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Biological Spectrum of Amyotrophic Lateral Sclerosis Prions

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Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD) are two neurodegenerative diseases with distinct clinical features but common genetic causes and neuropathological signatures. Ten years after the RNA-binding protein TDP-43 was discovered as the main protein in the cytoplasmic inclusions that characterize ALS and FTLD, their pathogenic mechanisms have never seemed more complex. Indeed, discoveries of the past decade have revolutionized our understanding of these diseases, highlighting their genetic heterogeneity and the involvement of protein-RNA assemblies in their pathogenesis. Importantly, these assemblies serve as the foci of protein misfolding and mature into insoluble structures, which further recruit native proteins, turning them into misfolded forms. This self-perpetuating mechanism is a twisted version of classical prion replication that leads to amplification of pathological protein complexes that spread throughout the neuraxis, offering a pathogenic principle that underlies the rapid disease progression that characterizes ALS and FTLD.

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD) are two major neurodegenerative diseases with devastating consequences for patients and their families. ALS and FTLD patients often live normal lives for several decades. When the disease strikes, it is so subtle that it frequently escapes initial diagnosis. ALS patients may first experience difficulty in walking, talking, performing fine motor tasks, or even breathing, depending on the subtype of the disease (Hardiman et al. 2011). In most cases, the disease progresses rapidly, leading to paralysis and death owing to respiratory failure, typically within 1–5 years from disease onset. First described and named more than a century ago by the French neurologist Jean-Martin Charcot (Rowland 2001), ALS is the most common motor neuron disease and was traditionally thought to spare cognitive functions. Discoveries of the past decade, however, have made a definitive link between ALS and FTLD (Polymenidou et al. 2012), the most common type of dementia in individuals of less than 60 years of age. Although ALS patients experience muscle deterioration over the course of the disease, FTLD patients lose their personality, with muscle function intact (Lashley et al. 2015). Progressive and severe atrophy of the frontal and temporal lobes causes variable behavioral dysfunctions, which ultimately result in the inabil-

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ity to think and speak (Ng et al. 2015). FTLD patients typically die 5–10 years postdiagnosis. An estimated 15%–25% of patients show an overlapping clinical presentation of ALS/FTLD, with either motor symptoms or behavioral changes occurring, and gradual decline as the disease progresses (Polymenidou et al. 2012; Ng et al. 2015). Approximately 10% of ALS and 30% of FTLD patients have a family history of the disease, whereas the majority of cases occur in the absence of apparent inheritance, called sporadic ALS (sALS) or FTLD.

Almost 150 years after the initial description of ALS and FTLD, it is sobering that both diseases remain incurable, without any effective therapy to alter disease course. Nevertheless, the past decade has been an unprecedented time of discovery of novel ALS and FTLD genetic causes and pathogenic mechanisms. Many of the "dogmas" in the field have been shaken, with the distinction between sporadic and familial disease fading (Renton et al. 2014). Mutations in many different genes (Table 1)—sometimes even concurrently (Rademakers and van Blitterswijk 2013)—cause ALS and/or FTLD, which are now recognized as two ends of the same disease spectrum.

Even though at first glance the different genetic causes of ALS and FTLD seem confusingly unrelated, a theme is emerging, suggesting that errors in two intertwined cellular pathways—namely, RNA metabolism and removal of pathological protein or protein-RNA assemblies—are central disease pathways. In the vast majority of ALS cases and a significant percentage (45%) of FTLD patients, these pathological assemblies contain the RNA-binding protein TDP-43 (TAR-DNA binding protein of 43 kDa) (Arai et al. 2006; Neumann et al. 2006), which undergoes a series of posttranslational modifications (Fig. 1). This predominance of TDP-43 pathology in most instances of ALS and FTLD supports a central pathogenic role of TDP-43 misaccumulation. Template-directed misfolding of TDP-43 (Furukawa et al. 2011; Nonaka et al. 2013) is suspected to propagate pathology across the nervous system (Brettschneider et al. 2013; Ravits 2014), suggesting a potential molecular mechanism underlying disease progression (Polymenidou and Cleveland 2011).

Despite this unifying TDP-43 pathology, the heterogeneity in clinical presentation and disease progression is paralleled by diversity in genetic causes and specific pathological findings. As we move forward, an important task will be to understand the origin and significance of this heterogeneity, so as to subcategorize ALS and FTLD patients and eventually handle them accordingly, both in the clinic and when modeling their disease at the bench.

GENETICS AND PATHOLOGY OF ALS AND FTLD SUBDIVIDE DISEASE INTO SOD1, TDP-43, FUSED IN SARCOMA, AND TDP-43/DPR PROTEINOPATHIES

The discoveries of gene mutations causing familial forms of ALS and FTLD in combination with neuropathological findings have provided crucial directions in initial research. Partially responsible for the delayed recognition of a linkage between ALS and FTLD was that the first genetic causes identified for ALS and FTLD (mutations in superoxide dismutase 1 [*SOD1*] (Rosen et al. 1993) and in the microtubule-associated protein tau [MAPT] (Hutton et al. 1998), respectively) exclusively caused either ALS or FTLD. In the current era of high-throughput sequencing, a growing list (Table 1) of genetic ALS and FTLD causes and risk factors has led to the realization that errors in RNA metabolism and protein degradation are instrumental in disease initiation. In this review, we discuss those genes and proteins that determine the distinct mechanistic pathway in the most prevalent subsets of ALS/FTLD patients.

SOD1 Is Most Commonly Associated with Pure ALS through a Pathogenic Mechanism Unique to this Subset of Disease

Discovery, Prevalence, and Clinical Presentation

Mutations in SOD1 were the first genetic causes identified in ALS (Rosen et al. 1993), and account for almost 20% of familial (Ling et al. 2013) and rare sporadic (Chio et al. 2008) ALS cases. The clinical presentation of SOD1 mutation carriers is that of classical ALS (Hardiman

