Stress granules as crucibles of ALS pathogenesis

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Amyotrophic lateral sclerosis (ALS) is a fatal human neurodegenerative disease affecting primarily motor neurons. Two RNA-binding proteins, TDP-43 and FUS, aggregate in the degenerating motor neurons of ALS patients, and mutations in the genes encoding these proteins cause some forms of ALS. TDP-43 and FUS and several related RNA-binding proteins harbor aggregation-promoting prion-like domains that allow them to rapidly self-associate. This property is critical for the formation and dynamics of cellular ribonucleoprotein granules, the crucibles of RNA metabolism and homeostasis. Recent work connecting TDP-43 and FUS to stress granules has suggested how this cellular pathway, which involves protein aggregation as part of its normal function, might be coopted during disease pathogenesis.

Stress granules, processing bodies, and RNA triage

Life is stressful. Eukaryotic cells have evolved sophisticated strategies to combat a barrage of cellular stresses, which include heat shock, chemical exposures, oxidative stress, and even aging (Morimoto, 2011). Under stress, a cell's top priority is to conserve energy and divert cellular resources toward survival and eventual recovery. One way to conserve resources is to limit the translation of cellular mRNAs and focus on producing just the essential proteins needed for survival (Lindquist, 1981). In eukaryotic cells, a powerful way of accomplishing this switch is by rapidly assembling nontranslating mRNAs and their associated RNA-binding proteins into aggregate-like structures. These structures, called RNP granules, include processing bodies (P-bodies) and stress granules (SGs; Anderson and Kedersha, 2008).

The localization, modification, and decay of mRNAs in RNP granules play a critical role in the cellular stress response (Anderson and Kedersha, 2008). P-bodies and SGs are sites where nontranslating mRNAs, as well as factors involved in translation repression and mRNA decay, preferentially localize when translation is stalled or impeded. SGs form in response to a variety of environmental stresses that impede translation (e.g., heat shock, glucose deprivation, oxidative stress, etc.) and may represent localized zones of stalled translation initiation (Kedersha et al., 2005; Kwon et al., 2007; Anderson and Kedersha, 2008). Indeed, SGs can be induced by pharmacologically inhibiting translation initiation, depleting mRNA translation initiation factors, or even by overexpressing certain RNA-binding proteins (Anderson and Kedersha, 2008).

Like SGs, P-bodies are also RNP granules that serve key roles in mRNA homeostasis (Jain and Parker, 2013). In contrast to SGs, P-bodies are not associated with regulation of translation initiation, but rather represent sites of mRNA degradation, translation repression, nontranslating mRNAs, and RNA-binding proteins. P-bodies mediate mRNA decay, including nonsense-mediated decay (NMD), and RNA interference by serving as sites of colocalization for RNA processing components such as the RNA decapping machinery, including the decapping enzymes DCP1/2; the activators of decapping Dhh1/RCK/p54, Pat1, Scd6/RAP55, and Edc3; the Lsm1-7 complex; and the exonuclease Xrn1 (Parker and Sheth, 2007). The mobilization of RNAs and RNA-binding proteins to RNP granules is differentially regulated (Jain and Parker, 2013; Shah et al., 2013) after induction by various sources of cellular stress. Although SGs and P-bodies are distinct foci of RNA-RNP localization, they can physically interact to facilitate the shuttling of RNA and protein species from one spatially localized compartment to another. Hence, SG and P-body formation are powerful protective mechanisms through which eukaryotic cells can dynamically triage RNA metabolism as they fight for survival from environmental stress (Anderson and Kedersha, 2008).

How to build an SG: the role of RNA-binding proteins with prion-like domains

In response to an unexpected cellular stress, RNP granules form very rapidly (within minutes) to minimize mRNA damage (Fig. 1; Chernov et al., 2009; Buchan et al., 2011). How are

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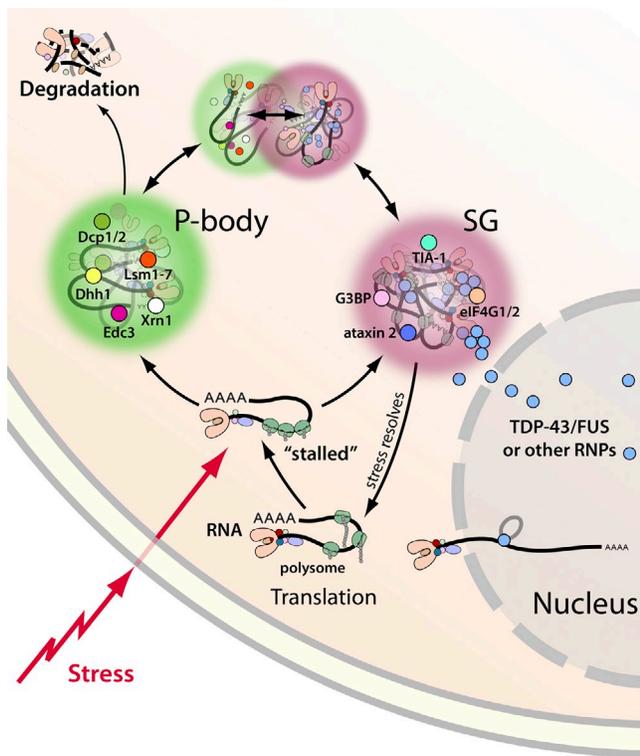
Figure 1. **SGs and P-bodies are sites of RNA triage.** Exposure to cellular stress can trigger a stress response that stalls translation initiation, resulting in the formation of SGs. SGs are dynamic cytoplasmic RNA–protein complexes that contain RNA-binding proteins, mRNAs, and translation initiation factors. When stress exposure dissipates, SGs disassemble and mRNA translation resumes. Nontranslating mRNAs can also be directed to P-bodies, distinct RNA-protein granules that are sites of stalled translation and mRNA degradation. SGs and P-bodies are differentially regulated and form independently, but they can and often do interact with each other. TDP-43, FUS, and other RNPs (e.g., TAF15, EWSR1, hnRNPA1, and hnRNPA2B1) reside predominantly in the nucleus, but stress exposure can trigger their recruitment to SGs. The implications for the recruitment of TDP-43, FUS, and others to SGs are explored in Fig. 3.

RNA-binding proteins and their associated RNAs able to coalesce to form SGs with such alacrity? Intriguingly, many SG- and P-body–associated RNA-binding proteins harbor prion-like domains (Gilks et al., 2004; Couthouis et al., 2011, 2012; Gitler and Shorter, 2011; King et al., 2012; Kim et al., 2013). A "prion-like domain" simply refers to a protein domain with a similar amino acid composition to yeast prion domains, which enable various yeast prion proteins such as Sup35 or Rnq1 to access the prion state (King et al., 2012). Prions are proteins capable of forming infectious amyloid conformations, which self-template their own assembly (Fig. 2, steps a–d). Prions can transmit heritable phenotypic changes from one cell to another, between individuals, or even between species (Shorter and Lindquist, 2005; Colby and Prusiner, 2011). In mammals, prions are the pathogenic agent responsible for deadly spongiform encephalopathies. Interestingly, yeast cells can transmit infectious phenotypes using a similar self-templating prion mechanism, and these phenotypes can sometimes even be beneficial, allowing yeast cells to adapt to and cope with varying environment conditions (Shorter and Lindquist, 2005; Malinovska et al., 2013).

