative splicing is a key mechanism during an insult, allowing the production of protein isoforms that enable the cell to better manage the stress and favour the repair of the damage caused [156]. Sam68, another RBP, also associates with insoluble chromatin and nuclear matrix fractions under stress [156]. Specifically, genotoxic stress induces redistribution of chromatin components with an enrichment of Sam68 and hnRNP A1 in transcriptionally active fractions. The subnuclear re-localization of Sam68 correlates with changes in alternative splicing of its mRNA targets. Therefore, during stress, TDP-43 may also play a role in specific splicing events, similar to other splicing regulators including TIA-1 and hnRNP A1, which show the same redistribution with stress [156]. Furthermore, Sam68 localizes to cytoplasmic SGs with genotoxic and oxidative stress, and interacts with both TIA-1 and G3BP in order to be recruited to SGs [156, 157]. An increase in the insoluble fraction of stress proteins, like TIA-1, during an insult is consistent with the formation of SGs. Therefore, TDP-43 redistribution to an insoluble fraction likely reflects its localization to cytosolic SGs during stress. Such localization may indicate cytoplasmic functions such as mRNA silencing, trafficking, storage, and sorting. Future studies are required to determine whether redistribution also involves stress-specific nuclear functions

such as transcription and splicing.

In our study, TDP-43 does not localize to PBs, which are sites of mRNA degradation, but is found to be in close proximity. Although TDP-43 does not directly localize to PBs, it may still be functionally associated. For example, TDP-43 could be involved in the tethering of SGs to PBs or could determine the fate of transcripts that arrive at SGs (e.g. storage or degradation). Indeed, hnRNP D demonstrates such a role, promoting decay during oxidative stress [13]. In response to prostaglandin A2, an oxidative stressor, hnRNP D lowers the stability of cyclin D1 mRNA, targeting it for decay. Although the mechanisms of degradation are not well understood, both the exosome and PBs are involved. Interestingly, TDP-43 co-localizes with SGs and PBs in ALS spinal cord motor neurons, which is consistent with the re-localization of TDP-43 under neuronal stress [93]. Therefore, we speculate TDP-43 does play a role in mRNA degradation during stress, albeit secondary.

hnRNP A2 localizes to SGs with oxidative stress

For the first time, we show endogenous hnRNP A2 forms cytoplasmic puncta in response to oxidative stress and these puncta localize to the same SGs as TDP-43. hnRNP A2 directly interacts with TDP-43, although functional links remain to be established [85]. hnRNP A2 is a nuclear-cytoplasmic shuttling protein with known functions is RNA processing and trafficking, telomere maintenance and oncogenesis [46, 158-161]. More recently, hnRNP A2 was described as a transcriptional co-activator of stress responsive genes in

the mitochondrial respiratory response pathway [162]. There are currently no reports that suggest a role for hnRNP A2 in the cellular stress response however, hnRNP A2 also directly interacts with PAPB1, which is a well-known SG component [26, 163]. Defining the role of hnRNP A2 during cellular stress remains to be investigated.

TDP-43 Regulates Stress Granule Dynamics

Our study demonstrates a novel role for TDP-43 in SG dynamics. SG formation is a transient event in which a cellular insult causes nuclear TIA-1 to translocate to the cytosol followed by the subsequent aggregation into SGs. The process occurs over approximately 15 minutes and once the insult is removed, SGs continue to coalesce for up to one hour [27]. SGs become larger and fewer over time, until eventually disassembling as the cell recovers. We show that TDP-43 depleted cells fail to form morphologically distinct SGs where SGs are fewer and smaller. Additionally, SGs in TDP-43 reduced cells are unable to persist for the same period as control. Therefore, although small SGs do form, and eventually attain a similar size and morphology as control, the structure dissipates quickly. These results indicate that TDP-43 is required for the proper formation and maintenance of SGs. Some SG-associated proteins serve as molecular scaffolds that define the SG domain. Indeed, fas-activated serine/threonine phosphoprotein, a TIA-1 binding protein, nucleates PBs and SGs and is stably associated with both [35]. While most SG proteins constantly shuttle through SGs, arguing against a rigid SG structural compartment, the composition of SGs remains relatively constant [29, 32, 35]. TDP-43 has