Table 1. Summary of ALS/FTLD-related genes and associated disease pathways

	Gene	Discovery	Protein function	Mutation type and inheritance	Mutation hotspot	Mutation effects	Frequency % fALS	Frequency % fFTD	Frequency % sALS	Frequency % sFTD	Pathology
ALS	*SOD1*	Roson et al. 1993	Superoxide dismutase, antioxidant enzyme	Missense, autosomal dominant	None, throughout the molecule	Disruption of normal SOD1 structure/ dimerization and misfolding	20	0	Rare, de novo	0	SOD1
	FUS	Kwiatkowski et al. 2009; Vance et al. 2009	RNA and DNA metabolism	Missense, autosomal dominant and recessive	Nuclear localization signal and low-complexity domain	Increased cytoplasmic localization	5		Rare, de novo		FUS
	TARDBP	Sreedharan et al. 2008; Kabashi et al. 2008; Van Deerlin et al. 2008	RNA and DNA motabolism	Missense, autosomal dominant	Glycine-rich or low-complexity domain	Increased aggregation propensity	5		Rare, de novo		TDP-43
	TAF15	Couthouis et al. 2011; Ticozzi et al. 2011	RNA and DNA metabolism	Missense and non-sense, autosomal dominant	Arginine-glycine-glycine box	Loss of RNA binding?	Rare				
	PFN1	Wu et at. 2012	Actin-binding protein and stress granule component	Missense, autosomal dominant	Not enough mutations known	Destabilize profilin	Rare				
	TUBA4A	Smith et al. 2014	Microtubulin component	Missense, autosomal dominant	Not enough mutations known	Destabilize the microtubule network	Rare		Rare		n.d.
	OPTN	Maruyama et al. 2010	Pleiotropic protein, including inflammation, autophagy, and vesicle trafficking	Nonsense and missense, autosomal dominant	Coiled coil and ubiquitin-binding domains	Defective autophagy and ubiquitin binding				~3	TDP-43
	VAPB	Nishimura et al. 2004	Unfolded protein response and vesicle trafficking	Missense, autosomal dominant	Not enough mutations known	Unknown					
	CHCHD10	Bannwarth et al. 2014	Mitochondrial protein located in the intermembrane space	Missense, autosomal dominant	Not enough mutations known	Unknown	Rare	Rare			
	MATR3	Johnson et al. 2014	RNA and DNA metabolism	Missense, autosomal dominant	Not enough mutations known	Unknown	Rare		Rare		

Continued

Table 1. *Continued*

	Gene	Discovery	Protein function	Mutation type and inheritance	Mutation hotspot	Mutation effects	Frequency % fALS	% fFTD	% sALS	% sFTD	Pathology
	SQSTM1/p62	Fecto et al. 2011; Rubino et al. 2012	Ubiquitin adaptor protein mediates proteasomal and autophagosomal degradation	Missense and nonsense, autosomal dominant	Not enough mutations known	Loss of function leading to impairment of autophagy					TDP-43
	C9ORF72	DeJesus-Hernandez et al. 2011; Renton et al. 2011	DENN protein important for endosomal trafficking	Hexanucleotide (G4C2) repeat expansion	Intron between noncoding exons 1a and 1b	Reduced expression and accumulation of repeat RNA and dipeptide proteins	40	20	7	7	TDP-43, DPRs, repeat RNA foci
	TBK1	Freischmidt et al. 2015; Cirulli et al. 2015	Kinase mediates autophagosomal degradation	Missense, autosomal dominant	None so far, throughout the molecule	Loss of function leading to impairment of autophagy	2	−3		−3	TDP-43
	UBQLN2	Deng et al. 2011	Ubiquitin ligase important for proteasomal and autophagosomal degradation	Missense, X-linked dominant	Proline-X-X repeat domain	Loss of function leading to impairment of protein degradation	1	1			TDP-43
FTD	*VCP*	Johnson et al. 2010	AAA-ATPase important for proteasomal degradation	Missense, autosomal dominant	None, throughout the molecule	Loss of function leading to impairment of proteasomal degradation	1	2	0	0	TDP-43
	CHMP2B	Skibinski et al. 2005; Parkinson et al. 2006	Endosomal ESCRTIII-complex subunit	C-terminal truncation, autosomal dominant	Not enough mutations known	Loss of function leading to impairment of autophagy/lysosomal pathway	0	1	0	0	TDP-43
	PGRN	Cruts et al. 2006	Pleiotropic protein, including inflammation and synaptic function	Missense, autosomal dominant	None, throughout the molecule	Loss of function	0	−20	0		TDP-43
	MAPT	Hutton et al. 1998	Promotes microtubule assembly and stability	Missense, autosomal dominant	Exons 9–13	Promote tau aggregation and abnormal phosphorylation	0	50	0	0	TAU

ALS/FTLD, Amyotrophic lateral sclerosis/frontotemporal lobar dementia; DENN, differentially expressed in normal and neoplasia; ESCRTIII, endosomal sorting complex required for transport III; fALS, familial ALS; fFTD, familial FTD; sALS, sporadic ALS; sFTD, sporadic FTD; n.d., not determined; DPRs, dipeptide repeats.