A unifying feature of the vast majority of known yeast prion proteins is the presence of a modular prion domain (Wickner et al., 2000). This domain enables the protein to which it is appended to form self-templating amyloid fibrils (Fig. 2, steps a–d; Li and Lindquist, 2000). Accessing the aggregated or prion form usually leads to a reduction of the protein's function. Importantly, these aggregation-associated phenotypes are reversible, and sophisticated disaggregation machinery helps to regulate the disassembly of prion aggregates (Glover and Lindquist, 1998; Shorter and Lindquist, 2004; Cashikar et al., 2005).

Strikingly, many of the RNA-binding proteins that make up SG and P-bodies harbor prion-like domains. Of the top 100 yeast proteins that harbor validated or predicted prion domains, ∼10% contain an RNA-recognition motif (RRM) domain, and ∼24% are annotated with a gene ontology (GO) accession of "RNA binding," several of which have been shown to contribute to SG and P-body formation. In the human proteome, there are ∼250 proteins with computationally predicted prion-like domains (King et al., 2012), of which ∼12% harbor an RRM domain and ∼20% are annotated with a GO accession of RNA binding (Table 1). Besides RNA binding and closely related categories, other functional categories enriched for proteins with prion-like domains include chromosome organization (GO:0051276), embryo development (GO:0009790), and protein-binding transcription factor activity (GO:0000988; GO Slim categories with <1,000 genes and an enrichment p-value $<10^{-5}$ by a Fisher's exact test even when RNA-binding proteins were excluded). The striking overrepresentation of prion-like domains in RNA-binding proteins strongly suggests an important role of this domain in RNA-binding protein function. Indeed, prion-like domains are ideal tools for RNA-binding proteins to deploy. Because they are able to self-associate, these domains confer the ability to rapidly coalesce to form P-bodies and SGs, and indeed many other RNP granules that serve as "crucibles" for many facets of RNA metabolism (Gilks et al., 2004). For example, TIA1, an RNA-binding protein that is required for SG formation in mammals, contains a prion-like domain that drives SG assembly (Gilks et al., 2004). Moreover, deletion of the prion-like domain from Lsm4 and Pop2 reduces their ability to accumulate in P-bodies in yeast (Reijns et al., 2008).

RNA-binding proteins equipped with prion-like domains are able to recruit target mRNAs with their RNA-binding domains (e.g., RRM, KH) and then round them up into RNP granules using their prion-like domains, where they wait for the cellular stress to subside. The reversibility of prion-like aggregation allows the RNP granules to rapidly dissociate when the stress dissipates, releasing the sequestered mRNAs and translation machinery to resume their normal functions. The precise machinery that drives SG disassembly remains to be fully elucidated, and it should be noted that metazoa lack direct homologues of Hsp104, which drives rapid prion dissolution in yeast (Shorter and Lindquist, 2004). However, it is not clear in the context of SGs whether prion-like domains access a highly stable amyloid form (Fig. 2). Indeed, the prion-like domains of some RNA-binding proteins access a more labile and dynamic cross-β structure, which can be tightly regulated by phosphorylation of serine residues in the prion-like domain
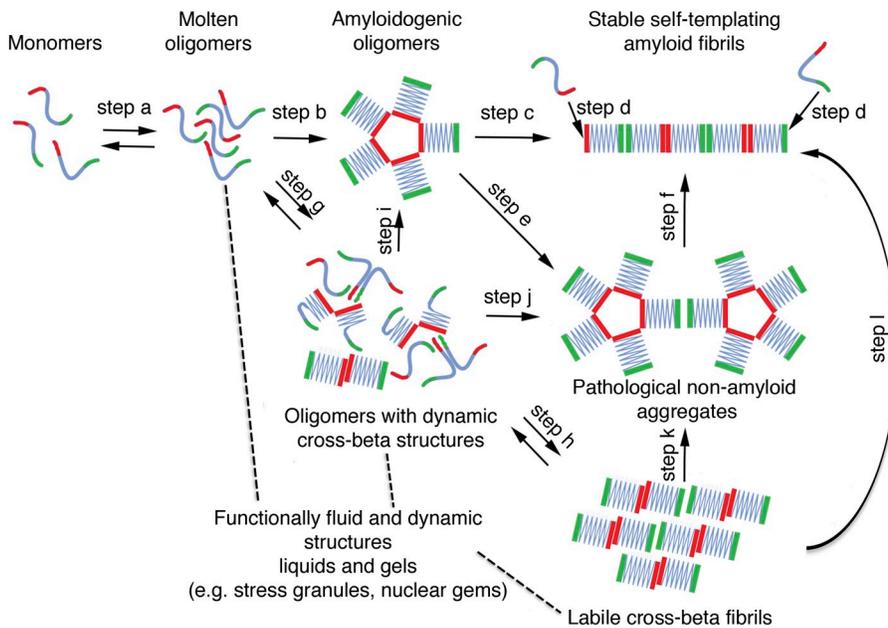
Figure 2. Prion and prion-like domains encode diverse conformational states and folding trajectories. Typically, prion and prion-like domains (depicted in blue, green, and red) populate a dynamic equilibrium comprised of soluble intrinsically unfolded monomers and molten oligomers (step a). These molten oligomers can subsequently evolve into multiple conformational states. In one trajectory, molten oligomers reorganize into more structured amyloidogenic oligomers (step b), which ultimately convert into a stable amyloid form (step c), which can self-template assembly (step d) and become infectious (i.e., a prion). Amyloidogenic oligomers can also cluster to form large pathological aggregates (step e), which might slowly convert to amyloid (step f). Alternatively, molten oligomers can partition into partially structured forms with dynamic cross-β structures that exhibit liquid-like properties (step g), and can rearrange further into labile cross-β fibrils with gel-like properties (step h). These liquid and gel-like collectives are likely critical structural components of various non–membrane-bound organelles including SGs and nuclear gems. Importantly, these transitions to liquid- and gel-like structures are readily reversible (steps a, g, and h). We suggest, however, that these structures are also prone to morph into amyloidogenic oligomers (step i), pathological nonamyloid aggregates (step j and k), and even stable self-templating amyloid (step l) connected with neurodegenerative disease.