structural characteristics that support a role in SG formation. For example, G3BP, a key factor in SG assembly has a dimerization domain that contributes to SG formation during arsenite treatment [21]. TDP-43 also has the capacity to dimerize [164]. Dimerization has been demonstrated by immunoprecipitation in which endogenous full-length TDP-43 and an exogenous flag-tagged protein interact via the N-terminus [164]. When the 86kDa dimerized protein is over-expressed in cells, it is sequestered in the cytoplasm and promotes the accumulation of TDP-43 immunoreactive proteins. Additionally, TDP-43 itself is inherently aggregate prone and contains a region (amino acids 343–360) rich in the amino acids glycine, glutamine and/or asparagine [28]. This type of sequence also exists in the PRD of TIA1-1, and has been shown to form specific aggregates that mediate the assembly of SGs. Therefore, TDP-43 may have a domain that is required for SG structure/assembly or mediates an interaction with SG components for proper aggregation. As such, Colombrita and colleagues have found that the selective lack of 100 amino acids in the C-terminal region of TDP-43, responsible for protein-protein interactions, results in the failure of TDP-43 to assemble into SGs [94]. Interestingly, the aggregation-prone domains of one protein can interact with other proteins to influence the transition between soluble and aggregation-prone confirmations [28]. In agreement with this, we show that TDP-43 regulates TIA-1 distribution.

TDP-43 regulation of TIA-1 and G3BP

The soluble form of TIA-1 acts as a translational silencer and the

insoluble aggregated form promotes stress-induced translational arrest via SG assembly. Therefore, TIA-1 solubility is critical for SG assembly. The exact mechanisms governing TIA-1 solubility are not well understood however, there is evidence suggesting HSP70 regulation is involved [28]. Gilks and colleagues have shown that HSP70 is required to maintain TIA-1 in a soluble (nuclear) form [28]. Therefore, during an insult, as HSP70 is recruited for protein renaturing, it is diverted from TIA-1, leading to TIA-1 aggregation and subsequent SG nucleation. Once the stress is removed, HSP70 once again solubilizes TIA-1, promoting SG disassembly. In TDP-43 depleted cells, we show an increase in the accumulation of soluble TIA-1 protein and an increase in mRNA with a concurrent decrease in insoluble TIA-1. TDP-43 regulation of TIA-1 provides a mechanism for impaired SG formation such that TIA-1 is hindered in transitioning to an insoluble form, thus delaying its aggregation into SGs. Therefore, we propose that TDP-43 is required for the initial homotypic aggregation of TIA-1, if not the heterotypic aggregation as well. However, SGs eventually attain a similar morphology and size as controls, suggesting that TIA-1, and perhaps other proteins, may be able to compensate or overcome TDP-43 deficits.

TDP-43 regulation of TIA-1 is also of particular interest since factors that regulate TIA-1 aggregation may influence cell survival. Normally TIA-1 aggregation is reversible, however when damage exceeds cellular thresholds, the aggregation is irreversible [27]. Thus, one could hypothesize that in an ALS disease context, the presence of TDP-43 mutants could enhance TIA-1

deregulation, contributing to a maladaptive stress response. Over time, this deregulation could lead to SGs that no longer dispersed, forming the initial pathological aggregates. In our model, we present an acute model of stress, whereas the disease context more likely represents chronic stress. Therefore, future studies will be aimed at investigating the role of TDP-43 in the CSR with chronic stress.

We also show that endogenous TDP-43 regulates SG dynamics via G3BP regulation. In TDP-43 depleted cells, there is a decrease in G3BP at both the mRNA and protein level. SG formation is inhibited in cells that are deficient in G3BP [31]. Therefore, TDP-43 induced reduction of G3BP is consistent with diminished SG assembly, providing a second mechanism for impaired SG dynamics. Interestingly, other studies have shown that TDP-43 functions as an mRNA stabilizing factor [75, 86]. Thus, TDP-43 may be required for the stabilization of G3BP mRNA, if not other mRNAs of SG components. Therefore, our evidence places TDP-43 upstream in the CSR in the regulation of SG components. Of note, eIF2α phosphorylation levels remain unchanged with TDP-43 depletion thus placing TDP-43 downstream of eIF2α phosphorylation. Furthermore, we suggest there is an interaction between TDP-43 and SG components that is crucial for proper SG formation. Consistently, we show both G3BP and hnRNP A2 fail to form discrete puncta in TDP-43 depleted cells. Both proteins have altered morphologies and distributions (e.g. more diffuse) within the cytosol following oxidative stress. Detailed studies examining other SG factors will provide insight as to whether this effect is a

specific effect of TDP-43 on hnRNP A2 and G3BP, or a global effect of TDP-43 on SGs, such that no SG components are able to aggregate properly. Moreover, determining whether ALS-linked mutations in TDP-43 affect TDP-43 regulation of SG proteins is an interesting next step.