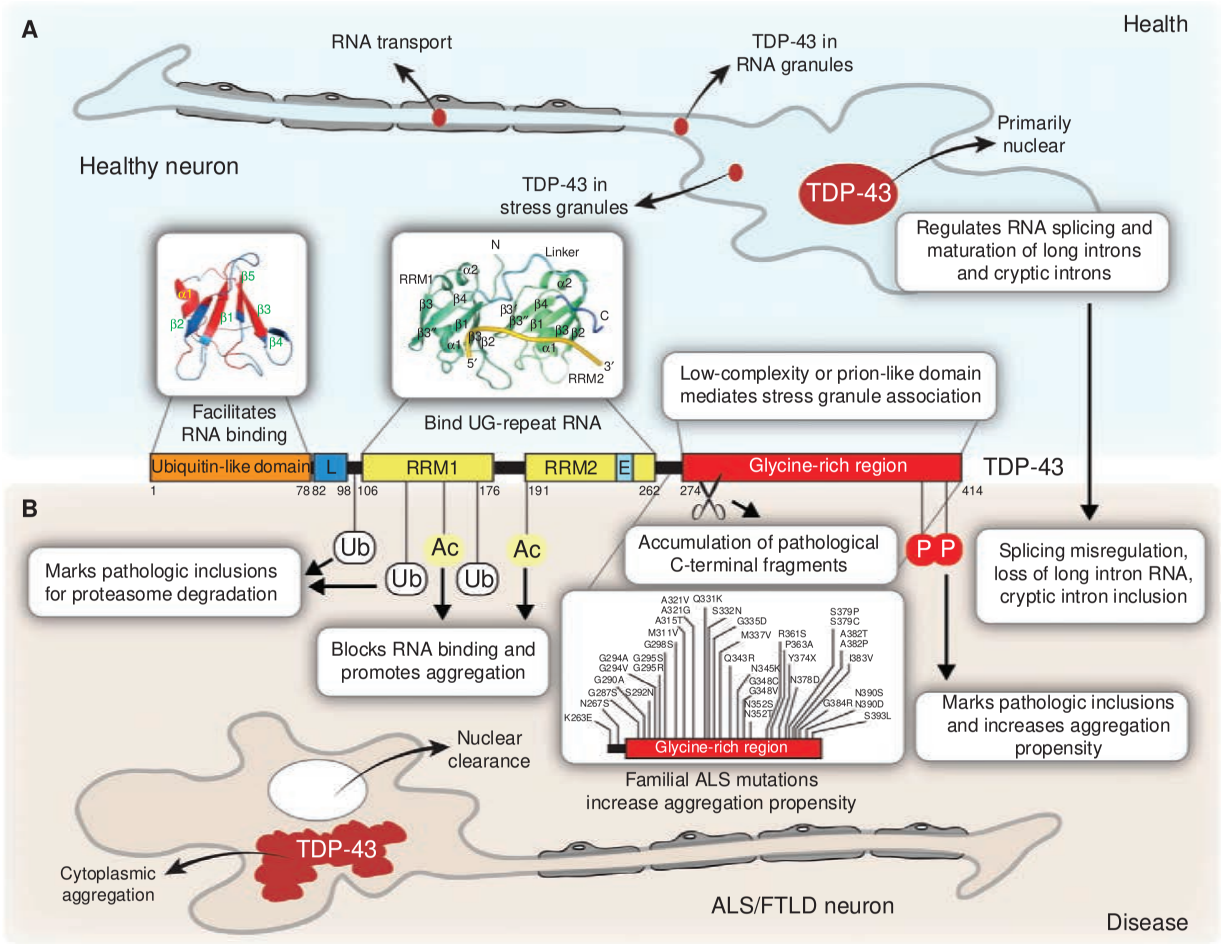

Figure 1. Structural and functional properties of physiological and pathological TDP-43. TDP-43, TAR-DNA binding protein of 43 kDa; RRM, RNA recognition motif; L, nuclear localization signal; E, nuclear export signal; ALS/FTLD, amyotrophic lateral sclerosis/frontotemporal lobar dementia.

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et al. 2011), and although rare cases of ALS with cognitive dysfunction have been reported (Stewart et al. 2006), SOD1-linked ALS is typically not associated with FTLD symptoms (Wicks et al. 2009).

Protein Structure and Function

SOD1 is a 153-amino-acid antioxidant enzyme, which detoxifies free superoxide radicals in the body. Normally localized in the cytoplasm and onto the mitochondrial intermembrane space (Vande Velde et al. 2008), SOD1 is one of the most abundant and stable proteins in the body. Indeed, wild-type (WT) SOD1 forms a protease-resistant homodimer (Li et al. 2010), which is important for its enzymatic activity.

Mutation Distribution and Effect

More than 170 ALS-linked missense mutations spanning the entirety of the SOD1 polypeptide have been reported (Ling et al. 2013), with each mutation disrupting the physiological conformation of at least a proportion of the mutant subunits, thereby leading to the misfolding and accumulation of abnormal forms. Although the misfolded protein is ubiquitinated, and thus directed for degradation (Basso et al. 2006), mutant SOD1 can escape rapid proteolysis, resulting in the formation of ubiquitin-positive cytoplasmic inclusions (Kerman et al. 2010), the pathological hallmark of ALS.

Pathological Findings

Although the crucial role of misfolded SOD1 in the pathogenesis of familial ALS (fALS) with *SOD1* mutations is unequivocal (Ilieva et al. 2009), the presence of misfolded SOD1 in sALS cases remains highly controversial. Whereas some studies have shown no evidence of misfolded SOD1 in sALS patients (Kerman et al. 2010; Brotherton et al. 2012), others have reported a diffuse cytoplasmic misfolded SOD1 in motor neurons in several sALS cases, which were not linked to any *SOD1* mutations (Rakhit et al. 2004; Bosco et al. 2010). This discrepancy may stem from the different anti-misfolded SOD1 antibodies and protocols used in these studies, as well as the inherent challenges in detecting misfolded proteins in postmortem autopsy tissue. Further studies are needed to resolve this issue, which is of great importance for our understanding of the commonalities in disease pathogenesis of ALS cases with seemingly distinct origins.

Pathogenic Mechanism

Although some ALS-linked mutations decrease the enzymatic activity of SOD1, others do not (Borchelt et al. 1994, 1995). In combination with the observations that loss of SOD1 in knockout mice does not lead to motor neuron degeneration (Reaume et al. 1996) and that disease from a transgene-encoded, dismutase-inactive mutation is unaffected by reduction or elimination of endogenous SOD1 (Bruijn et al. 1998), the evidence argues strongly against a loss-of-function mechanism in SOD1-linked ALS. In contrast, expression of mutant SOD1 in transgenic mice (Gurney et al. 1994; Bruijn et al. 1997) and rats (Howland et al. 2002), or naturally occurring in dogs with canine degenerative myopathy (Crisp et al. 2013), leads to late-onset, progressive paralysis with gliosis and ubiquitinated misfolded SOD1 deposition. Importantly, the propensity for SOD1 aggregation was reported to correlate with disease duration, with a higher aggregation propensity associated with a shorter disease course (Prudencio et al. 2009), a finding that highlights the likely pathogenic significance of protein aggregation. Near elimination of large aggregates of mutant SOD1 provided no change in disease onset or progression in SOD1 mutant mouse models (Parone et al. 2013), without affecting smaller misfolded SOD1 species, which are likely the active toxic agents.

TDP-43: Rare Mutations but Prevalent Aggregation Suggest a Causative Link between Protein Aggregation and Disease

Discovery, Prevalence, and Clinical Presentation

The finding that TDP-43 is the major component of the ubiquitin-positive cytoplasmic inclusions found abundantly in patients with

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sALS (Arai et al. 2006; Neumann et al. 2006) was a major breakthrough. This discovery was rapidly followed by the finding that mutations in TDP-43 cause ALS in ~5% of familial cases (Gitcho et al. 2008; Kabashi et al. 2008; Sreedharan et al. 2008; Van Deerlin et al. 2008; Yokoseki et al. 2008). Most patients with TDP-43 mutations develop a classical ALS phenotype without cognitive deficit, with some variability within families in the site and age of onset. Although initial studies failed to find mutations in TDP-43 in FTLD patients, rare TDP-43 mutations in patients displaying FTLD, with or without motor neuron disease, were reported in 2009 (Benajiba et al. 2009; Borroni et al. 2009; Gitcho et al. 2009; Kovacs et al. 2009).