(Fig. 2, steps a, g, and h; Sun et al., 2011; Han et al., 2012; Kato et al., 2012). Importantly, these labile fibrillar assemblies enable a phase transition to hydrogel structures that are capable of retaining RNAs and RNA-binding proteins that are specific to the protein forming the gel (Han et al., 2012; Kato et al., 2012). In *Caenorhabditis elegans*, P granules exhibit liquid-like behaviors, including dripping, wetting, fusion, rapid dissolution, and condensation (Brangwynne et al., 2009). More broadly, interactions between diverse multivalent macromolecules (including multidomain proteins and RNA) can elicit sharp liquid–liquid demixing phase transitions that give rise to micrometer-scale liquid droplets in aqueous solution, which can also be regulated by phosphorylation (Li et al., 2012). These phase transitions likely underpin RNP granule biogenesis and represent a fundamental mechanism for organizing non–membrane-bound compartments in the cytoplasm (Fig. 2, steps a, g, and h; Hyman and Simons, 2012; Weber and Brangwynne, 2012). Similar self-organizing events underpin the de novo formation of subnuclear organelles, such as Cajal bodies (Kaiser et al., 2008; Shevtsov and Dundr, 2011). Remarkably, certain RNA-binding proteins, such as TDP-43 and FUS, which have been linked to neurodegenerative diseases, are also required for formation of nuclear gems, subnuclear organelles that likely participate in small nuclear RNP (snRNP) biogenesis and are lost in amyotrophic lateral sclerosis (ALS; Yamazaki et al., 2012; Tsuiji et al., 2013).

The ability to rapidly form RNP granules by deploying prion-like domains does not come without a price. Indeed, the ability to phase transition to hydrogel-like structures likely also simultaneously increases the propensity to form more intractable,

pathogenic aggregates (Fig. 2, steps i–l). At least two RNA-binding proteins that harbor prion-like domains, TDP-43 and FUS, can contribute to deadly human neurodegenerative diseases when mutated or when subcellular localization and aggregation becomes unchecked. And, as discussed next, these two proteins may represent just the tip of the iceberg for aggregation-prone RNA-binding proteins connected with neurodegenerative disease (King et al., 2012).

TDP-43 and FUS: an emerging role for RNA-binding proteins in neurodegenerative disease

ALS is a fairly common neurodegenerative disease caused by a selective loss of motor neurons from the brain and spinal cord (Cleveland and Rothstein, 2001). Typically, this neurodegeneration leads to paralysis and death within 2–5 years of diagnosis. Like several other neurodegenerative diseases, the degenerating neurons of ALS patients are characterized by the accumulation of protein aggregates (Forman et al., 2004). In 2006 it was discovered that the major protein component of many of these inclusions is the 43-kD TAR-DNA–binding protein (TDP-43; Arai et al., 2006; Neumann et al., 2006). TDP-43 is a DNA- and RNA-binding protein with two RRMs and a C-terminal prion-like domain, which is also referred to as a Gly-rich domain (Cushman et al., 2010). TDP-43 normally localizes to the nucleus, where it has roles in regulating splicing and mRNA stability (Lagier-Tourenne et al., 2010; Da Cruz and Cleveland, 2011). In the degenerating neurons and glia of ALS patients, TDP-43 is depleted from the nucleus and accumulates in large cytoplasmic aggregates. TDP-43 also undergoes several disease-specific posttranslational

Table 1. **Human RNA-binding proteins with prion-like domains**

Protein	RRM domain (Pfam PF00076)	RNA binding (GO:0003723)	Prion domain score	Prion domain rank	P-bodies and/or SGs?	Neurodegenerative disease?
TNRC6A	−	+	42.1	9	+	
FUS	+	+	38.5	12	+	ALS, FTLD
ATXN1	−	+	35.5	15		SCA1
TAF15	+	+	33.2	22	+	ALS, FTLD
EWSR1	+	+	32.3	25	+	ALS, FTLD
HNRPDL	+	+	31.5	27		
HNRNPD	+	+	30.7	29.5		
HNRNPA2B1	+	+	29.9	32	+	IBMPFD
ILF3	−	+	29.8	33		
HNRNPUL1	−	+	28.5	37		
HNRNPA1	+	+	28.2	38	+	IBMPFD, ALS
HNRNPAB	+	+	27.8	39		
HNRNPA3	+	+	27.2	41	+	C9orf72 ALS/FTLD
TARDBP	+	+	26.5	43	+	ALS, FTLD
HNRNPU	−	+	24.9	49		
TIA1	+	+	23.4	53	+	Welander distal myopathy
HNRNPA1L2	+	+	22.8	57		
HNRNPH1	+	+	22.2	63		
DDX5	−	+	21.2	73	+	
PSF	+	+	20.8	79		FTLD
HNRNPA0	+	+	20.5	81		
HNRNPH2	+	+	17.5	101		
DAZ2	+	+	16.2	119		
RBM14	+	+	16.0	122		
CSTF2	+	+	15.7	126		
TNRC6C	−	+	15.4	128	+	
SOX2	−	+	15.0	135		
CAPRIN1	−	+	14.9	136	+	
DROSHA	−	+	14.8	137		
DDX17	−	+	14.8	139		
DAZ3	+	+	14.6	144.5		
DAZ2	+	+	14.6	144.5		
DAZ1	+	+	14.1	148		
HNRNPH3	+	+	14.0	151		
CSTF2T	+	+	14.0	153		
CELF4	+	+	13.8	156		
TIAL1	+	+	13.6	162	+	
RBM33	+	+	12.9	178		
DLX2	−	+	12.5	188		
DAZAP1	+	+	11.7	203		
SUPT6H	−	+	11.6	206		
ATXN2	−	+	10.2	227	+	SCA2, ALS
DHX9	−	+	10.1	230		
PSPC1	+	+	10.0	231		
GAR1	−	+	9.4	235		
SF1	−	+	9.4	236		
FUBP1	−	+	9.2	237		
EIF4G3	−	+	8.5	243	+	
EIF4G1	−	+	8.3	246	+	PD

49 human RNA-binding proteins (containing the Pfam RRM [PF00076] and/or annotated with the GO term for RNA-binding [GO:0003723]) also harbor predicted prion-like domains. Prion score, based on Alberti et al. (2009), indicates the maximum log-likelihood ratio for prion-like amino acid composition vs. non–prion-like amino acid composition in any 60 consecutive amino acid window contained in a region parsed as prion-like by the hidden Markov model. Prion domain rank is from 21,873 human proteins, 250 of which had positive prion score. Localization to P-bodies or SGs is based on literature searches, especially Buchan and Parker (2009). Human disease connections for select proteins are indicated.

modifications, including hyperphosphorylation, ubiquitination, and cleavage (Kwong et al., 2007). Because TDP-43 is both depleted from the nucleus and accumulates in cytoplasmic aggregates, it raises the question of whether loss of TDP-43 nuclear function, a gain of toxic function in the cytoplasm, or some combination of both contributes to disease (Fig. 3). Shortly

after the discovery of a major role of TDP-43 in ALS pathological lesions, in 2008 human geneticists converged on the TDP-43 gene (*TARDBP*), identifying more than 30 different TDP-43 mutations in sporadic and familial ALS patients (Lagier-Tourenne et al., 2010). Together, these discoveries, and the convergence of pathology and genetics on TDP-43, completely revolutionized ALS research and focused attention on RNA-binding proteins and RNA processing pathways (Lagier-Tourenne et al., 2010). Beyond ALS, TDP-43 was also found to be the proteinaceous building blocks of the cytoplasmic inclusions found in a large subset of frontotemporal lobar degeneration (FTLD) patients, and indeed this subtype is now subsumed under the classification FTLD-TDP (Mackenzie et al., 2011).