hnRNP A2 redistribution

Our study demonstrates that TDP-43 regulates hnRNP A2 distribution. In cells depleted of TDP-43, hnRNP A2 redistributes from a soluble to an insoluble form. It is not uncommon for hnRNP proteins to interact and collaborate in order to carry out specific functions. Indeed, hnRNP A1 and hnRNP H interact directly to enforce specific splicing decisions, providing an important mechanism of splicing control [165]. Moreover, several hnRNP related molecules can replace each other, particularly in the case of the hnRNP A/B family, to carry out functions such as alternative splicing. For instance, hnRNP A1 is involved in mouse hepatitis virus replication and transcription [59]. In the absence of hnRNP A1, mouse hepatitis virus RNA interacts with several alternate proteins, all belonging to the hnRNP A/B family in RNA replication. This event is attributed to the structural similarities between these proteins, particularly the homology of their RBDs. Further evidence for this phenomenon is demonstrated in the mouse erythroleukemia cell line CB3 which is an hnRNP A1-deficient cell line. The deletion is not lethal, suggesting critical hnRNP A1 functions are replaced by other alternative factors [166]. TDP-43 is most closely related to the hnRNP A/B family and directly interacts with hnRNP A2. Therefore, hnRNP A2 redistribution with TDP-43 depletion

may represent a compensatory mechanism where hnRNP A2 replaces, and carries out, the functions of TDP-43 in its absence.

TDP-43 mutations

Our results provide evidence for impaired SG formation in human patient lymphoblastoid cells expressing the disease-causing mutation TDP-43^{R361S}. This observation is not the result of decreased protein expression since we show both TDP-43^{D169G} and TDP-43^{R361S} have increased steady-state expression levels. An increase in mutant TDP-43 protein levels is not surprising, considering that TDP-43 mutants have increased protein stability [167]. The defect conferred by the TDP-43^{R361S} mutation provides an interesting new avenue to explore involving HDAC6. HDAC6, a multifunctional deacetylase that plays a role in deciding the fate of polyubiquitinated proteins, is also a component of SGs and is required for SG assembly [160, 170]. In cells lacking HDAC6, TIA-1 remains largely nuclear, failing to translocate to the cytoplasm [170]. Moreover, HDAC6 deficient mouse embryonic fibroblasts fail to form morphologically discrete SGs. Fiesel and colleagues have shown, in a cell culture model, that TDP-43 regulates HDAC6 levels such that silencing of TDP-43 results in specific down-regulation of HDAC6 (both at the protein and mRNA level) [168]. Moreover, the expression of various TDP-43 mutants is able to restore HDAC6 levels to control levels, except expression of the R361S mutant. Therefore, given these data, the TDP-43^{R361S} mutation may be hindered in its ability to form SGs in our study due to deregulation of HDAC6.

More recently, TDP-43 has been shown to form a complex with FUS/TLS

to coregulate HDAC6 [86]. Therefore, mutations in either TDP-43, or FUS/TLS, may result in a dysfunctional complex, deregulating its target mRNA, and thus impairing SG formation. As such, it would be interesting to investigate whether the TDP-43:FUS/TLS complex is important in the regulation of other components of the stress response, such as TIA-1 or G3BP. The relationship between TDP-43, FUS/TLS, HDAC6 and SGs requires further investigation. Future studies are also required to properly understand how different ALS-linked mutants may perturb the normal stress response and components of the pathway.

E. CONCLSUION

Cellular recovery from stress is not successful when damage exceeds certain thresholds, or when one or more components of the stress-activated cascade is impaired. The SG pathway is increasingly implicated in disease. For example, it has been demonstrated that SMN, a protein associated with autosomal recessive motor neuron disease and spinal muscular atrophy, facilitates SG formation and may influence neurodegeneration via the disruption of RNA stability through SG function [152]. Furthermore, in Parkinson's disease it is proposed that large pathological aggregates arise due to disrupted SG dynamics [169]. Taken together, our data demonstrate an important role for TDP-43 in response to acute cellular stress. During an insult, we propose that TDP-43 may function in stress-specific splicing and transcription, ultimately influencing gene regulation and cellular survival. We provide evidence for TDP-43 localization to SGs with various types of insults and show TDP-43 is not merely a resident of SGs, but participates in the stress response. TDP-43 functions as an integral component in SG assembly and an upstream regulator of key SG proteins. Furthermore, we provide the first description of hnRNP A2 in the CSR, localized t SGs, and we hypothesize that hnRNP A2 may compensate for TDP-43 when TDP-43 is silenced. One method, in the context of this study, to test this hypothesis is to determine if the expression of hnRNP A2 could restore defects in both SG dynamics and TIA-1/G3BP levels in the absence of TDP-43. Confirming this hypothesis presents an interesting mechanism of how to overcome deficits resulting from TDP-43

mutations.