Protein Structure and Function

TDP-43, a highly conserved and essential protein that belongs to the ribonucleoprotein family, was initially identified by its binding to transactivation response DNA, a regulatory element of human immunodeficiency virus type 1 (Ou et al. 1995). A multifunctional protein involved in multiple steps of RNA processing (Lagier-Tourenne et al. 2010; Polymenidou et al. 2012), TDP-43 contains two RNA recognition motifs (RRM1 and RRM2), which cooperatively bind to UG-repeat RNAs (Polymenidou et al. 2011; Lukavsky et al. 2013), an interaction that is potentially stabilized via an atypical ubiquitin-like domain residing in the N-terminal part of the protein (Qin et al. 2014). TDP-43 regulates alternative splicing, as well as the levels of long intron-containing RNAs, which are necessary for neuronal activity (Polymenidou et al. 2011). Moreover, TDP-43 has been reported to act as a repressor of cryptic exons, which when included lead to the degradation of respective mRNAs (Ling et al. 2015).

TDP-43 in healthy cells is primarily nuclear, as most of its physiological functions in RNA processing take place in the nucleus. However, TDP-43 is naturally a shuttling protein, as it contains both a nuclear localization signal (NLS) and a nuclear export signal (NES) (Fig. 1A). Indeed, TDP-43 also plays an important role outside of the nucleus, including RNA transport to distal locations for local translation (Alami et al. 2014), a function particularly important for neurons. Moreover, TDP-43 associates with stress granules (Liu-Yesucevitz et al. 2010; Dewey et al. 2011; Bentmann et al. 2012), which are nonmembranous cytoplasmic foci composed of nontranslating messenger ribonucleoprotein (mRNP) complexes that rapidly aggregate in cells upon stress, allowing selective RNA inactivation (Anderson and Kedersha 2009). The C-terminal domain, which contains a glycine-rich region (Fig. 1A) important for protein–protein interactions, is necessary for the recruitment of TDP-43 in stress granules, as deletion of this region was shown to inhibit the process (Dewey et al. 2011).

Mutation Distribution and Effect

The majority of ALS-linked missense mutations in TDP-43 are localized in the C-terminal, glycine-rich domain (Lagier-Tourenne et al. 2010; Ling et al. 2013), which contains a glutamine/asparagine-rich low-complexity or prion-like domain that shares similarities with yeast prions (Cushman et al. 2010; Fuentealba et al. 2010). The latter are proteins showing ordered, self-perpetuating aggregation, which are transmissible from an affected cell to its progeny (reviewed in Chien et al. 2004; Shorter and Lindquist 2005; Cushman et al. 2010). The known yeast prion domains can switch their conformation between two states: An intrinsically unfolded state and an aggregated one that imposes its conformation on its unfolded counterpart in a mechanism similar to mammalian prions. Importantly, ALS-linked point mutations significantly increase the aggregation propensity of the already aggregation-prone prion-like domain of TDP-43 (Johnson et al. 2009; Guo et al. 2011; Molliex et al. 2015), which indicates a causative link between TDP-43 aggregation and disease (Fig. 1B). Despite this finding, expression of ALS-linked TDP-43 mutants in transgenic mice (Wegorzewska et al. 2009; Zhou et al. 2010; Igaz et al. 2011; Huang et al. 2012; Arnold et al. 2013) has largely failed to reproduce the characteristic TDP-43 pathology seen in ALS

patients, and although these mice develop various abnormalities with aspects of motor neuron disease, they do not fully reproduce the spectrum of ALS symptoms. Although early reports have found that mutant TDP-43 displays normal activity in splicing regulation of a reporter gene (D'Ambrogio et al. 2009), genome-wide analysis of mice expressing mutant TDP-43 showed broad aberrant splicing events (Arnold et al. 2013). Moreover, TDP-43 mutants show defective transport of their RNA cargo along neuronal axons (Alami et al. 2014).

Pathological Findings

Although TDP-43 localizes predominantly in the nucleus in healthy cells, in affected neurons and glial cells, TDP-43 mislocalizes to the cytoplasm, where it forms pathogenic inclusions (Fig. 1B). This protein redistribution and cytoplasmic aggregation prevent TDP-43 from returning to the nucleus, which leads to its nuclear clearance (Arai et al. 2006; Neumann et al. 2006; Igaz et al. 2008; Winton et al. 2008). Moreover, disease-associated TDP-43 presents a biochemical signature (Fig. 1B) characterized by acetylation (Cohen et al. 2015), polyubiquitination, hyperphosphorylation, and proteolytic cleavage (Arai et al. 2006; Neumann et al. 2006). The latter gives rise to highly aggregative C-terminal fragments that deposit in the cytoplasm and recruit all functional full-length TDP-43 (Igaz et al. 2008; Neumann 2009).

The above pathological findings characterize the vast majority of ALS cases, with notable exceptions for patients with mutations in SOD1 and fused in saracoma (FUS), which lack TDP-43 inclusions but harbor misfolded SOD1 (Mackenzie et al. 2007; Tan et al. 2007; Kerman et al. 2010) or FUS (Kwiatkowski et al. 2009; Vance et al. 2009) protein accumulations, respectively.

Pathogenic Mechanism

TDP-43 toxicity may be caused by either loss of its normal function because of protein redistribution and aggregation in the cytoplasm or because the misfolded protein assemblies in the cytoplasm are toxic per se. It is important to note that the relative contribution of each mechanism is still not determined. Nevertheless, these two mechanisms are not mutually exclusive and together likely cause motor neuron degeneration in combination (Lagier-Tourenne et al. 2010; Polymenidou et al. 2012; Ling et al. 2013), especially because they are intimately linked in the cells. Indeed, TDP-43 levels are controlled by a tight auto-regulatory mechanism (Ayala et al. 2011; Polymenidou et al. 2011), mediated by TDP-43-dependent splicing of its own pre-mRNA (Polymenidou et al. 2011; Bembich et al. 2014).

After an initiation phase, the formation of TDP-43 pre-inclusions has a dual effect in cells. First, their presence causes cellular stress, leading to increased nuclear export of TDP-43, which then gets trapped by the existing cytoplasmic TDP-43 aggregates, leading to a sustained decrease of nuclear TDP-43, which in turn leads to increased levels of stable TDP-43 mRNAs. Consequently, the protein levels of TDP-43 increase, providing abundant substrate for seeded aggregation and therefore contributing to the growth of pathogenic TDP-43 deposits. Taking into account that increased levels of TDP-43 mRNA were observed in the motor neurons of ALS patients (Rabin et al. 2010), such a feed-forward mechanism could explain the aggregate propagation underlying ALS.