Almost immediately after the identification of TDP-43 mutations as a cause of ALS, another RNA-binding protein, remarkably similar to TDP-43, was implicated in ALS pathogenesis. Mutations in *FUS* (fused in sarcoma; also known as *TLS*, translocated in liposarcoma) were identified in familial and sporadic ALS patients (Lagier-Tourenne et al., 2010). Like TDP-43, FUS is normally a nuclear protein, and ALS-causing FUS mutations result in cytoplasmic FUS accumulations. Interestingly, these inclusions do not contain TDP-43, and TDP-43 inclusions do not contain FUS (Vance et al., 2009).

Identification of prion-like domains in TDP-43, FUS, and beyond

A fascinating and potentially revolutionary new concept is emerging in several neurodegenerative diseases. It involves the transmission or propagation of protein aggregates from cell to cell and from one brain region to another during the onset and progression of disease (Aguzzi and Rajendran, 2009; Cushman et al., 2010; Frost and Diamond, 2010). Compelling in vitro and in vivo experimental evidence now indicates that diverse neurodegenerative disease proteins are able to enter cells, seek out their cognate proteins, and template their conversion to aggregated conformations. This "bucket-brigade"-like form of propagation helps to explain how disease pathology seems to emerge from epicenters in the brain and spread along anatomically interconnected brain regions during disease progression (Braak et al., 2003; Ravits and La Spada, 2009). It should also be noted that cases in which disease pathology initially arises at disconnected brain regions does not necessarily exclude a prion-like mechanism of spread. Pathology that originates in discontiguous brain regions could represent independent nucleation sites from which pathology can then spread. Thus, even pathology that originates at disparate sites could be caused by prion-like nucleation that is then followed by propagation.

But what is the molecular basis for the prion-like spread of neurodegeneration? How can and do these proteins access an altered conformation that is able to recruit and then template the conversion of nonaggregated protein to the altered disease-associated conformation? Prion proteins in fungi accomplish this by using prion domains (Fig. 2, steps a–d). These domains are typically enriched in uncharged polar amino acids (such as asparagine, glutamine, and tyrosine) and glycine (Alberti et al., 2009; King et al., 2012). Based on the salient features of prion domains in yeast prion proteins, a bioinformatics approach recently identified novel "prion-like" domains in the C-terminal domain of TDP-43 (amino acids 277–414) and in the N-terminal domain of FUS (amino acids 1–239; Cushman et al., 2010). Disease-associated mutations in these domains might cause disease by increasing the propensity of TDP-43 or FUS to aggregate. Indeed, the ALS-linked TDP-43 mutations, Q331K and M337V, fall in the prion-like domain and directly accelerate TDP-43 misfolding (Johnson et al., 2009).

Beyond TDP-43 and FUS, more than 40 additional RNA-binding proteins in the human proteome also contain predicted prion-like domains (Table 1), raising the intriguing possibility that these proteins may also contribute to ALS and related neurodegenerative diseases (King et al., 2012). Indeed, TAF15 and EWSR1 are now connected with ALS and FTLD, where they were found to be aggregated in the cytoplasm of degenerating neurons (Couthouis et al., 2011, 2012; Neumann et al., 2011). Mutations in TAF15 and EWSR1 are connected with sporadic ALS and accelerate misfolding of the pure protein in vitro (Couthouis et al., 2011, 2012). Moreover, in a subset of FTLD cases, PSF is aberrantly localized to the cytoplasm of oligodendrocytes and forms detergent-insoluble structures (Seyfried et al., 2012). A mutation in TIA1, a critical protein for SG formation, was recently found as the cause of Welander distal myopathy, an adult-onset autosomal dominant disease associated with distal limb weakness (Klar et al., 2013). Mutations in the prion-like domains of hnRNPA1 and hnRNPA2/B1 cause familial forms of inclusion body myopathy with frontotemporal dementia, Paget's disease of bone, and ALS (sometimes called "IBMPFD/ALS"), and mutations in the prion-like domain of hnRNPA1 are also connected with familial and sporadic ALS (Kim et al., 2013). Disease-linked mutations in the prion-like domain of hnRNPA1 and hnRNPA2/B1 introduce a potent steric zipper, which can form two self-complementary β sheets that comprise the spine of an amyloid fibril (Sawaya et al., 2007). The mutant steric zippers dysregulate and accelerate hnRNPA1 and hnRNPA2/B1 misfolding (Kim et al., 2013). Indeed, these mutations likely divert hnRNPA1 and hnRNPA2/B1 away from physiological folding trajectories connected with RNP granule assembly (Fig. 2, steps a, g, and h) and promote formation of pathological amyloid forms (Fig. 2, steps a–d). Finally, mislocalized hnRNPA3 was recently identified as a constituent of cytoplasmic inclusions in ALS and FTLD cases harboring *C9orf72* hexanucleotide repeat expansions (Mori et al., 2013). Collectively, these findings suggest that RNA-binding proteins with prion-like domains stand on the edge of a precipice that can collapse to yield devastating neurodegenerative disorders.

The discovery of prion-like domains in TDP-43, FUS, and many more RNA-binding proteins presents a conundrum: if the prion-like domains of these proteins directly contribute to their dysfunction and cause lethal neurodegenerative diseases, why are these domains so highly conserved through evolution? It seems that the aggregation-prone nature of these proteins is harnessed so that they can perform essential cellular functions, such as being able to rapidly coalesce to form SGs under situations of cellular stress (Fig. 2, steps a, g, and h).