We describe an effect for one TDP-43 mutation, which is deficient in the formation of SGs. The effects of TDP-43 mutations on its proper functioning in the CSR can be numerous. For instance, mutations representing a loss or gain of function can range from incomplete repression of mRNAs or de-repression at inappropriate timing, defects in splicing and transcription, alterations in cell stress signaling pathways and also changes in the regulation of gene transcription.

In conclusion, our study provides a model for how TDP-43, as a key player in the CSR, can contribute to disease. Disruptions of TDP-43 lead to impairments in the stress response via different mechanisms. Two of which are TIA-1 and G3BP regulation, essentially resulting in improper SG formation and maintenance (Fig 1). We believe a muted stress response over time, paired with successive encounters of oxidative stress, may lead to an overall inefficiency of cells to manage and recover from stress, ultimately leading to death. Defining the relevance of this study of TDP-43 biology specifically in motor neurons and ALS is now an ensuing challenge.

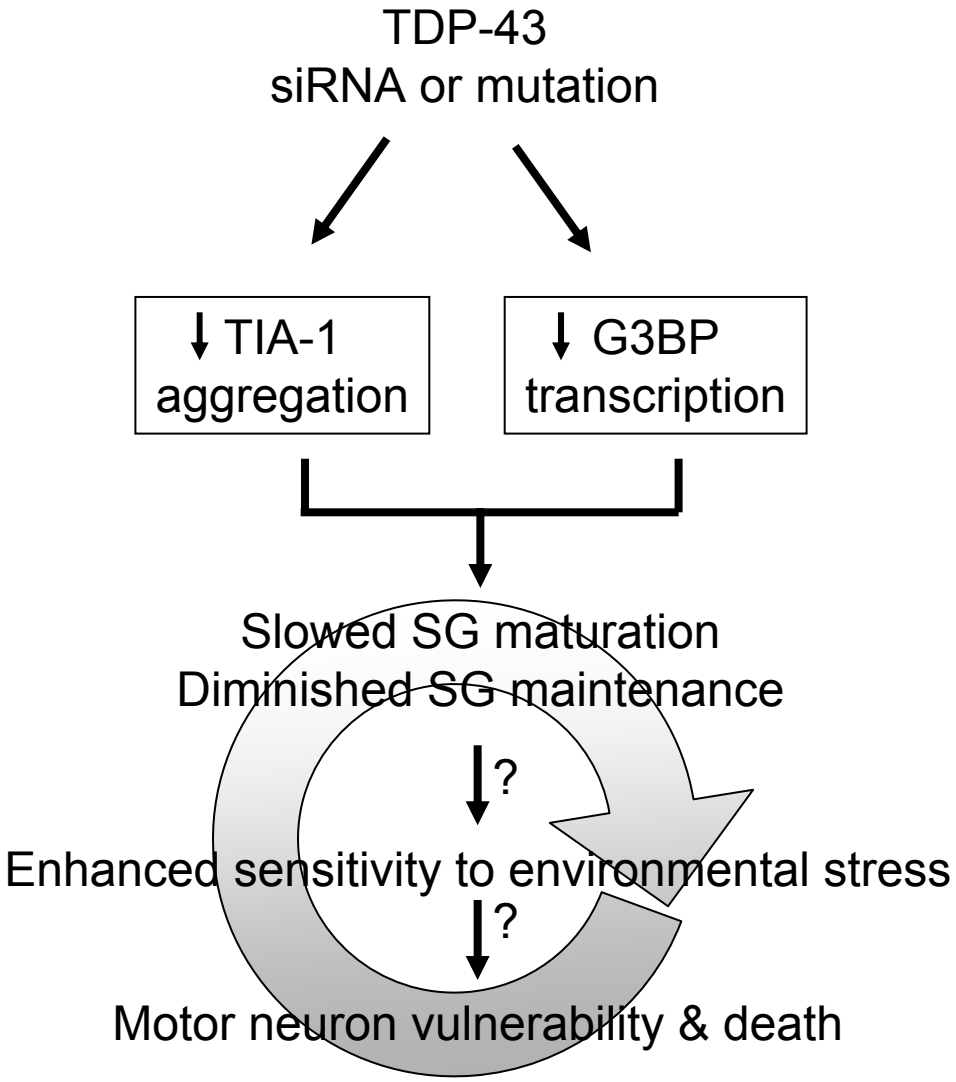

Figure 1. Model of TDP-43 in regulation of SGs. (Figure adapted from McDonald, K.K., Aulaus, A., et al. (2010). *Manuscript under revision*)