Fused in Sarcoma/Translocated in Liposarcoma: Cytoplasmic Retention Leading to Aggressive ALS

Discovery and Prevalence

Soon after the identification of TDP-43, ALS-causing mutations were found in another DNA/RNA-binding protein, fused in sarcoma (FUS) or translocated in liposarcoma (TLS) (Kwiatkowski et al. 2009; Vance et al. 2009). Missense and nonsense mutations in the FUS gene account for ~ 4% of fALS cases and rare sporadic cases (Kwiatkowski et al. 2009; Vance et al. 2009). In the absence of mutations, pathologic accumulations of WT FUS occur in ~10% of FTLD cases (Neumann et al. 2009a).

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Clinical Presentation and Heterogeneity

Most patients with FUS mutations develop a classical ALS phenotype without cognitive defect; however, rare cases with ALS-FTLD (Kwiatkowski et al. 2009) or pure FTLD (Blair et al. 2010; Van Langenhove et al. 2010) have also been reported. Interestingly, several patients with the FUS-R521C mutation developed an unusual presentation, including an early onset drop-head syndrome (Ticozzi et al. 2009; Blair et al. 2010; Corrado et al. 2010; Tateishi et al. 2010). This is an atypical phenotype, as only about 1% of ALS patients present with severe weakness of the neck extensor muscles in the early stage of the disease (Gourie-Devi et al. 2003). Moreover, mutations in FUS have been associated with some of the most aggressive ALS forms, presenting with juvenile onset and unusually rapid disease progression (Baumer et al. 2010; Yan et al. 2010; Zou et al. 2013). In the absence of FUS mutations, WT FUS misaccumulates in distinct clinical subgroups of frontotemporal dementia patients (Munoz et al. 2009; Neumann et al. 2009a,b), without TDP-43 or tau pathology (Urwin et al. 2010), which are now collectively referred to as "FUS-proteinopathies."

Protein Structure and Function

FUS is a 526-amino-acid-long ribonucleoprotein that binds nucleic acids through its C-terminal part, which comprises an RNA recognition motif, two glycine-arginine (R/G)-rich motifs, and a zinc finger domain. The N-terminal part, which shows high aggregation propensity, contains a glycine-rich region and a domain enriched in glutamine, glycine, serine, and tyrosine residues (Sun et al. 2011). FUS contains NLS and NES sequences, which allow its shuttling between the nucleus and the cytoplasm, suggesting that FUS is involved in RNA metabolism pathways that take place in both cellular compartments (Lagier-Tourenne et al. 2010).

FUS and TDP-43 show significant structural and functional homology (Lagier-Tourenne et al. 2010). Like TDP-43, FUS has the ability to bind nucleic acids and comprises a prion-like domain. The nucleic acid binding of FUS is caused by the presence of R/G-rich motifs, an RNA recognition motif, and a zinc finger domain. The first half of the protein, which contains a glutamine-glycine-serine-tyrosine (Q/G/S/Y)-rich region and the R/G-rich motifs, is responsible for the high-aggregation propensity of FUS (Sun et al. 2011), and similarly to TDP-43, FUS is predicted to have prion-like properties (Cushman et al. 2010; Polymenidou and Cleveland 2011). Moreover, like TDP-43, FUS is mainly nuclear and uses a transportin shuttle to reach the cytoplasm (Dormann et al. 2010), where it partitions in stress granules (Bentmann et al. 2012; Dormann et al. 2012).

Mutation Distribution and Effect

Most of the ALS-linked FUS mutations are localized in the NLS (Lagier-Tourenne et al. 2010; Ling et al. 2013) and do not influence aggregation per se (Dormann et al. 2010; Sun et al. 2011) but rather promote redistribution of FUS to the cytoplasm (Dormann et al. 2010; Ito et al. 2011; Sun et al. 2011), which consequently may initiate its aggregation.

Pathological Findings

FUS predominantly localizes to the nucleus of healthy cells, whereas in ALS patients with FUS mutations, it is redistributed to the cytoplasm of affected cells where it accumulates and aggregates. This mislocalization leads to partial loss of nuclear protein (Vance et al. 2009; Mackenzie et al. 2010), similarly to TDP-43 pathology, albeit to a lesser extent. Strikingly, although the FUS aggregates are immunoreactive for p62 and ubiquitin, in contrast to the characteristic pathological signature of most ALS cases, FUS inclusions are TDP-43 negative (Vance et al. 2009; Suzuki et al. 2010; Tateishi et al. 2010).

Pathogenic Mechanism

The functional and structural similarities of TDP-43 and FUS (Lagier-Tourenne et al. 2010) suggest that the two proteins trigger ALS by an independent initiating event, but potentially

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through common downstream pathways, one of which involves their common RNA targets. Indeed, although TDP-43 and FUS bind a distinct spectrum of RNAs, they have high preference for mRNAs derived from genes with exceptionally long introns (Polymenidou et al. 2011; Lagier-Tourenne et al. 2012). Importantly, 45 common TDP-43 and FUS RNA targets, the majority of which are important for normal neuronal function, are significantly downregulated upon depletion of either TDP-43 or FUS in the adult mouse brain and in cultured human neurons (Polymenidou et al. 2011; Lagier-Tourenne et al. 2012). High abundance in the brain of these long intronic genes (Ameur et al. 2011; Polymenidou et al. 2011), which are bound with very high affinity by both TDP-43 and FUS, presents a possible explanation for why neurons are mostly susceptible to ALS pathogenic processes.

Intronic Hexanucleotide Repeats in *C9ORF72* Are the Most Common Genetic Cause of ALS/FTLD and Lead to Complex Pathology

Discovery and Prevalence

One of the most exciting discoveries in the ALS field was the identification of intronic hexanucleotide repeat expansions in the *C9ORF72* gene of ALS patients, which is now recognized as the most frequent genetic cause of ALS and FTLD, accounting for ~40% of fALS and ~30% of familial FTLD (fFTLD) cases (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Gijselinck et al. 2012; Majounie et al. 2012). Moreover, up to 90% of families with concurrent ALS and FTLD have hexanucleotide repeat expansions in *C9ORF72* (Majounie et al. 2012; Rademakers and van Blitterswijk 2013; Renton et al. 2014). Unexpectedly, *C9ORF72* hexanucleotide repeat expansions were also found in approximately 7% or 5% of apparent sALS or FTLD cases, respectively, suggesting that the fraction of ALS and FTLD cases with genetic origin may be larger than anticipated.