Figure 3. **How TDP-43 and FUS might interface with SGs during pathogenesis.** (A) In normal neurons, TDP-43 and FUS are localized to the nucleus, and SGs form and dissipate normally. TDP-43 or FUS localization becomes abnormal during ALS pathogenesis and may interface with SGs in several different (and non-mutually exclusive) ways: (B) TDP-43 or FUS exit from the nucleus and begin to accumulate in the cytoplasm as preinclusions, where they interact and might colocalize with SGs. Here, TDP-43 and FUS might become modified by kinases, proteases, and ubiquitin-modifying enzymes, which are all present in SGs. These pathological modifications could accelerate TDP-43 and FUS aggregation or prevent return to the nucleus. Thus, SGs could serve as an obligate conduit for TDP-43 and FUS aggregation. Ubi, ubiquitin. (C) TDP-43 and FUS aggregation in the cytoplasm might interfere with SG function, perhaps by interfering with their ability to regulate RNAs targeted to these structures. (D) TDP-43 and FUS might be required in the nucleus for the regulation of SG genes, and therefore depletion from the nucleus could lead to a dysregulation of SG genes and decreased SG formation and function.
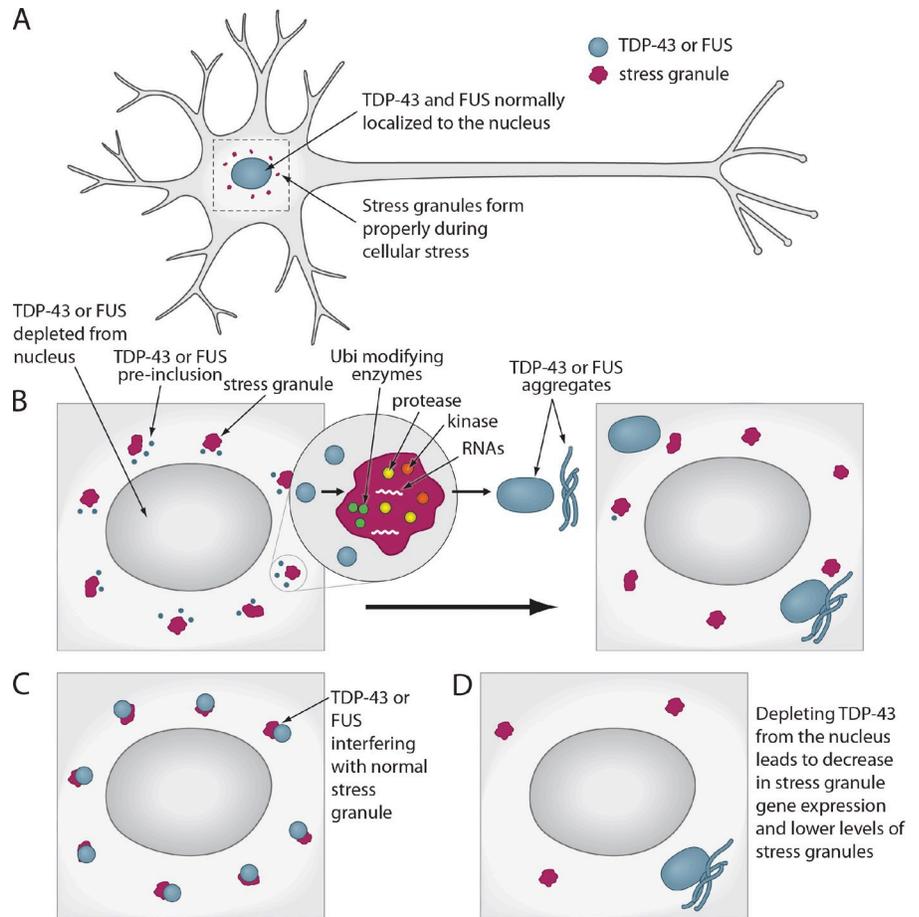
TDP-43 and FUS associate with SGs

Not only do TDP-43 and FUS possess prion-like domains that appear to mediate pathological protein aggregation, both proteins also harbor conserved RRMs. The RRMs of TDP-43 and FUS contribute to both proteins localizing to SGs and P-bodies (Colombrita et al., 2009) and might also synergize with the prion-like domains to promote aggregation. Such interactions are crucial for physiological regulation of RNA stability, processing, and decay, as well as pathological cellular stress responses. For example, cytoplasmic TDP-43 inclusions have been shown to colocalize with several SG markers (e.g., TIA-1, TIAR) in cultured cells and primary neurons in response to cellular stress, and in postmortem samples from ALS and FTLD patients (Liu-Yesucevitz et al., 2010; Wolozin, 2012). In addition to ALS and FTLD, TIA-1 has been show to interact with tau and to localize with neurofibrillary pathology in Alzheimer's disease and other tauopathies (Vanderweyde et al., 2012).

TDP-43 and FUS are nuclear proteins that can quickly be shuttled to the cytoplasm upon stress induction. Once in the cytoplasm, they rapidly associate with SGs (Ayala et al., 2008b; Dormann et al., 2010). In normal cells, once the instigating stress resolves, SGs dissolve and TDP-43 and FUS return to the nucleus. Thus, the nuclear-cytoplasmic shuttling of TDP-43 and FUS, as well as their association with cytoplasmic SGs, are physiological and reversible processes.

However, in pathological conditions, the nuclear-cytoplasmic shuttling of TDP-43 and FUS becomes dysregulated, possibly owing to diverse genetic and environmental factors, or perhaps even aging itself. For example, ALS-linked TDP-43 mutations promote persistent cytoplasmic aggregation of the mutant TDP-43 and result in increased SG association (Fig. 3), which does not appear to be reversible after stress induction (Liu-Yesucevitz et al., 2010). Indeed, wild-type (WT) TDP-43 is also prone to localize to SGs and access aggregated structures that are not readily cleared after severe stress (Parker et al., 2012). Cells expressing mutant TDP-43 appear to either exhibit attenuated SG formation or be prone to abnormal SG assembly (Liu-Yesucevitz et al., 2010; McDonald et al., 2011). Although TDP-43 does not appear to be necessary for SG formation, siRNA knockdown of WT TDP-43 has been shown to significantly reduce average SG size, arguing that TDP-43 may play a direct role in the SG assembly process (McDonald et al., 2011).

FUS, like TDP-43, demonstrates stress-induced SG localization, and ALS-linked FUS variants are more prone to localize to SG compartments (Bosco et al., 2010; Dormann et al., 2010; Gal et al., 2011; Sun et al., 2011). Indeed, it has been proposed that the mechanism through which FUS mutations act to promote ALS may be directly related to mislocalization of FUS to the cytoplasm, leading to abnormal interactions with SGs and consequent dysregulation of SG physiology (Bosco et al., 2010; Sun et al., 2011). Most of the bona fide ALS-causing FUS mutations cluster in a small region in the extreme C-terminal domain of FUS, which encodes a PY-NLS motif (Dormann et al., 2010; Zhang et al., 2012). This motif is both necessary and sufficient

for FUS nuclear localization, and the most pathogenic mutations in FUS have the most severe effect on binding to the nuclear import factor karyopherin-β2 and consequent FUS nuclear localization (Dormann et al., 2010; Zhang et al., 2012). The most aggressive ALS-linked FUS mutations such as P525L and R495X (which removes the entire PY-NLS) are associated with earlier age of ALS onset, cause FUS to be localized entirely to the cytoplasm, and greatly perturb binding to karyopherin-β2 (Dormann et al., 2010; Zhang et al., 2012). Once FUS starts to accumulate in the cytoplasm, it has a greater propensity to associate with SGs, and various environmental stressors accentuate this process (Dormann et al., 2010; Fig. 3).