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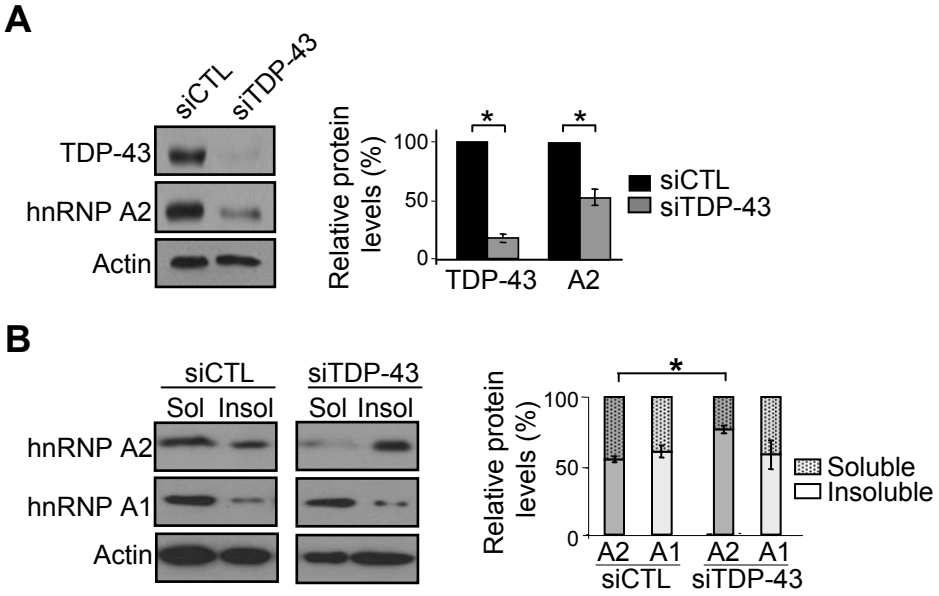
APPENDIX

I. Results Omitted from Manuscript

Figure 1. Endogenous TDP-43 regulates hnRNP A2 solubility

Given our data that TDP-43 regulates SG components, both hnRNP A2 and TDP-43 localize to SGs, and that TDP-43 and hnRNP A2 are reported to directly interact [85], we hypothesized that TDP-43 may regulate hnRNP A2 directly. Using TDP-43 siRNA, we established conditions which consistently yield an average 84% reduction of TDP-43 protein levels compared to control siRNA 72 hours post-transfection (**Fig. 1A**). Interestingly, the pool of soluble hnRNP A2 was also found to be reduced 56% in TDP-43 depleted cells suggesting that TDP-43 directly regulates hnRNP A2 (**Fig. 1A**). hnRNP proteins commonly exist in RIPA-soluble and insoluble compartments, likely due to their ability to form large ribonucleoprotein complexes and/or interact with nuclear matrix components. Thus, we sought to determine whether the apparent reduction in hnRNP A2 was due to TDP-43 regulation of hnRNP A2 distribution in RIPA solubilized cells. We noted that in control siRNA cells, 45% of total hnRNP A2 is soluble (**Fig. 1B**). However, in the absence of TDP-43 there is a two-fold decrease in the available soluble pool of hnRNP A2 and a concomitant increase in hnRNP A2 in the insoluble fraction of RIPA-solubilized cells such that 77% of hnRNP A2 is insoluble (**Fig. 1B**). The impact of TDP-43 on hnRNP A2 distribution was specific since the distribution of the related molecule hnRNP A1 was unchanged in the absence of TDP-43. Thus, TDP-43 is required to maintain a soluble pool of hnRNP A2.


A
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hnRNP A2
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Relative protein levels (%)
100
50
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TDP-43
A2
siCTL
siTDP-43
B
siCTL
siTDP-43
Sol Insol
Sol Insol
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hnRNP A1
Actin
Relative protein levels (%)
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50
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Soluble
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