Clinical Presentation and Heterogeneity

Patients with repeat expansions in *C9ORF72* may present with classical ALS, mixed ALS-FTLD, or FTLD without motor neuron disease (Hsiung et al. 2012; Simon-Sanchez et al. 2012; Snowden et al. 2012b). The frequency of the mixed ALS-FTLD phenotype is higher in C9ORF72 carriers compared with non-C9ORF72 patients. FTLD patients with *C9ORF72* expansions most frequently develop behavioral variant FTLD, which is associated with clinical characteristics such as psychosis and other neuropsychiatric features (Snowden et al. 2012a; Cooper-Knock et al. 2014), which suggests that distinct pathophysiological mechanisms are operating in this subgroup of patients.

Protein Structure and Function

The normal function of C9ORF72 is not well understood, but recent studies suggest that it may be important for endosomal trafficking (Zhang et al. 2012; Levine et al. 2013; Farg et al. 2014). C9ORF72 is homologous to proteins related to differentially expressed in normal and neoplasia (DENN), which is a GDP/GTP exchange factor (GEF) that activates Rab-GTPases (Zhang et al. 2012; Levine et al. 2013). Furthermore, C9ORF72 was found in the extracellular space and in cytoplasmic vesicles, where it colocalizes with Rab proteins that are implicated in autophagy and endocytic transport in mouse and human neurons (Farg et al. 2014). Importantly, the C9ORF72 protein seems to be necessary for normal macrophage maturation and function, as *C9orf72* knockout mice develop splenomegaly and enlarged lymph nodes (Jiang et al. 2016; O'Rourke et al. 2016).

Mutation Distribution and Effect

In normal healthy controls, the intronic GGGG CC repeat in the *C9ORF72* gene is shorter than 25 units, whereas in ALS or FTLD patients, it can expand up to 800–4400 units (DeJesus-Hernandez et al. 2011; Gijselinck et al. 2012), with major consequences (Majounie et al. 2012; Rademakers and van Blitterswijk 2013; Renton et al. 2014). First, these large intronic expansions interfere with transcription of *C9ORF72* RNA, thereby decreasing its levels (DeJesus-Hernandez et al. 2011; Gijselinck et al. 2012).

Moreover, the repeat RNA accumulates in nuclear foci (DeJesus-Hernandez et al. 2011; Lagier-Tourenne et al. 2013; Zu et al. 2013; Haeusler et al. 2014) and can also be translated via repeat-associated non-ATG (RAN) translation (Ash et al. 2013; Mori et al. 2013; Zu et al. 2013), leading to the production and accumulation of abnormal dipeptide proteins.

Pathological Findings

C9ORF72-linked ALS cases show a complex pathological signature that, besides the typical TDP-43 aggregation, is characterized by p62-positive and TDP-43-negative ubiquitinated inclusions in the cerebellar and hippocampal regions (Al-Sarraj et al. 2011; DeJesus-Hernandez et al. 2011; Troakes et al. 2012), which are usually free of pathology in non-C9ORF72 ALS (Geser et al. 2008, 2009). These inclusions contain one of five different aggregating dipeptide-repeat (DPR) proteins—namely, poly-GA, poly-GP, poly-GR, poly-PR, and poly-PA (Ash et al. 2013; Mori et al. 2013; Zu et al. 2013). These DPR proteins are generated by an unconventional type of translation called RAN translation (Zu et al. 2011), which operates on sense (Ash et al. 2013; Mori et al. 2013) and antisense (Zu et al. 2013) transcripts originating from the hexanucleotide repeat RNA. Moreover, hexanucleotide repeats themselves were also found to accumulate in nuclear RNA foci in neurons (DeJesus-Hernandez et al. 2011) and glial cells (Lagier-Tourenne et al. 2013) of C9ORF72 ALS and FTLD patients.

Pathogenic Mechanisms

Repeat expansions have been previously reported to disrupt RNA metabolism in other neurodegenerative diseases (Ranum and Cooper 2006). Several cytotoxic mechanisms have been described for *C9ORF72* hexanucleotide repeat expansions, but their relative contributions to ALS pathogenesis are not established. First, patients with *C9ORF72* expansions show typical TDP-43 cytoplasmic inclusions with nuclear clearance (Al-Sarraj et al. 2011; DeJesus-Hernandez et al. 2011; Gijselinck et al. 2012; Troakes et al. 2012), which suggests that all of the mechanisms described above for TDP-43 are relevant for this type of ALS. Moreover, patients carrying hexanucleotide repeat expansions were shown to produce reduced levels of *C9ORF72* RNA (DeJesus-Hernandez et al. 2011; Gijselinck et al. 2012), caused by the inactivation of the repeat-containing allele via promoter hypermethylation (Xi et al. 2013), or transcriptional abortion of repeat RNAs (Haeusler et al. 2014). Reduced *C9ORF72* RNA and, by extension, the protein may not be enough to perform its normal function, a mechanism known as haploinsufficiency. However, the lack of an ALS/FTLD-like phenotype in mice with heterozygous (Panda et al. 2013; Suzuki et al. 2013) or homozygous (Koppers et al. 2015) disruption of the mouse *C9orf72* homolog strongly argues against the role of haploinsufficiency in C9ORF72-ALS/FTLD pathogenesis.

Another possibility is that the repeat RNA is toxic per se. Indeed, the DNA and RNA hexanucleotide repeats form complex structures, which results in transcriptional hindrance and in accumulation of abortive transcripts that contain the hexanucleotide repeats (DeJesus-Hernandez et al. 2011; Lagier-Tourenne et al. 2013; Zu et al. 2013; Haeusler et al. 2014). Those abortive transcripts form G-quadruplexes and hairpins and bind essential proteins, including nucleolin, whose sequestration leads to nucleolar stress and other downstream defects (Haeusler et al. 2014). However, recent evidence suggests that RNA foci might also be neutral, or even protective (Tran et al. 2015), because retention of repeat RNA in the nucleus prevents its unconventional translation and production of toxic protein products (Ash et al. 2013; Mori et al. 2013; Gendron et al. 2015; Mackenzie et al. 2015; van Blitterswijk et al. 2015).