Models of TDP-43/FUS in SG biology

The molecular mechanisms by which FUS and TDP-43 mutations contribute to ALS/FTLD are not fully understood. The formation of TDP-43– or FUS-positive subcellular aggregates that colocalize with SGs in neurons from ALS patients supports the hypothesis that cellular mistargeting of these RRM-containing proteins has a key role in ALS pathophysiology (Fig. 3). The loss of TDP-43/FUS from the nucleus or their increased cytoplasmic localization to SGs could serve as a critical pathway for ALS. Hence, in the context of ALS pathophysiology, two central questions remain: (1) Do mutations in TDP-43 and FUS cause a loss or gain of function in these proteins? and (2) Is altered SG assembly a consequence or cause of TDP-43 and FUS mislocalization? The answers to these questions will enhance our mechanistic understanding of ALS and will help guide the development of potential therapeutic strategies.

Several models have been proposed to explain the role of FUS, TDP-43, and SG biology in ALS/FTLD pathogenesis. We will briefly highlight evidence supporting three hypotheses that are not mutually exclusive, and it is probable that they even synergize to varying degrees in specific cases of ALS and FTLD.

Model 1: Gain of function in SGs model. The gain-of-function (GOF) toxicity model asserts that pathological TDP-43 or FUS aggregate in SGs, impeding normal SG-mediated RNA homeostasis (Fig. 3 B). In this model, the physiological localization of TDP-43 to SGs during cell stress is a consequence of normal stress response but is not required for normal SG assembly, an observation in line with SG formation in the absence of TDP-43 expression (Colombrita et al., 2009; Liu-Yesucevitz et al., 2010; McDonald et al., 2011). In contrast, the excessive SG localization of FUS and TDP-43 (Bosco et al., 2010; Liu-Yesucevitz et al., 2010) might drive dysregulated assembly of inappropriate fibrillar aggregates (Johnson et al., 2009; Sun et al., 2011), which perturb the localization of messenger RNPs (mRNPs) required for RNA processing and decay, thereby interfering with RNA sorting. If this process continues unabated, ultimately the assembly of translation initiation complexes could be affected by preventing the increase in local concentration of mRNAs and translation factors (Buchan and Parker, 2009). Finally, insoluble FUS and TDP-43 aggregates alter SG dynamics even after stress resolution (Parker et al., 2012), resulting in SG persistence as has been observed in cells expressing ALS-associated TDP-43 or FUS mutants (Bosco et al., 2010; Dormann et al., 2010; Liu-Yesucevitz et al., 2010;

Dewey et al., 2011, 2012; Wolozin, 2012). Together, this model describes how the stress-provoked phenotypes of cells expressing pathological FUS and TDP-43 may result from accumulation of these RNPs in SGs, consequently inhibiting physiological stress responses.

It remains unclear, however, whether cytoplasmic mislocalization and SG aggregation of TDP-43 or FUS is causative in ALS/FTLD patients, or whether these cytopathological phenotypes represent the end stage consequence of cellular stress responses within degenerating neurons. Evidence for the former has come from experiments demonstrating that SG formation itself during cell stress response appears to be cytoprotective by helping to shift translation toward heat shock proteins (Lindquist, 1981; Wolozin, 2012). Notably, mRNAs that are highly translated during stressful growth conditions often use noncanonical translational mechanisms (Spriggs et al., 2008). For instance, mRNAs containing internal ribosome entry sites (IRES) require trans-activating factors for ribosomal recruitment, including hnRNPA1 and PCBP2, which are both recruited to SGs (Bonnal et al., 2005; Guil et al., 2006; Fujimura et al., 2010). Among IRES-containing transcripts are many keys to cell proliferation, survival, and apoptosis. Here, the aggregation of mutant FUS or TDP-43 in SGs may inhibit or "out-compete" the localization of RNAs encoding antiapoptotic factors or proliferative factors required to promote cell recovery after cell injury. Therefore, exuberant accumulation of aggregation-prone FUS or TDP-43 in SGs might inhibit translation of mRNAs critical to cell survival and recovery, and consequently increase the rate of cell death and neuron loss (Nevins et al., 2003).

It is important to note that TDP-43 and FUS must bind RNA and aggregate to confer toxicity in multiple model systems. Indeed, mutation of the RRMs of FUS and TDP-43 to forms that are incompetent for RNA binding greatly reduces toxicity without affecting aggregation (Elden et al., 2010; Voigt et al., 2010; Sun et al., 2011; Daigle et al., 2013). Thus, mere misfolding or aggregation driven by a prion-like domain is not sufficient for toxicity. These observations indicate the finding that RNA binding might enable TDP-43 and FUS to access specific misfolded conformations that are intrinsically toxic. Alternatively, or in addition, RNA-binding activity might enable TDP-43 and FUS to sequester essential RNAs and other RNA-binding proteins in inclusions. Because TDP-43 and FUS regulate the splicing of myriad mRNAs with large introns that are critical for neuronal viability (Polymenidou et al., 2011; Colombrita et al., 2012; Lagier-Tourenne et al., 2012), it seems probable that sequestration of these mRNAs might be particularly damaging for specific neurons. Thus, strategies to release these RNAs from aggregated FUS or TDP-43 traps could have therapeutic utility.

Model 2: Loss of function in SGs model. A GOF model for TDP-43 and FUS in ALS pathogenesis would be consistent with the broad and apparently pervasive role of toxic protein aggregation across several neurodegenerative diseases (Forman et al., 2004). However, it is not clear how the formation of insoluble fibrils of FUS and TDP-43 causes neuronal dysfunction and death. Moreover, FUS- or TDP-43–containing SGs can disperse in healthy neurons after stress induction,

which suggests, superficially at least, that their aggregation alone is not sufficient for cytotoxicity. Indeed, it will be important to determine whether FUS and TDP-43 localized to SGs adopt the same conformation as FUS and TDP-43 in pathological inclusions. That is, do FUS and TDP-43 form a continuum of aggregated structures, some of which are functional and beneficial and others that are toxic and deleterious? The propensity of yeast prion domains to access a range of distinct self-templating structures, termed strains (Shorter, 2010), suggests that FUS and TDP-43 are also likely to access an entire spectrum of distinct aggregated conformers that could exert beneficial or deleterious effects (Fig. 2).

Significant cellular damage and death is induced by TDP-43 or FUS knockdown in neurons, primary cell lines, and model organisms, which suggests a loss-of-function (LOF) model (Hicks et al., 2000; Ayala et al., 2008a). Moreover, although the physiological roles of TDP-43 and FUS are still being clarified, two distinct possibilities have been proposed for LOF toxicity. Specifically, the nuclear LOF model suggests that the cytoplasmic mislocalization of FUS or TDP-43 impedes their ability to perform essential nuclear functions, whereas the cytoplasmic LOF model emphasizes the fact that FUS or TDP-43 play a physiological role in cytoplasmic SGs during cell stress but that ALS-associated conformers or mutants have impaired protein–protein or protein–RNA interactions. Again, these possibilities are not mutually exclusive and may synergize to promote ALS or FTLD pathogenesis.