Indeed, the repeat-containing C9ORF72 RNA transcripts can escape the nucleus and associate with ribosomal complexes in the cytoplasm, where they are subjected to RAN translation (Ash et al. 2013; Mori et al. 2013; Zu et al. 2013), leading to the production and accumulation of abnormal dipeptide proteins. The relative abundance of dipeptide repeat proteins (Mori et al. 2013; Gendron et al. 2015; Macken-

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zie et al. 2015; van Blitterswijk et al. 2015), as well as their contribution to disease (Gomez-Deza et al. 2015), is not settled. Some investigators have reported poly-GA aggregates as the most prominent DPRs in patients (Ash et al. 2013; Mori et al. 2013; Gendron et al. 2015; Mackenzie et al. 2015) and in rat primary neurons (May et al. 2014), suggesting a direct role in neurodegeneration. Poly-GA DPRs were indeed shown to cause neurotoxicity in vitro by inducing endoplasmic-reticulum stress (Zhang et al. 2014). However, conflicting reports have proposed that the most common DPRs present in both upper and lower motor neurons are the poly-GP (Zu et al. 2013), which were subsequently shown to be present in the cerebrospinal fluid of C9ORF72 patients (Su et al. 2014). Moreover, the relatively short synthetic sense poly-GR and antisense poly-PR DPRs were reported to be highly toxic when exogenously applied to human astrocytes because they entered the nucleus bound to nucleoli and killed the cells in culture (Kwon et al. 2014). Finally, expression of "protein-only" poly-GR and poly-PR constructs in *Drosophila* was associated with progressive neuronal death in vivo, which is perhaps attributable to the basic nature or a common structural motif of these DPRs (Mizielinska et al. 2014). Moreover, DPRs were shown to compromise nucleocytoplasmic transport (Freibaum et al. 2015; Jovicic et al. 2015), another potential toxic mechanism of these aberrant proteins.

Each of the Major Pathological Proteins of ALS and FTLD Has the Potential to Behave Similarly to Prions

Template-Directed Misfolding and Cell-to-Cell Spread of SOD1

Despite the high stability of the native WT protein, recombinant SOD1 can form fibrils spontaneously, most potently under conditions that disrupt its dimerization, such as reducing agents or high temperature (Chia et al. 2010; Münch et al. 2011), whereas ALS-linked mutations in SOD1 promote aggregation (Prudencio et al. 2009; Chia et al. 2010). Furthermore, aggregates reconstituted in vitro from recombinant WT or mutant SOD1 trigger aggregation of endogenously expressed protein in cell culture (Münch et al. 2011; Furukawa et al. 2013). Importantly, the induced SOD1 aggregates persisted within cells when the original pathogenic seed was no longer present (Grad et al. 2011; Münch et al. 2011). Therefore, the newly induced aggregates can act as new misfolding templates and show properties consistent with prion-like propagation (see Sibilla and Bertolotti 2017). Curiously, human misfolded SOD1 is not a competent template for mouse SOD1, an observation that was attributed to a single amino-acid difference between the human and mouse protein position 32 (Grad et al. 2011), pointing to a likely interacting region for this conformational conversion. This finding explains the inactive role of mouse SOD1 in human mutant SOD1 transgenic mice, which neither affects any aspect of disease course (Bruijn et al. 1998) nor coaggregates with the human mutant protein (Deng et al. 2006).

The mechanism of intracellular aggregate secretion has puzzled researchers for a number of years, and the details of the molecular machinery involved in disease remains largely unknown and may well be specific for each protein. Indeed, mutant SOD1 has been proposed to be actively secreted with neurosecretory vesicles through an aberrant interaction with chromogranins A and B (Urushitani et al. 2006). Moreover, protein aggregates released from dying cells may enter cells through macropinocytosis (Münch et al. 2011), whereas living cells have been proposed to secrete misfolded SOD1 via exosomes (Grad et al. 2014), which are suspected to act as "Trojan horses" for a number of intracellular aggregates involved in neurodegeneration (Aguzzi and Lakkaraju 2016).

Evidence for In Vivo Transmissibility of Misfolded SOD1

The first implication for the in vivo transmissibility of SOD1 pathology was recently reported (Ayers et al. 2014). In this study, genetically vulnerable mice expressing a G85R-SOD1:yellow fluorescent protein (YFP) fusion protein were intraspinally injected with homogenates from

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terminally affected, paralyzed mutant mice. The injected mice developed motor neuron disease and showed a widespread redistribution of endogenous SOD1 to protein inclusions, found abundantly throughout the spinal cord, brainstem, and thalamus. Although these data strongly support the prion-like propagation of misfolded SOD1 in vivo, the lack of transmissibility to mice overexpressing WT human SOD1 suggests that the process is extremely inefficient and depends on the loosening of the stable SOD1 dimeric structure by the presence of the YFP tag.

Further supporting seeded aggregation and spread of misfolded SOD1 in vivo, transgenic mutant human SOD1-expressing mice accelerated disease progression and shorter life span upon coexpression of WT human SOD1 ($hSOD1^{WT}$) (Deng et al. 2006). The conversion of unaffected to pathogenic phenotype may be explained by prion-like misfolding of human WT SOD1 by the A4V mutant in the double-transgenic mouse. Indeed, disease in these mutant/WT co-expressing mice is accompanied by high levels of insoluble WT SOD1, suggesting that misfolded, mutant SOD1 assemblies can recruit and convert WT SOD1 to an aberrant isoform in a template-directed manner. Interestingly, transgenic mice greatly overexpressing $hSOD1^{WT}$ were also shown to exhibit SOD1 aggregation, neurotoxicity, and shortened life span (Graffmo et al. 2013). These data suggest that both mutant and WT human SOD1 may be involved in the development of ALS in humans (Deng et al. 2006; Graffmo et al. 2013). Indeed, mutant WT coaggregates were found in fALS patients (Bruijn et al. 1998).

TDP-43 and FUS Aggregation: The Complex Life of Low-Complexity Domains

The high propensity of TDP-43 and FUS to aggregate is linked to the presence of a low-complexity (Kato et al. 2012), or prion-like (Cushman et al. 2010), domain in both proteins—namely, in the N-terminal part of FUS (amino acids 1–239) and the C-terminal part of TDP-43 (amino acids 274–414). Curiously, such domains, which are frequently found in RNA-binding proteins (King et al. 2012), show no similarity whatsoever to mammalian prion proteins, which in their native form are stable globular proteins (Christen et al. 2013). Yet, TDP-43 and FUS seem to fulfill the in vitro prion test because small amounts of aggregated TDP-43 (Furukawa et al. 2011) or FUS (Nomura et al. 2014) were able to induce misfolding and aggregation of their corresponding natively folded protein, implicating template-dependent propagation. Recently, the insoluble TDP-43 aggregates derived from brain homogenates of ALS patients were shown to seed protein misfolding and accumulation in TDP-43 transfected cultured cells (Nonaka et al. 2013).

The prion-like domains of TDP-43 (Johnson et al. 2009; Nonaka et al. 2009; Furukawa et al. 2011; Guo et al. 2011) and FUS (Sun et al. 2011) apparently mediate protein aggregation and contribute to their seeding ability. Indeed, increased expression of the TDP-43 glycine-rich domain induced protein accumulation and cell toxicity (Johnson et al. 2009; Zhang et al. 2009; Liu-Yesucevitz et al. 2010; Furukawa et al. 2011; Guo et al. 2011; Pesiridis et al. 2011). In contrast, deletion of TDP-43 (Johnson et al. 2009; Furukawa et al. 2011) or FUS (Sun et al. 2011) prion-like domains prevented aggregate formation, which indicates that these domains are required for efficient seeding.

The implication of both TDP-43 and FUS/TLS in stress-granule assembly (Andersson et al. 2008; Wang et al. 2008; Colombrita et al. 2009; Moisse et al. 2009; Liu-Yesucevitz et al. 2010; Ito and Suzuki 2011; McDonald et al. 2011) offers a plausible mechanism for aggregation initiation and seeding as a response to a variety of cellular stresses. Indeed, the very formation of stress granules is mediated by the ordered aggregation of TIA1, an integral stress-granule protein component that possesses a Q/N-rich, prion-like domain (Gilks et al. 2004). Seeded aggregation of TIA1 through its prion-like domain seems to be the driving force of stress-granule formation, because this domain is not only indispensable for nucleation of TIA1 but also can be replaced by another prion-like domain from a yeast prion protein (SUP35) without visibly affecting the size or number of stress granules (Gilks

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et al. 2004). Aggregated TIA1 within cytoplasmic foci recruits mRNAs and other proteins, including TDP-43 and FUS (Andersson et al. 2008; Wang et al. 2008; Colombrita et al. 2009; Moisse et al. 2009; Liu-Yesucevitz et al. 2010; Ito and Suzuki 2011; McDonald et al. 2011).

Because increased protein concentration is expected to be a main determinant for protein aggregation, the increase of local TDP-43 and FUS concentration within stress granules could facilitate the initiation of their aggregation. This may be further assisted by the presence of RNA that can act as a scaffolding molecule mediating the ordered aggregation of TDP-43 and FUS within these cytoplasmic foci. Indeed, the scaffolding capacity of RNA has been established in the in vitro aggregation of the mammalian prion protein (PrP) (Deleault et al. 2003), as generation of infectious prions with purified PrP has been achieved only by the addition of RNA and phospholipids (Wang et al. 2010). Indeed, RNA was shown to mediate the formation of fibril-like assemblies in vitro (Schwartz et al. 2013).

The hypothesis (Polymenidou and Cleveland 2011) that the functional prion-like conformational changes of TDP-43 and FUS associated with stress-granule formation may transform into pathogenic, self-perpetuating, irreversible aggregation in disease has now been supported by a number of recent, elegant biophysical studies (Burke et al. 2015; Molliex et al. 2015; Patel et al. 2015). Indeed, FUS (and other RNA-binding proteins carrying low-complexity domains) forms dynamic liquid-like assemblies in vitro (Han et al. 2012; Kato et al. 2012; Burke et al. 2015; Molliex et al. 2015; Patel et al. 2015), which somewhat resemble cellular stress granules and which remarkably can "mature" into rigid, large protein assemblies, reminiscent of pathological protein aggregates seen in patients (Burke et al. 2015; Molliex et al. 2015; Patel et al. 2015). In other words, stress granules may be operating to facilitate FUS and/or TDP-43 "seeding" within the cytoplasm of diseased cells. The observation that stress-granule proteins partition in the TDP-43 and FUS pathological inclusions found in ALS patients supports this view (Fujita et al. 2008; Liu-Yesucevitz et al. 2010; Dormann and Haass 2011).

Unresolved is whether specific cellular RNAs are sequestered within the cytoplasmic FUS and/or TDP-43 inclusions, thereby depleting the cell of essential RNA components. The latter, if true, could explain the observation that although the RNA-recognition motifs of TDP-43 are not required for its aggregation (Johnson et al. 2008; Pesiridis et al. 2011), binding to RNA seems to be indispensible for its cytotoxicity (Elden et al. 2010; Voigt et al. 2010).

Although the above observations, coupled with the unequivocally important role of the TDP-43 prion-like domain in disease (Igaz et al. 2008; Neumann 2009), have focused attention on the C-terminal part of TDP-43, two recent studies highlight the significance of an N-terminal segment for both its normal function and aggregation. Indeed, the N-terminal part of TDP-43 was reported to facilitate RNA binding (Qin et al. 2014), thereby ensuring proper protein function such as splicing regulation (Zhang et al. 2013; Qin et al. 2014), as well as promoting aggregation (Zhang et al. 2013; Qin et al. 2014). In addition, RNA-FUS amyloid-like assemblies in vitro are mediated via its RGG domain (Schwartz et al. 2013), highlighting the importance of other protein regions, besides the low-complexity domains, in both health and disease.

IMPLICATION OF PRION-LIKE SPREAD FOR DISEASE PROGRESSION

Neurodegeneration in ALS typically begins focally and then spreads spatiotemporally until the loss of the motor neurons of the respiratory system (Ravits et al. 2007a,b; Ravits and La Spada 2009). The most plausible model for this progression of disease from a focal start would be the spreading of toxic aggregates from a focal site. The gradual distribution of pathological TDP-43 accumulation across the nervous system in ALS (Geser et al. 2008; Brettschneider et al. 2014a) and FTLD (Geser et al. 2009; Brettschneider et al. 2014b) supports this concept. As attractive as the prion model is, an alternative—one might almost call it "antiprion"—

theory was recently proposed based on evidence that a human endogenous retrovirus-K is activated in some ALS patients, which leads to the expression of a viral protein that is neurotoxic (Li et al. 2015). Future studies are needed to dissect the contribution of the different mechanisms to disease initiation and progression.

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