The cytoplasmic LOF model suggests that FUS and TDP-43 are critical mediators of nuclear-cytoplasmic RNA transport and shuttling, which affect the physiology of SG formation during stressful conditions. TDP-43 has been repeatedly demonstrated to have pronounced effects on SG dynamics (Fig. 3 C), as delayed formation, reduced size, altered morphology, and limited stability of SGs are observed in HeLa cells and neuroblastoma cell lines depleted of TDP-43 (McDonald et al., 2011; Aulas et al., 2012).

In addition, WT FUS appears to be recruited to dendrites by mGluR5 activation in neurons, where it mediates a rise in local RNA content, and FUS-null neurons demonstrate abnormal spine morphology and density (Fujii et al., 2005), underscoring that loss of normal FUS function can contribute to pathogenesis. The role of FUS in SG biology is still being delineated, but FUS has been shown to bind RNAs encoding actin-stabilizing proteins such as Nd1-L, which may be important for mRNA transport to dendritic spines (Fujii et al., 2005). Thus, the subcellular localization of TDP-43 and FUS to SGs during stress induction, and subsequent dispersal after stress, might indicate that the trafficking of FUS and TDP-43 to SGs is cytoprotective and possibly critical for RNA triage and resolution of translational stall.

Model 3: Nuclear LOF model. Although the cytoplasmic LOF model is consistent with many aspects of TDP-43 and FUS pathological interactions, both proteins have well-established important nuclear roles as well, including pre-mRNA splicing, RNA stability, and transcriptional regulation (Lagier-Tourenne et al., 2010). Indeed, TDP-43 was originally discovered as a transcriptional repressor that binds to TAR DNA of HIV-1 (Ou et al., 1995) and was subsequently found to repress the transcription of SP-10 required for spermatogenesis in mice (Tan and Manley, 2010). Furthermore, TDP-43 has been shown to play an important role in regulating the splicing of alternative isoforms of proteins, including cystic fibrosis transmembrane regulator (CFTR), apolipoprotein A-II, and survival of motor neuron (SMN), possibly related to its association with several other protein involved in splicing (e.g., SC35; Buratti et al., 2001; Mercado et al., 2005; Fiesel et al., 2012). Thus, depletion of nuclear pools of TDP-43 and FUS is likely to impact these essential functions. Moreover, ALS-linked mutations might even subtly impair nuclear TDP-43 or FUS function in a manner that is selectively toxic for motor neurons upon aging and may not even require depletion of nuclear pools (Polymenidou et al., 2011; Lagier-Tourenne et al., 2012; Schwartz et al., 2012; Arnold et al., 2013).

Moreover, TDP-43's role in SG biology may actually depend on its nuclear regulation of SG genes (Fig. 3 D). Specifically, the role of TDP-43 in SG assembly has recently been shown to strongly depend on its regulation of G3BP transcription (Aulas et al., 2012). G3BP, a protein required for normal SG assembly, is a transcriptional target of TDP-43 and is severely depleted in TDP-43–depleted cells (Dammer et al., 2012). Restoration of G3BP by overexpression rescues the SG phenotype observed in TDP-43–depleted cells, which suggests that TDP-43 is an upstream regulator of G3BP and that this could explain a significant proportion of TDP-43's role in SG dynamics. However, this is entirely dependent on TDP-43's nuclear, not cytoplasmic, function. It will be important to determine whether restoration of FUS and TDP-43 function in the nucleus or cytoplasm, or both, is sufficient to rescue neurodegenerative disease phenotypes.

Ataxin 2, SGs, and disease: integrating environmental stress and disease?

Ataxin 2 is another SG-associated RNA-binding protein that contributes to neurodegenerative disease (Elden et al., 2010; Orr and Zoghbi, 2007). Ataxin 2 interacts with polyA-binding protein (PABP1) to regulate mRNA polyadenylation and is required for SG assembly (Kaehler et al., 2012). Ataxin 2 harbors a polyglutamine (polyQ) tract and is a member of a family of polyQ disease proteins, which include the Huntington's disease protein huntingtin. The ataxin 2 polyQ tract normally contains 22 or 23 Qs. PolyQ tract expansions >34 Q cause spinocerebellar ataxia type 2 (SCA2), an autosomal dominant hereditary ataxia (Orr and Zoghbi, 2007). Interestingly, ataxin 2 has recently been shown to associate with TDP-43 in an RNA-dependent manner and to modify TDP-43 cytotoxicity in multiple model systems (Elden et al., 2010). Ataxin 2 protein accumulates abnormally in discrete foci in the degenerating motor neurons of ALS patients, structures likely to represent SGs and harbor other key SG components. Ataxin 2 has also been connected to ALS through genetics. Intermediate-length ataxin 2 polyQ expansions (27–33 Qs), longer than normal but not past the threshold for SCA2, were recently associated with increased risk for ALS (Elden et al., 2010). Together, these findings suggest mechanistic links between the diseases ALS and SCA2 and implicate a key building block of SGs, ataxin 2, in disease pathogenesis. It is also tempting to interpret these findings by
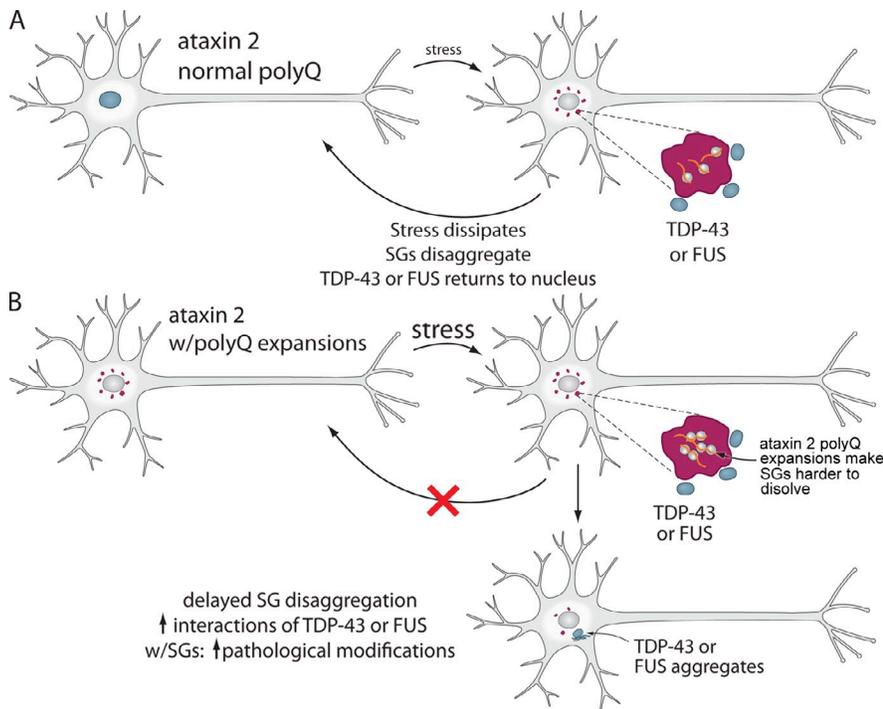
Figure 4. **How ataxin 2 polyQ expansions might affect SGs in ALS.** Ataxin 2 is a component of SGs and is required for their formation and function. Pathogenic polyQ expansions in ataxin 2 underlie spinocerebellar ataxia 2 and ALS. (A) SGs form upon cellular stress. The ataxin 2 polyQ length is normally 22 Q. When the stress dissipates, SGs dissolve. (B) In the presence of a pathogenic ataxin 2 polyQ expansion, the SGs might be more difficult to dissolve, perhaps owing to increased ataxin 2 stability. Persistent SGs would have a greater chance to interface with TDP-43 or FUS in the cytoplasm (TDP-43 and FUS normally shuttle in and out of nucleus). Thus, increased interactions of TDP-43 or FUS with SGs could lead to enhanced pathological modifications, resulting in TDP-43 or FUS aggregate formation.

speculating that the pathogenesis of ALS and related neurodegenerative diseases could be intimately tied to SG formation pathways.

How could different mutations in ataxin 2 (long vs. intermediate length polyQ expansions) contribute to distinct phenotypic consequences in SCA2 versus ALS? One potential explanation is that intermediate-length polyQ expansions in ataxin 2 result in enhanced stress-induced caspase activation, which leads to increased TDP-43 pathological modifications, including cleavage and hyperphosphorylation (Fig. 4). In surprising contrast, neither normal-length nor long, SCA2-length polyQ expansions result in this type of stress response (Hart and Gitler, 2012). Given ataxin 2's pivotal role in SG assembly and function and the ability of polyQ expansions to enhance the interaction between ataxin 2 and TDP-43, one way that ataxin 2 could link TDP-43 to SGs and ALS pathogenesis is by promoting the recruitment of TDP-43 to SGs. Once associated with SGs, TDP-43 would be exposed to kinases, proteases, and ubiquitin-modifying enzymes, all of which are regulators of SG dynamics. This might increase the likelihood that one or more of these enzymes could subject TDP-43 to modifications such as phosphorylation, cleavage, and ubiquitination, which are all hallmarks of TDP-43 pathology in disease (Neumann et al., 2006).

Another way that polyQ expansions in ataxin 2 could contribute to ALS is by hampering the ability of SGs to dissolve properly and/or by reducing the efficiency by which TDP-43 returns to the nucleus (Fig. 4 B), with the cumulative effect being a greater propensity for TDP-43 to abnormally accumulate in the cytoplasm. This concept suggests that ALS pathogenesis is deeply rooted in basic cell biology, and a better understanding of the regulators of SG assembly and disassembly could provide new insight into disease mechanisms and suggest novel therapeutic approaches.

Finally, ataxin 2 and SGs, with their roles in sensing and responding to environmental stresses, might help to explain the connection between environmental exposures, including traumatic injury, and the pathogenesis of ALS and related neurodegenerative disorders. Upon exposure to stress, TDP-43, FUS, and other RNA-binding proteins move from the nucleus to the cytoplasm and associate with ataxin 2–containing SGs (Bosco et al., 2010; Dormann et al., 2010; Liu-Yesucevitz et al., 2010). Once the stress subsides, SGs no longer aggregate, and the RNA-binding proteins translocate back to the nucleus. This repeated cycle of aggregation and disaggregation, over the course of a lifetime, perhaps more so when exposed to specific environmental exposures and traumas, can become misregulated and can lead to the improper cytoplasmic localization of one or more of these proteins. This failure to restore nuclear localization of these proteins could cause the subsequent disease pathology (Fig. 4). Age-dependent breakdown in cellular proteostasis may lead to further defects in maintaining SG protein quality control and likely underpins the late-onset aspect of disease. In individuals with genetic risk factors for ALS, such as intermediate-length ataxin 2 polyQ expansions, the threshold for disease-causing responses to such stresses might be lower than normal. Genetic screening of contact sport athletes and military personnel for genetic risk factors such as ataxin 2 polyQ expansions might be an important way of prescreening individuals who could potentially be at an elevated risk for more severe clinical consequences from such injuries.

Potential therapeutic approaches to mitigate SG-mediated processes

This connection between ataxin 2, TDP-43, and SG biology illuminates the concept that the pathogenesis of ALS and related diseases might be tightly linked to an ancient core cell

biological pathway (SG formation), and raises the possibility of therapeutic interventions aimed at targeting SGs. First, it may be useful to identify factors that promote recruitment of FUS and TDP-43 into SGs. If specific inhibitors of interaction between primary aggregation factors and FUS or TDP-43 could be identified, this may be of therapeutic potential. Interestingly, Liu-Yesucevitz et al. (2010) recently showed that stably expressed WT and mutant TDP-43 can be coimmunoprecipitated with TIA-1 in HEK293 cells. In addition, this interaction appears to be RNA-dependent, as RNase treatment eliminates this interaction (Liu-Yesucevitz et al., 2010). Moreover, elimination of either the first or both RNA-binding domains of TDP-43 reduces basal aggregation (Liu-Yesucevitz et al., 2010). FUS recruitment to SGs is clearly RNA-dependent, as an engineered FUS mutant lacking the RNA-binding domain showed low levels of localization with TIA-1 in response to arsenite (Bentmann et al., 2012). Blocking this type of interaction could potentially impede SG localization and mitigate disease.

Conclusions

Several emerging links now tie SG assembly and RNA-binding proteins with prion-like domains to neurodegenerative disease (Gitler and Shorter, 2011; King et al., 2012; Wolozin, 2012; Table 1). This convergence suggests that common solutions might be developed to mitigate the pathogenesis of several disorders, including some forms of ALS, which are caused by dysfunctional RNA and protein proteostasis. For example, although for the most part FUS and TDP-43 control the splicing of different pre-mRNAs (Polymenidou et al., 2011; Lagier-Tourenne et al., 2012), there are a small number of genes whose expression levels depend on TDP-43 or FUS, including PARK2, KCNIP4, and SMYD3, which are essential for neuronal survival and function (Lagier-Tourenne et al., 2012). Likewise, genetic modifiers of TDP-43 and FUS toxicity in yeast display little overlap, but there are exceptions (Elden et al., 2010; Sun et al., 2011; Armakola et al., 2012). We expect that model organisms will play a major role in identifying common solutions for correcting defective RNA and protein proteostasis that is critical for developing drugs with broad efficacy.

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